Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Ankita Chowdhury Date

Regulation of antiviral immune responses in SIV infection

By

Ankita Chowdhury
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____

Guido Silvestri, M.D.
Advisor

_____ _____

Rama Amara, Ph.D.
Committee Member

Tony Moody, M.D.
Committee Member

_____ _____

Cynthia Derdeyn, Ph.D.
Committee Member

Mirko Paiardini, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date:

Regulation of antiviral immune responses in SIV infection

By

Ankita Chowdhury

B.S. Molecular and Cellular Biology, University of Maine, 2010

Advisor: Guido Silvestri, M.D.

An Abstract of

A Dissertation submitted to the Faculty of the Graduate School of Emory University

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

Program in Immunology and Molecular Pathogenesis

Graduate Division of Biological and Biomedical Sciences

2015

Human Immunodeficiency Virus (HIV) is still among the most lethal human pathogens, causing 1.6 million deaths this year. Recent progress in understanding the biology of broadly neutralizing antibodies and mechanisms responsible for latent reservoirs has fueled the search for effective HIV vaccine and eradication strategies. The development of vaccine and eradication strategies will require efficient induction of host antiviral responses which requires a better understanding of the immune response to HIV. This thesis elucidates the regulation of antiviral immune responses in the context of Simian Immunodeficiency Virus (SIV) infection of rhesus macaque. First, experimental CD8 T cell depletion led to significantly higher fold increase of virus in blood in controllers compared to progressors (chapter two). While cell-associated virus increased in all CD4 compartments in progressors, it decreased in central memory CD4 T cells in controllers. These results provide strong evidence that CD8 T cells contribute heavily to the suppression of virus in controllers via differential mechanisms in cellular compartments. The next research project led to a detailed analysis of T follicular regulatory (T_{FR}) cells found within germinal centers (GC) that share phenotypic markers with, but are distinct from, T follicular helper (T_{FH}) and T regulatory cells (chapter three). Chronic SIV infection was associated with a decreased T_{FR} to T_{FH} ratio. These data point to the suppressive function of T_{FR} cells and may be one of several mechanisms of immune control within GC with important consequences on the quality of antibody response and on the level of virus replication. Finally, this thesis describes a previously undiscovered aspect of interaction between GC B and T cells resulting in single-celled lymphocytes with surface expression of both T and B cell markers (chapter four). These lymphocytes likely arise as a consequence of membrane exchange following high-affinity interactions

and may serve as an additional tool to study vaccine-elicited immune responses within lymph nodes. Taken together, these findings expand on our understanding of the complex CD4 and CD8 T cell responses in the context of SIV infection and may contribute to the development of protective and prophylactic HIV therapeutics.

Regulation of antiviral immune responses in SIV infection

By

Ankita Chowdhury

B.S. Molecular and Cellular Biology, University of Maine, 2010

Advisor: Guido Silvestri, M.D.

A Dissertation submitted to the Faculty of the Graduate School of Emory University
In partial fulfillment of the requirements for the degree of Doctor of Philosophy

Program in Immunology and Molecular Pathogenesis
Graduate Division of Biological and Biomedical Sciences

2015

Table of Contents

List of Tables **3**

List of Figures **4**

Abstract **1**

Chapter One: Introduction **3**

Epidemiology 3

Structure and Phylogeny of HIV 4

HIV infection and progression to AIDS 6

Non-human primate models of HIV infection 7

HIV Pathogenesis 8

HIV treatment and cure 10

HIV vaccine development 11

Immune Responses to HIV 15

Chapter Two: Differential impact of *in vivo* $CD8^+$ T lymphocyte depletion in controller versus progressor SIV-infected macaques. **27**

Abstract 27

Introduction 29

Materials and Methods 32

Results 36

Discussion 42

Acknowledgments 47

Figures 48

Chapter Three: Decreased T_{FR}/T_{FH} ratio in SIV-infected rhesus macaques may contribute to accumulation of T_{FH} cells in chronic infection **59**

Abstract 60

Introduction 61

Materials and Methods 65

Results 71

Discussion 81

Acknowledgements 86

Chapter Four: Identification of single celled lymphocytes with surface expression of T and B cell markers within primate lymph nodes. **101**

Abstract 102

Introduction 103
Materials and Methods 106
Results 111
Discussion 117
Figures **120**
Chapter Five: Discussion **133**
References **142**

List of Tables

Table 3.1 Quantification of T_{FH} and T_{FR} cells by immunohistochemistry 100

Table 4.1 List of human subject samples 119

List of Figures

Figure 2.1 Experimental design and effective depletion of $CD8^+$ T lymphocytes 48

Figure 2.2 Plasma and tissue viral loads following CD8 T cell depletion 51

Figure 2.3 T-bet expression in $CD8^+$ T cells is the best correlate of the level of viral load after CD8 depletion 53

Figure 2.4 SIV-DNA in $CD4^+$ T_N, T_{CM} and T_{EM} increases post-depletion in progressor but not controller RMs 55

Figure 2.5 $CD8^+$ T lymphocyte depletion is associated with greater increase in $CD4^+$ T cell activation in controllers than in progressors 57

Figure 3.1 T_{FR} can be defined by flow cytometry and identified by confocal microscopy within the germinal centers of RM 87

Figure 3.2 T_{FR} share phenotypical features of both T_{FH} and T_{REG} populations 89

Figure 3.3 RNA expression patterns confirm that T_{FR} share T_{FH} and T_{REG} like phenotype 91

Figure 3.4 Kinetics of T_{FR}, T_{FH} and T_{REG} after SIV infection 93

96

Figure 3.5 Comparable levels of SIV infection in T_{FH}, T_{FR} and T_{REG} isolated from the spleen of chronically SIV-infected RM 94

Figure 3.6 T_{FR} frequencies negatively correlate with T_{FH} and GC B-cell frequencies in the lymph nodes of RM 95

Figure 3.7. RNA expression within T_{FR} cells in uninfected and infected RM 96

Figure 3.8 Supplementary Figure: RNA expression within TFR in uninfected and SIV infected RM 98

Figure 3.9 Supplementary Figure: PCR and flow cytometry validation of RNA-sequencing data 99

Figure 4.1 A distinct lymphocyte population identified by the expression of CD3 and CD20 on the cell surface is found in secondary lymphoid organs 120

Figure 4.2 $CD3^+CD20^+$ cells in secondary lymphoid organs have enriched expression of markers associated with T_{FH} 122

Figure 4.3 $CD3^+CD20^+$ cells express several B cell surface markers associated with the TCR-MHC synaptic complex 124

Figure 4.4 CD40L surface expression identifies cells of T_{FH} origin within $CD3^+CD20^+$ population 124

Figure 4.5 $CD3^+CD20^+$ cells increase in frequency following SHIV 126

Figure 4.6 $CD3^+CD20^+$ population can be found within human lymphoid organs and reflect phenotype of $CD3^+CD20^+$ population in RM 127

Figure 4.7 Supplementary Figure: Representative flow cytometry plot showing gating strategy 129

Figure 4.8 Supplementary Figure: Representative cell conjugates and doublets 131

Figure 4.9 Supplementary Figure: Representative histograms showing relative expression of T_{FH} like makers 132

Abstract

Human Immunodeficiency Virus (HIV) is still among the most lethal human pathogens, causing 1.6 million deaths this year. Recent progress in understanding the biology of broadly neutralizing antibodies and mechanisms responsible for latent reservoirs has fueled the search for effective HIV vaccine and eradication strategies. The development of vaccine and eradication strategies will require efficient induction of host antiviral responses which requires a better understanding of the immune response to HIV. This thesis elucidates the regulation of antiviral immune responses in the context of Simian Immunodeficiency Virus (SIV) infection of rhesus macaque. First, experimental CD8 T cell depletion led to significantly higher fold increase of virus in blood in controllers compared to progressors (chapter two). While cell-associated virus increased in all CD4 compartments in progressors, it decreased in central memory CD4 T cells in controllers. These results provide strong evidence that CD8 T cells contribute heavily to the suppression of virus in controllers via differential mechanisms in cellular compartments. The next research project led to a detailed analysis of T follicular regulatory (T_{FR}) cells found within germinal centers (GC) that share phenotypic markers with, but are distinct from, T follicular helper (T_{FH}) and T regulatory cells (chapter three). Chronic SIV infection was associated with a decreased T_{FR} to T_{FH} ratio. These data point to the suppressive function of T_{FR} cells and may be one of several mechanisms of immune control within GC with important consequences on the quality of antibody response and on the level of virus replication. Finally, this thesis describes a previously undiscovered aspect of interaction between GC B and T cells resulting in single-celled lymphocytes with surface expression of both T and B cell markers (chapter four). These lymphocytes

likely arise as a consequence of membrane exchange following high-affinity interactions and may serve as an additional tool to study vaccine-elicited immune responses within lymph nodes. Taken together, these findings expand on our understanding of the complex CD4 and CD8 T cell responses in the context of SIV infection and may contribute to the development of protective and prophylactic HIV therapeutics.

Chapter One: Introduction

Epidemiology

The CDC reported the first cases of what would later be named Acquired Immunodeficiency Syndrome (AIDS) in young men in the United States of America in 1981(1, 2). In 1983 and 84, several independent reports identified Human Immunodeficiency Virus (HIV) as the causative agent for AIDS (3-5). In the 30 years since, researchers worldwide have worked to understand HIV and control the HIV/AIDS pandemic. In 2013, the UNAID estimated 2.1 million new HIV infections and a total of 23 million people living with HIV (6). Since the start of the epidemic an estimated 39 million people have died of AIDS-related diseases. Sub-Saharan Africa accounts for nearly 70% of the global burden of HIV infections. At its height in 1985, HIV and AIDS infected 130,000 new individuals in the US annually. Today, after 30 plus years of dedicated scientific research and public health campaigns the transmission rates have stabilized and people with HIV are living longer. Worldwide, the rate of new infections has fallen by 38% since 2001 and the rate of AIDS-related deaths has fallen by 35% since 2005.

HIV spreads through sexual, percutaneous and perinatal routes with heterosexual contact being the major route of transmission (7). In the US, HIV continues to disproportionately affect men who have sex with men (MSM), transgendered people, sex workers, and

injection drug users. In the US, 63% of new cases still occur among MSM while 25% occur through heterosexual contact. Gender-based inequality, discrimination and violence against females, particularly in Sub-Saharan African countries, contribute to the high rates of infection among women.

Structure and Phylogeny of HIV

HIV is a primate lentivirus of the Retroviridae family (8). Its genetic material consists of two copies of single-stranded positive sense RNA that is 9kb long and encodes 9 genes-gag, pol, env, vpu, tat, rev, nef, vif, vpr. The viral capsid houses the ssRNA and three viral enzymes: integrase, protease and reverse transcriptase. The viral capsid is covered in an envelope that consists of a host-derived lipid bilayer and the virally encoded gp120 and gp41 envelope proteins. The viral envelope protein complex binds the CD4 receptor and a co-receptor, CCR5 or CXCR4 on the surface of target host cells(9-12). This binding leads to the fusion of the viral lipid envelope with the cell membrane and allows the virus to enter the host cell. Next, the viral reverse transcriptase transcribes the viral RNA into DNA which is then integrated into the host chromosomal DNA. Transcription of viral DNA into RNA is followed by splicing of the RNA, proteolytic cleavage of proteins and assembly of new virions. The extremely low fidelity of reverse transcriptase (3.4×10^{-5} mutations per basepair per cycle) contributes to extremely high genetic variability of HIV (13).

There are two types of HIV that infect humans, HIV-1 and HIV-2. HIV-1 and HIV-2 share approximately 40% nucleotide sequence homology (14). The transmissibility and pathogenicity of HIV-2 is lower than HIV-1 and infected individuals have better outcomes and longer survival (15, 16). Zhu et al. found evidence of human HIV-1 infection as early as 1959 in the Democratic Republic of Congo (17). In 1985, researches isolated Simian Immunodeficiency Virus (SIV) from macaques that first pointed towards a link between the human and simian lentiviruses (18). Phylogenetic analyses suggest that HIV-1 arose as a result of cross species transmissions from chimpanzees (SIVcpz) while HIV-2 most likely resulted from a transmission from sooty mangabey (SIVsmm)(19, 20). Since it entered the human population, HIV-1 has diversified into a vast range of subtypes that are mainly confined by geography. HIV-1 can be subdivided in to 4 groups -M, N, O and P. The M group has the widest prevalence and can further be classified into 9 clades: A-D, F-H, J-K and other circulating recombinant forms. Clade C accounts for 52% of all global infections while clade B, which is most common in Western Europe and the Americas accounts for only 10% of all infections. Genetic diversity is recognized as a hallmark of HIV and is caused mainly by the low fidelity of reverse transcription, the occurrence of super-infection, and high rate of viral replication (13) This high genetic variability has important implications for diagnostic testing, disease progression and treatment outcomes (21).

HIV infection and progression to AIDS

Mucosal transmission is followed by rapid spread of the virus to target cells in the mucosal tissue. Infected cells and antigen presenting cells (APCs) carry the virus to lymphoid organs where the virus replicates at very high levels. This stage of the infection results in flu-like symptoms and is associated with very high plasma viral loads, referred to as the acute stage. At this point, the virus is usually homogenous and CCR5-tropic (22). Development of virus specific immune responses reduces the viral loads and establishes a set point viremia after which chronic infection ensues. Continued virus replication during the chronic stage, mainly in lymphoid organs, leads to genetic diversification and development of escape variants (23). HIV infection leads to progressive damage of the immune system, ultimately leading to CD4 T cells counts lower that 200 cells/ml of blood, increasing viral load, and manifestation of opportunistic infections and tumorous growths defined as AIDS (24).

Time to an AIDS diagnosis varies greatly in infected individuals ranging from 1 year to nearly 20 years. Based on the rate of progression to AIDS, individuals can be grouped into four categories: 1) rapid progressors, who progress to AIDS within 3 years of infection 2) intermediate progressors, within 3-10 years of infection and 3) long term nonprogressors who maintain low detectable plasma viremia (<5000 HIV-RNA copies/ml) after 10 years of infection and 4) elite or natural controllers who maintain plasma HIV-RNA levels below 50 copies/ml. Studies of long-term nonprogressors and

elite controllers have led to several important discoveries about genetic and immunological factors that play a role in control of HIV.

Non-human primate models of HIV infection

Over 40 species of African non-human primates are infected by species-specific SIV (25). Of these, the best-studied viruses include those that infect African green monkeys (SIVagm), sootey managbey monkeys (SIVsm), mandrills (SIVmnd), sykes monkeys (SIVsyk), and chimpanzees (SIVcpz). SIV infections in these 'natural hosts' do not cause pathological symptoms and do not lead to any AIDS-like disease (25). Experimental infection of rhesus macaque (RM) leads to AIDS- like disease (26, 27). Animal models are invaluable tools as they allow researchers to perform experiments that would be impossible or unethical in humans. Non-human primates, close genetic relatives of humans, serve as models for studying many human diseases and SIV-infection of RM serves as an excellent animal model to study pathogenesis and vaccine development strategies (26, 27). The two most common virus strains used to experimentally infect RM are SIVmac239 and SIVmac251, both of which are derived from SIVsmm (26). The chimeric SHIV virus contains the SIVmac239 backbone along with *env* and other genes from HIV and is extremely useful for the study of neutralizing antibodies to HIV envelope proteins (28).

Several similarities exist between natural infection of African monkeys and non-natural infection in RM and humans (reviewed in (25)) including 1) high-viremia throughout infection 2) strong innate and adaptive immune activation during acute infection 3) short

life span of infected cells 4) loss of CD4 T cells from mucosal sites in acute infection. However, comparative studies have been able to identify key factors that seem to protect natural-hosts from progressing to AIDS like immunopathology. These key strategies include: 1) resolution of acute immune activation 2) restriction of infection to non-central memory T cell subsets and 3) prevention of mother to infant transmission (25). These mechanisms of control provide key insights into how best to elicit protection against HIV infection and control HIV replication.

HIV Pathogenesis

HIV and SIV primarily infect activated CD4 memory T cells expressing the main virus co-receptor CCR5 (29). During pathogenic infection, these CD4 T cells are progressively depleted in both blood and mucosal tissues (30). A series of studies have led to the formulation of a pathogenic model in which the pattern of infected CD4 T cells is the key determinant of H/SIV pathogenesis (31-35). In this view, infection of central memory $CD4^+$ T cells (T_{CM}) is a strong correlate of pathogenesis, while infections in which T_{CM} are relatively spared (i.e., SIV-infected SMs or HIV-infected "non-progressors") are typically non-pathogenic (36-38).

While the direct role of HIV in infecting and killing $CD4^+$ T cells is clearly central to HIV/AIDS pathogenesis, several "indirect" mechanisms of immune deficiency have been described, including chronic immune activation and inflammation, bystander death of

uninfected cells, and ineffective T cell regeneration (reviewed in (39)). The pathogenic role of immune activation is confirmed by the observations that HIV-infected individuals with undetectable viremia but significant immune activation can progress to AIDS (40) and that individuals with preserved $CD4^+$ T cell counts despite persistently high viremia (i.e., viremic non-progressors) show limited immune activation (41). Even prior to infection, systemic immune activation is a possible determinant of viral acquisition (42-45).

A hallmark of HIV/AIDS pathogenesis is the loss of mucosal immune integrity with consequent translocation of microbial products from the intestinal lumen to the portal and systemic circulation, where they cause persistent innate immune activation (46). Recent advances in this area include the observations that pathogenic SIV infection is associated with major changes in the intestinal virome (47) and that probiotic/prebiotic supplementation of antiretroviral treatment improves the gastrointestinal immunity in SIV-infected macaques (48).

An additional important feature of pathogenic H/SIV infections is the functional and structural impairment of lymph nodes due to (i) virus trapping in follicular dendritic cells and virus replication in $CD4^+$ follicular T helper cells (35, 49, 50), (ii) disruption of the fibroblastic reticular network with increased collagen deposition (51), and (iii) failure of $CD4^+$ T cell homeostasis (51). In SIV-infected macaques, the structural impairment of lymph nodes can be partially restored by the administration of TNF-a antagonists during the acute phase of infection (52).

HIV treatment and cure

The advent of antiretroviral therapy (ART) has greatly reduced the mortality and morbidity associated with HIV infection due to its ability to dramatically suppress HIV viral replication and prevent progression to AIDS. Typical ART comprises of a combination of two or more drugs of the following classes: nucleoside or non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors and entry inhibitors (53). A growing body of evidence suggests that early initiation of ART regardless of CD4 counts can prevent transmission of HIV and leads to better immune outcomes (54, 55). Recently, antiretrovirals have been used as tools to prevent acquisition of infection in the form of post-exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) (56-59).

While ART results in dramatically reduced active viral replication, the currently available treatments do not achieve a full recovery from the immune dysfunction induced by the virus (60, 61). Even under ART, persistent virus in blood and tissues positively correlate with markers of immune and low CD4 T cell counts (62). Many studies have shown that residual immune activation and/or incomplete immune reconstitution occur, at various levels of severity, in ART-treated HIV-infected individuals, and are involved in the development of the so-called "end-organ disease" which includes cardiovascular disease, HIV-associated neurocognitive dysfunction, metabolic and kidney abnormalities, bone disease, and others (reviewed in (63)). Moreover, ART does not lead to the eradication of virus and soon after ART interruption there is a rapid rebound of virus (64). This is due to

the establishment of latently infected cells that are long-lived, invisible to the immune system and unaffected by ART (65, 66). Studies in macaque have demonstrated that the latent reservoir is established just days after infection (67). Several studies show that long-lived resting memory CD4 T are the main contributors to this reservoir (reviewed in (68)). Recent studies have also suggested that T memory stem cells (T_{scm}) may be another subset that when infected serve as a long-term virus reservoir (69, 70). For these reasons ART treatment, as it is available now, does not represent a cure.

Several innovative approaches have been proposed for the eradication of virus fuelled by some instances of success under special conditions (71). Chief among these are inducing the reactivation of latent virus by administering reacting agents such as histone de-actylase (HDAC) inhibitors. Following the reversal of latency cytotoxic cells are required to recognize and kill these infected cells. This will undoubtedly require boosting of cellular antiviral immune responses as most viruses in latent cells are resistant to preexisting antiviral responses (72). Further studies that lead to an improved understanding of latent reservoirs and CD8 responses capable of eradicating virus from such latently infected cells are critical for the development of a cure.

HIV vaccine development

Vaccine development represents the best long-term hope in combating the HIV pandemic (6). Unfortunately, all classical strategies to develop a vaccine have failed. HIV presents several unique challenges for the development of an effective vaccine (73). These

include: high genetic variability of the virus, no reliable correlates of immune protection, the ability of the virus to establish a latent reservoir and the creation of preferential targets for infection upon activation of the immune system. Unlike other vaccines, the requirements for an HIV vaccine are also vastly different. An ideal HIV vaccine must elicit sterilizing immunity in order to prevent establishment of a latent viral reservoir. Alternatively, a strong vaccine-induced cellular response is also highly desirable and may effectively suppress viral replication to very low levels.

Several recent studies have demonstrated the ability of immunization to elicit strong suppressive cellular immunity in macaque. Live-attenuated SIV vaccines elicit activated effector memory $CD8^+$ T cells in lymph nodes that can suppress or even completely contain early SIV replication at these sites (74). Further, rhesus cytomegalovirus (rCMV)-based vectors expressing SIV antigens confer strong protection from highly pathogenic challenge through the persistent induction of effector $CD8^+$ T cells in mucosal tissues (75). In another vaccine study, macaques expressing "protective" MHC class I alleles (a model for HIV elite controllers) that were immunized against the appropriately MHC-restricted Nef and Vif epitopes potently suppressed virus replication after pathogenic SIV challenge (76). Collectively, these elegant studies directly demonstrate the potential of vaccine-elicited antiviral $CD8^+$ T cell responses. These findings can also be applied to the development of therapeutic vaccines that boost CD8 T cell responses to uncommon, subdominant epitopes and effectively eliminate latently infected cells following reversal of latency (72).

Perhaps the most striking recent advances in our understanding of HIV-specific immune responses are in the area of HIV-specific neutralizing antibodies. A series of breakthrough studies conducted independently in several laboratories have shown that a 10-30% of HIV-infected individuals produce very potent and broadly reactive neutralizing antibodies (bnAbs) (77-83). The naturally occurring bnAbs, in most cases, are produced several years after the initial infection and in the context of chronic virus replication, which they are incapable of suppressing likely due to escape mechanisms (84-86).

Structural studies have revealed the key targets for neutralization in the HIV Envelope protein i.e., CD4 binding sites, the so-called membrane-proximal external region or MPER, and the N-linked glycans in positions N160 and N332 (77-83). These studies have also elucidated some previously unrecognized structural and genetic features of these bnAbs i.e., long complementary determining region-H3 with ~30 aminoacids, high rate of somatic mutation, and the presence of genetic insertions or deletions (77-83). Intriguingly, the "germline" versions of a subset of these bnABs (i.e., those directed against the CD4 binding site) do not seem to recognize HIV Envelope proteins (87), therefore suggesting that a complex, multi-step process of sequential antigenic stimulation of the relevant B cell clones is required.

Viral and antibody evolution studies provide valuable insight into the interplay between antibody lineage development and viral evolution (88). As described by Liao et al., CH103 CD4-binding site antibody develops relatively early in infection and more

significantly- the unmutated common ancestor of CH103 avidly binds the transmitted/founder virus. However, increased neutralizing breadth occurs after significant diversification of viral variants and the induction of further mutations in CH103 lineage antibodies. In contrast, Moore et al. elucidate the case of two HIV-individuals wherein the transmitted/founder virus develop an escape mutation resulting in a glycan-shift from Asn 334 to Asn 332 on the viral envelope; this in turn presents the epitope to stimulate PGT128-like glycan-dependent bnAb in these individuals (89). These reports provide evidence that viral evolution within the infected host facilitates development of bnAbs. Moore et al. also found that while the conserved viral epitope for glycan-dependent bnAB arises quite frequently in infected individuals, this does not guarantee the development of the bnAb in that individual.

Passive transfer experiments in macaques have convincingly demonstrated that HIV-specific bnAbs can provide complete protection against transmission of chimeric SHIVs expressing the HIV envelope glycoprotein (90, 91). These experiments strongly suggest that a vaccine designed to elicit and to maintain such antibodies would protect against HIV. Most recently, Moldt et al. demonstrated that PGT121, one of the most highly potent bnAbs identified so far, is able to provide sterilizing immunity to macaques against high-dose mucosal SHIV challenge after passive administration of only 1 mg/kg concentrations of the Ab (92). This work provides direct evidence that vaccines may only be required to elicit and maintain single digit mg/ml concentrations of serum antibody in order to be effective.

An improved understanding of the structural details and antibody evolution of bnAbs highlight the challenge associated with the design of immunogens capable of specifically and robustly inducing potent bnAbs. Jardine et al. have designed gp120 outer domain immunogens that are able to activate both germline and mature VRC01-class B cells thus providing a viable immunogen that can be tested as a vaccine prime (93). Further elucidation of the genetic, structural, and functional properties of these naturally occurring bnAbs provides essential information to be used for the rational design of novel and more effective immunogens (94). Eliciting bnAbs is also likely to require sequential immunization with engineered immunogens that first activate naïve B cells and then focus evolution of antibodies to conserved epitopes (95).

Immune Responses to HIV

Several components of the host immune system can suppress virus replication *in vivo*, including host-restriction factors, cellular immunity, neutralizing antibodies, and possibly other mechanisms as well. Even CD4 T cells, despite being the main target of infection, mount a significant response to HIV and may contribute to control of the infection to some extent (96, 97). Here I will provide an overview of aspects of the host immune responses that are most relevant to the development of a prophylactic and therapeutic vaccines.

CD8 T cell responses

Cytotoxic CD8 T cells recognize infected cells by the binding of their T cell receptor (TCR) with cognate peptide MHC-I complex on the infected cell (98). The principal mechanism of killing is through the release of cytotoxic granules carrying perforin and granzymes into the target cell that leads to target cell lysis. Another mechanism includes the binding of FAS Ligand (FASL) to FAS expressed on the target cell that results in the activation of caspases and apoptosis.

CD8 T cells inhibit viral replication in HIV infected individuals as evidenced by several findings (99-101). CD8 T cells play an essential role in establishing and maintaining viral set point, at 2-3 logs lower than the peak viremia during acute infection (102). Further, analysis of the viral sequence indicates that HIV is constantly under pressure from CD8 T cells resulting in escape mutants (103, 104). On a population level, genetic analyses of both humans and rhesus macaque have provided evidence that individuals with particular HLA types have predictably worse or better disease outcomes. Humans with HLA-B27 and B57 alleles have delayed progression to AIDS while individuals with HLA-B35 and B7 progress rapidly (105). Polyfunctional CD8 T cell responses are also positively correlated with slower diseases progression (106).

A powerful tool in analyzing the function and mechanism of CD8 T cells in H/SIV infection is the *in vivo* depletion of CD8 T cells by infusion of depleting antibody (107). Previous depletion studies have shown that CD8 depletion is associated with a marked increase in viral load and does not affect, the increase if viral load occurred before

detectable increase in CD4 T cells activation and the life span of productively infected cells during SIV infection of RM (108). Findings from previous CD8 depletion studies suggest that CD8 T cells exert a significant control of viral load by non-cytolytic function either blocking infection of new cells or decreasing production of virus from infected cells (109, 110). Further experiments using the CD8 depletion model can provide a better understanding of the exact mechanisms by which CD8 T cells contribute to suppression of viral replication that can then be used to develop therapeutic interventions to eradicate virus.

Germinal center response

Germinal centers are specialized structures within secondary lymphoid organs that are the site of the T cell-dependent humoral response known as the germinal center (GC) reaction (111). Within GCs, B cells undergo the process of somatic hypermutation (SHM), class switch recombination (CSR) and affinity maturation as they differentiate into high-affinity plasma cells and memory cells (112). The resulting plasma cells and memory B cells produce class-switched, high affinity antibodies specific for the infecting pathogen (113).

Secondary lymphoid structures are broadly divided into T cell zones and B cell zones. Follicular dendritic cells (FDCs) reside within B cell zones and express several complement receptors that allow antigen capture and display (114). Naïve B cells recognize cognate antigen captured by FDCs and migrate towards the inter-follicullar zone where they interact with antigen-specific T cells (115). Following this, some B cells

move towards the medullary chords and differentiate into short-lived plasmablasts (116). Other fully activated B cells upregulate Bcl6, a day later, and migrate back towards the center of the B cell follicle (117). Seven days after infection, the GC is fully established and organized into a dark zone and a light zone. The dark zone is densely packed with rapidly proliferating B cells called centroblasts (118). This proliferation is accompanied with the diversification of the BCRs by rearrangement of Ig genes. Centroblasts can be identified by their high level of expression of CXCR4, the chemokine receptor for CXCL12 produced by reticular cells (119). The production of CXCL12 drives the polarization of GCs and is critical for an efficient GC response(119). The light zone consists of centrocytes ($CXCR4^{lo}$), FDCs, macrophages and most importantly, T follicular helper (T_{FH}) cells. The light zone is the site for selection of high-affinity B cells (120). Re-circulation between the dark and light zone results in several rounds of SHM and affinity maturation (121). Most GC B cells are programmed to die and depend on survival signals from T cells to bypass the apoptosis pathway. The B cell receptor (BCR) allows B cells to bind and internalize antigen which are then processed and presented on the cell surface as peptide:MHC complexes. The higher affinity BCRs are able to capture more antigen which results in higher density of peptide:MHC complexes being presented on their surface. T_{FH} cells within GCs move rapidly within the light zone and form several contacts with GC B cells. The longest engagements occur with B cells that present the highest density of antigen (121-123). B cells that receive the most help from T_{FH} cells survive. These B cells are able to proliferate more upon re-entry to the dark zone, which begins a new cycle of SHM and affinity maturation. GCs are thought to be

oligoclonal (111, 124), however T_{FH} cells are able to migrate to and from different GC resulting in maximal diversification of antigen specificity (122).

T Follicular Helper cell response

In 1986, T cell were first identified within GCs by Velardi et al and by 2000, they were understood to be a distinct CD4 T cell subset crucial to the development of T-dependent antibody response. Today, T follicular helper (T_{FH}) cells are recognized as critical for the development and maintenance of an optimal GC response (125). Bcl6 is the master regulator of T_{FH} cells and as a transcriptional repressor blocks Blimp-1 driven transcription and differentiation (126, 127). Bcl6 is also highly expressed in GC B cells and antagonizes Blimp-1 that drives B cell differentiation to plasma cells thus maintaining the GC B cell state. Dendritic cell mediated priming of T cells begins the activation and differentiation of CD4 T cells into T_{FH} cells (128). Engagement with activated B cells of the same antigen-specificity reinforces Bcl6 upregulation and the T_{FH} differentiation program. In addition, ICOS ligation on the T_{FH} cell surface and IL-6 stimulation are both critical to the differentiation of T_{FH} cells (129). T_{FH} cells and GC B cells have and intimate relationship depending on each other for optimal functioning and maintenance (125, 130). After continued interaction with cognate GC B cells, T_{FH} cells upregulate ICOS, PD1 and the transcriptional factor c-maf all of which define a fully differentiated GC T_{FH} cells (131). Interactions between T_{FH} and GC B cells are mediated via numerous cell-surface molecules: ICOS and ICOSL, SAP and SLAM, CD40L and CD40, and PD1 and PDL1 (132). The ligation of CD40 on the B cell surface drives

activation of activation induced cytidine deaminase (AID), which in turn drives SHM and CSR (133). Finally, IL-21 the key cytokine secreted by T_{FH} cells is also an essential survival signal for GC B cells (134).

A series of important recent studies in both HIV-infected humans and SIV-infected macaques have independently shown that while T_{FH} are a preferential target for direct virus infection they are not depleted as compared to other memory $CD4^+$ T cell subsets (35, 50, 135-137). PD-1, a cell-surface marker often associated with decreased functionality and directly co-related with increased periods of antigen exposure, is a key phenotypic marker of T_{FH} cells. Recent *in vitro* analysis of human T_{FH} cells demonstrates that the ligation of PD1 on T_{FH} cells reduces IL-21 production and ICOS expression suggesting an impaired ability of T_{FH} cells to provide helper function to GC B cells in chronic stages of HIV infection (138). The full extent to which T_{FH} contribute to the generation of HIV-specific bnAbs- in the context of either natural infection of immunization of uninfected individuals- is the object of intense investigation.

GC T_{FH} are the limiting factor for optimal B cell responses (139). Adoptive transfer experiments in mice have shown that transfer of antigen specific T cells leads to the proliferation of GC B cells and spontaneous formation of GCs. Several studies in mice and humans have suggested that excessive T_{FH} cells are associated with auto immunity. The sanroque mice model has aberrant mRNA regulation that leads to the accumulation of ICOS, IFN-g, TNF-a and OX-40 molecules in the cells (140, 141). This in turn leads to accumulation of T_{FH} cells and the occurrence of an SLE like phenotype in the mice. In

humans, studies have found positive correlations between circulating frequencies of $CD4^+CXCR5^+$ T cells and titers of auto antibodies in patients with SLE, rheumatoid arthritis and myasnthia gravis further pointing to a connection between T_{FH} cell numbers and aberrant of regulation of B cell responses (142-145).

The consequences of dysregulated or dysfunctional T_{FH} cells in HIV infection are manifold (133, 146). Firstly, in combination with high antigen availability, the high frequency of T_{FH} cells could lower the threshold for selection among competing B cells in the GC resulting in low quality antibodies. A loss of function of T_{FH} cells post infection could also lead to poor survival or differentiation signals to B cells. Secondly, as the preferred site of infection and replication, a high frequency and high-activated status of T_{FH} cells leads to higher production of virus further driving overall immune activation. Consequently, the regulation of T_{FH} cell number and function in natural infection or in response to immunization is of special interest to the field.

T Regulatory Cells

T regulatory cells (T_{REG}) regulate several aspects of the immune system including activation, proliferation and effector functions and ultimately contribute to the maintenance of self-tolerance and immune homeostasis (147, 148). Forkhead box p3 (FoxP3) is the master regulator of T_{REG} and in humans, lack of FoxP3 is associated with a severe autoimmune disease called immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (149, 150). Natural T_{REG} cells differentiate in the thymus but peripheral or induced T_{REG} can develop in the periphery at the site of inflammation in a

TGF-b dependent manner (151, 152). T_{REG} cells can mediate their suppressive effects through several contact based or antigen specific and contact independent or antigen non-specific methods (153-155). CTLA-4 mediated suppression is contact specific and it is constitutively expressed on T_{REG} cells. CTLA4 deficient mice have a severe autoimmune phenotype (156-160). T_{REG} cells also express PDL1 and suppress effector T cells function by ligating PD1 (161, 162). The various immunosuppressive cytokines IL-10, TGF-b and IL-35 mediate contact independent suppression (163). T_{REG} cells express high levels of the high affinity IL-2 receptor alpha (CD25) and can also compete for IL-2 binding with other T cells and mediate suppressive effects in an antigen non-specific manner (164, 165).

Overall, T_{REG} cells can have two broad effects on HIV infection (166). As a suppressive cell type, T_{REG} can suppress antigen specific CD4 and CD8 T cells responses thus inhibiting the immune system's ability to control the virus (167). Alternatively, T_{REG} cells may also play a beneficial role by decreasing the level of immune activation that is one of the key drivers of HIV immunopathology (168). Further by controlling the level of proliferation, T_{REG} cells may also suppress viral replication. The role of T_{REG} merits further investigation as T_{REG} could potentially suppress vaccine induced cellular and antibody responses (169).

A population of lymph node resident regulatory T cells called T follicular regulatory (T_{FR}) cells has been recently described in various mouse studies (170, 171). $FoxP3^{+}$ cells with suppressive function have previously been found in human tonsils (172, 173).

Congenically marked thymic derived CD45.2 FoxP3^{+} CD4 cells when transferred into CD45.1 mice gave rise to T_{FR} cells but transfer of thymic derived FOXP3^{-} CD4 cells did not, thus showing that T_{FR} cells differentiate from natural T_{REG} (171). Further, much like T_{FH} cells, the development of T_{FR} cells was found to require interaction with B cells and upregulation of Bcl6, CXCR5, ICOS and PD1 (174). SAP deficient Sh2d1a$^{-/-}$ mice did not develop T_{FH} or T_{FR} cells indicating that like T_{FH} cells, T_{FR} cells also require SAP (Sh2d1a) (171). Further mixed bone-marrow chimera and adoptive transfer studies in mice showed that T_{FR} cells suppress T_{FH} cell proliferation *in vitro* and also control the numbers of GC B cells *in vivo*(175, 176). Wollenberg et al. co-transferred CD4 T cells with FoxP3^{+} T_{REG} cells into TCRa$^{-/-}$ mice and noticed decreased antigen specific antibody titers following immunization (175). The exact mechanisms by which T_{FR} cells mediate suppression is not yet understood although several mechanisms similar to those used by T_{REG} cells have been proposed. These include i) secretion of immunmodulatory cytokines including IL-10 and TGF-b, ii) direct inhibitory signaling through PDL1, iii) competitively disrupting T_{FH} and GC B cell interaction via CTLA4 binding of B7-2 and iv) downregulation of co-stimulatory molecules on T_{FH} and B cells. Further study of this newly discovered population, especially in the context of chronic infection, could lead to vital information about how to fine tune germinal center response to achieve the desired quality of antibodies following immunization.

Immunological Synapse

T cells activation is initiated by the recognition of cognate antigen in association with the MHC complex by the T cell receptor (TCR) at the immunological synapse (177, 178). Antigen recognition is followed by actin reorganization, clustering of TCR and peptide:MHC molecules, co-stimulatory molecules, and various adhesion molecules in a concentric formation knows as the supramolecular activation complex (SMAC) (179-181). The SMAC can further be divided into the central, c- and peripheral, p- SMAC. While the c-SMAC and p-SMAC are organized quickly, the distal or d-SMAC takes several minutes to form (181). Phosphatase CD45, CD43 and CD4 involved in downstream signaling all localize to the d-SMAC (182). This clustering allows intra-cellular signaling molecules to be in close proximity and interact with each other for longer duration (182). The formation of the immunological synapse stabilizes binding between interacting cells and allows for directed release of cytotoxic granules or effector molecules (183). Imaging studies have also found that the immunological synapse and associated cytoskeletal restructuring slows down rapidly migrating T cells allowing cognate cells to interact for longer (181, 184). Interestingly, in the context of HIV infection, immune synapse formation enhances spread of virus via enhanced cell-cell transfer or formation of a virological synapse (185).

Trogocytosis

Trogocytosis is the rapid exchange of membrane and membrane-associated molecules between two cells. Studies have described trogocytosis occurring between T cells and antigen presenting cells (APCs), B cells, NK cells and dendritic cells. Trogocytosis in T

cells is antigen-dependent and requires TCR:pMHC interaction and immunological synapse formation (186). In B cells however, trogocytosis appears to be less antigen specific. Several molecules including TCR, p:MHC, co-stimulatory molecules such as CD80, CD86, OX40 and adhesion molecules such as ICAM-1 and LFA-1 are exchanged during trogocytosis. Blocking either actin polymerization or src kinase activity inhibits trogocytosis again indicating the need for immunological synapse formation (187). Further, trogocytosis occurs more frequently in activated cells than in naïve cells, perhaps as a consequence of higher levels of adhesion molecule expression on activated cells (188). A proposed mechanism of trogocytosis is through the downmodulation and internalization of the TCR that follows its engagement (189). This downmodulation can be coincident with phagocytosis of cell membrane and membrane associated molecules. While the integration of acquired molecules can be visualized by flow cytometry, it is not evident how stable the expression of such acquired molecules is.

The functional consequence of trogocytosis is not well defined (190). In APCs, the acquisition of molecules from the cell surface of T cells may lead to sustained signaling promoting survival and activation. For T cells, the same sustained signaling has been described by Osbourne et al. and can lead to activation and proliferation of the T cell (191). Alternatively, acquisition of the pMHC molecule can allow the T cell to present antigen like an APC and perhaps receive further co-stimulatory signals from other T cells. Another possibility is that these acquired molecules are not stably expressed on the cell surface. In this case, trogocytosis will effectively sequester costimulatory molecules or p:MHC and TCR molecules from cells. Further study of lymphocytes that have

undergone trogocytosis, especially in the context of H/SIV infection may reveal additional means to identify antigen specific and activated cells at the site of immune activation or inflammation.

Chapter Two: Differential impact of *in vivo* CD8$^+$ T lymphocyte depletion in controller versus progressor SIV-infected macaques.*

Ankita Chowdhury[a], Timothy Lee Hayes[a&], Steven E. Bosinger[a], Benton O. Lawson[a], Thomas Vanderford[a], Joern E. Schmitz[b], Mirko Paiardini[a], Michael Betts[c], Ann Chahroudi[a], Jacob D. Estes[d], and Guido Silvestri[a#]

[a]Emory Vaccine Center and Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329

[b] Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.

[c]Institute for Immunology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104; Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

[d]AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory, Frederick, Maryland, USA.

[&]Present Address: Bayer HealthCare, 455 Mission Bay Blvd S, San Francisco, CA 94158

*Originally published in *The Journal of Virology*. Chowdhury et al. Differential Impact of In Vivo CD8+ T Lymphocyte Depletion in Controller versus Progressor Simian Immunodeficiency Virus-Infected Macaques. J. Virology. 2015 . 89(17):8677-86

Available at: http://jvi.asm.org/content/early/2015/06/05/JVI.00869-15

Abstract

Numerous studies have demonstrated that $CD8^+$ T lymphocytes suppress virus replication during HIV/SIV infection. However, the mechanisms underlying this activity of T cells remain incompletely understood. Here we conducted $CD8^+$ T lymphocyte depletion in 15 rhesus macaques (RMs) infected i.v. with SIV_{mac239}. At day 70 post-infection the animals (10 progressors with high viremia and 5 controllers with low viremia) were CD8-depleted by i.v. administration of the antibody M-T807R1. As expected, CD8 depletion resulted in increased virus replication, more prominently in controllers as compared to progressors, which correlated inversely with pre-depletion viremia. Of note, the feature of $CD8^+$ T cells pre-depletion that correlated best with the increase in viremia post-depletion was the level of $CD8^+$T-bet^+ cells. We next found that CD8 depletion resulted in a homogenous increase of SIV-RNA in superficial and mesenteric lymph nodes, spleen, and the gastro-intestinal tract of both controllers and progressors. Interestingly, the level of SIV-DNA increased post-depletion in both central-memory (T_{CM}) and effector-memory (T_{EM}) $CD4^+$ T cells in progressor RMs, but decreased in the $CD4^+$ T_{CM} of 4 out of 5 controllers. Finally, we found that CD8 depletion is associated with a greater increase in $CD4^+$ T cell activation (measured by Ki-67 expression) in controllers as compared to progressors. Overall, these data reveal a differential impact of $CD8^+$ T lymphocyte depletion between controller and progressor SIV-infected RMs, thus emphasizing the complexity of the *in vivo* antiviral role of $CD8^+$ T cells.

Introduction

Several lines of evidence indicate that $CD8^+$ T lymphocytes mediate control of virus replication during both HIV infection of humans and SIV infection of rhesus macaques (RMs). First, the post-peak decline of viremia in acute HIV infection is coincident with the expansion of HIV-specific T cells (192, 193). Second, during both acute and chronic HIV/SIV infection, immune pressure mediated by HIV/SIV-specific $CD8^+$ T cells is manifested by viral escape mutations (194). Third, there is a clear association between the presence of certain MHC class I alleles and disease progression during both HIV infection of humans and SIV infection of RMs (195, 196). Fourth, HIV-1-infected individuals with polyfunctional HIV-1-specific T cells appear to progress less rapidly compared to those whose T cells have more limited functionality (106). While compelling, all these studies are correlative in nature and fail to establish a direct cause-effect relationship. In this context, the most convincing evidence for a direct effect of $CD8^+$ T lymphocytes in suppressing virus replication came from a series of studies in which these cells were depleted *in vivo* in SIV-infected RMs (107, 108, 197, 198). These studies clearly demonstrated that antibody-mediated *in vivo* depletion of $CD8^+$ T lymphocytes is consistently associated with increased virus replication and faster disease progression (199). Despite this strong evidence indicating that $CD8^+$ T lymphocytes suppress virus replication during HIV/SIV infections, these cells ultimately fail to prevent disease progression in the vast majority of HIV-infected individuals and SIV-infected RMs.

The mechanisms by which $CD8^+$ T lymphocytes exert an anti-viral effect *in vivo* are still incompletely understood. Conceivably, these mechanisms can be summarized by two major categories: (i) $CD8^+$ T cells may reduce production of virions on a per cell basis by either direct killing of infected cells or by decreasing the rate of virus production via non-cytolytic mechanisms, and/or (ii) $CD8^+$ T cells may reduce the number of productively infected cells by either inhibiting the spread of infection (i.e., via production of β-chemokines or other cytokines) or by limiting the number of targets (i.e., activated $CD4^+$ T cells) available for infection. While the exact *in vivo* contribution of these non-mutually exclusive antiviral effects by $CD8^+$ T lymphocytes has not yet been defined, there is preliminary evidence that they all may be involved (101, 110, 200, 201). Further, there are several basic aspects of how depletion of $CD8^+$ T lymphocytes affects SIV replication that have not yet been fully elucidated. These aspects include (i) the kinetics of virus replication post-CD8 depletion in progressor (i.e., high viremia) vs. controller (i.e., low viremia) animals; (ii) the anatomic location of productively infected cells that support increased viremia post CD8 depletion; (iii) how CD8 depletion impacts the frequency of infected cells within the main memory $CD4^+$ T cell subsets; (iv) the features of the SIV-specific $CD8^+$ T cell response prior to depletion that correlate best with the increase of virus replication post-depletion; and (v) the impact of $CD8^+$ T lymphocyte depletion on $CD4^+$ T cell activation.

In this study, we set out to investigate these aspects of the antiviral effect of $CD8^+$ T lymphocytes in 15 SIV-infected RMs (10 progressors and 5 controllers) that underwent

$CD8^+$ T lymphocyte depletion at day 70 post-infection. The main results of this study are that (i) $CD8^+$ T lymphocyte depletion is followed by an increase in virus replication that was more prominent in SIV-infected controllers as compared to progressors, and that this increase in virus replication was relatively homogenous in various lymphoid organs and tissues, (ii) the levels of T-bet in $CD8^+$ T cells prior to depletion predicted the increase in virus replication post-depletion, (iii) the levels of SIV-DNA in central-memory (T_{CM}) $CD4^+$ T cells increased post-depletion in progressor RMs, but decreased in controllers, and (iv) $CD8^+$ T lymphocyte depletion was associated with a greater increase in $CD4^+$ T cell activation in controllers as compared to progressors. We concluded that these data reveal a complex role of $CD8^+$ T lymphocytes in controlling virus replication in SIV-infected RMs that includes significant differences between controllers and progressors.

Materials and Methods

Animals. Eighteen female adult rhesus macaques were infected i.v. with 3,000 $TCID_{50}$ of SIV_{mac239}. All RMs were Mamu-B*08 and -B*17 negative; three Mamu-A*01 positive RMs were assigned to each experimental group and two Mamu-A*01 RMs were assigned to the control group. Five animals each were sacrificed at day 3, day 7, and day 14 post CD8 depletion. Mock depleted RMs were sacrificed 7 days post-depletion. Blood and tissues were collected throughout the study period, and plasma viral loads were monitored on a weekly basis. All animals were housed at the Yerkes National Primate Research Center and maintained in accordance with NIH guidelines. Studies were approved by the Emory University Institutional Animal Care and Use Committee.

$CD8^+$ T lymphocyte depletion. Ten weeks after SIV infection, 15 RMs were depleted of $CD8^+$ T lymphocytes by i.v. treatment with 50 mg/kg of the rhesus recombinant mAb M-T807R1 (National Institutes of Health Nonhuman Primate Reagent Resource). Three non-depleted control animals were mock depleted with a primatized control IgG1 OKT3 antibody reactive against the human CD3 molecule (NIH Nonhuman Primate Resource Reagent). The efficacy of the depletion in blood was determined by flow cytometric analysis and complete blood cell counts at multiple time points after administration of the depleting reagent. Depletion efficiency in tissues was determined by flow cytometric analysis as a fraction of $CD8^+$ T cells from samples obtained pre-depletion.

Sample collection and processing. Peripheral blood mononuclear cells (PBMC) were isolated from blood by gradient density centrifugation (Ficoll). Lymphocytes were isolated from freshly obtained lymph node and rectal biopsies by passing them through a 70 μm cell strainer and lysing red blood cells with ACK lysis buffer (Life Technologies).

Immunophenotyping, cytokine responses and flow cytometry. Multicolor flow cytometric analysis was performed on lymphocytes isolated from peripheral blood and tissues according to standard procedures. The Abs used were as follows: anti-CD4 APC-Cy7 (clone OKT4; BioLegend), anti-CD4 Pacific Blue (clone OKT4; BioLegend), anti-CD8 QDot 705 (clone 3B5; Invitrogen), anti-CD8 Brilliant Violet 711 (clone RPA-T8; BioLegend), Ki-67 FITC (clone B56; BD Biosciences), anti-CD3 Pacific Blue (clone SP34-2; BD Biosciences), anti-CD3 APC-Cy7 (clone SP34-2; BD Biosciences), anti-CD95 PE-Cy5 (clone DX2; BioLegend), anti-CCR7 (clone 3D12; BD Biosciences), anti-CCR5 (clone 3A9; BD Biosciences), anti-CD28 ECD (clone 28.2; Beckman Coulter), anti-CD28 PE-Cy7 (clone 28.2; eBioscience) anti-CD16 Alexa 700 (clone 3G8; Biolegend) anti-CD56 QDot 605 (clone MEM-188; Invitrogen), anti-CD20 eFluor 650 (clone 2H7; eBioscience), anti-CD62L PE (clone Sk11; BD Biosciences), anti-IL2 (clone RAT; Life technologies), anti-MIP1a PE (clone D21; Fisher), anti-MIP-1b PE (clone 11A3; Fisher), IFNg APC (clone B27, BD Pharmingen), TNF-a Alexa 700 (clone mab 11, BD Pharmingen). Samples assessed for Ki-67 expression were surface stained first with the appropriate Abs, then fixed and permeabilized using BD Perm 2 (BD Pharmingen), and stained intra-cellularly with Ki-67. Cytokine staining was performed on frozen PBMCs after stimulation with pools of 15-mer peptides spanning the sequences of three major antigenic proteins of SIV_{mac239} (gag, pol,

env) as described previously (202). Flow cytometric acquisition and analysis of samples were performed on an LSRII flow cytometer driven by the BD FACSDiva software package (version 6.1.3; BD Biosciences). Analysis of the acquired data was performed using FlowJo software (version 9.6.3; Tree Star). Further statistical analyses were performed using PRISM (GraphPad) and Excel (Microsoft Office 2011) software.

Plasma viral loads. Quantitative real-time reverse-transcriptase (RT)-PCR assay to determine SIV_{mac239} plasma viral load was performed as previously described (203).

Sorting of $CD4^+$ T cell subsets. Freshly isolated PBMC were stained with anti-CD4 Pacific Blue (clone OKT4; BioLegend), anti-CD3 APC-Cy7 (clone SP34-2; BD Biosciences), anti-CD95 PE-Cy5 (clone DX2; BioLegend), anti-CD28 PE-Cy7 (clone 28.2; eBioscience), anti-CD62L PE (clone Sk11; BD Biosciences). $CD3^+CD4^+$ T cells were characterized as either naive ($CD28^+CD95^-CD62L^+$), central memory ($CD28^{+/-}CD95^+CD62L^+$), or effector memory ($CD28^{+/-}CD95^+CD62L^-$) and sorted on a FACS Aria III Cell Sorter (BD Biosciences).

Quantitative PCR for SIV gag DNA. Quantification of SIV_{mac} *gag* DNA from sorted naïve, central memory, and effector memory $CD4^+$ T cells was performed as previously described (204) Simultaneous PCR was performed for monkey albumin gene copy number and used to determine cell number quantifications. Samples with undetectable SIV DNA were assigned a level of half of the lower limit of detection for graphical purposes and statistical analysis.

SIV in situ hybridization and quantitative image analysis. SIV *in situ* hybridization was perfomed on 5-μm tissue sections with SIV-digoxigenin-labeled anti-sense riboprobes as previously described (205). Quantification of SIV-RNA positive cells was performed by counting positive cells detected by NBT/BICP (purple-black color) in ten randomly selected fields totaling a minimum of 120 mm^2.

Statistical Analyses. Statistical analyses were conducted using GraphPad Prism 5.0. Kruskal-Wallis one-way analysis of variance tests were performed to determine differences between groups in all the tissues (Figure 2). Mann-Whitney U tests were used to perform analyses between pre- and post-depletion frequencies (Figure 2, 4, 5). Spearman rank correlation tests were used to analyze all correlations (Figure 2, 3, 4). All p values less than 0.05 were defined as significant.

Results

Experimental design. To investigate the *in vivo* role of $CD8^+$ T lymphocytes as mediators of antiviral immune responses during SIV_{mac239} infection of RMs, we depleted these cells from a cohort of 15 adult Indian RMs. All animals were infected intravenously with 3,000 $TCID_{50}$ of SIV_{mac239} and $CD8^+$ T lymphocytes were depleted by s.c. administration of the primatized monoclonal antibody M-T807R1 at day 70 post-infection. Ten RMs were defined as "progressors" with high viremia at the time of CD8 depletion and 5 RMs were defined as "controllers" due to low viral loads pre-depletion. The study also included three control SIV infected, mock-depleted animals. In all animals, several tissues, including blood, bone marrow (BM), lymph nodes (LN), and rectal mucosa via rectal biopsy (RB) were sampled at multiple time points throughout the study (see Figure 1A for details). The SIV-infected CD8-depleted RMs then underwent elective necropsy at day 3-14 after depletion to extensively investigate the effect of this procedure in various lymphoid tissues and cell subsets. As shown in Figures 1B and 1C for two representative RMs and for the whole group of animals, respectively, treatment with M-T807R1 resulted in depletion of > 99% of circulating $CD8^+$ T cells. As expected based on previous studies, the level of depletion was lower in tissues than in peripheral blood, and ranged between 50-70% in lymph nodes, and between 86-96% in the rectal mucosa (data not shown).

$CD8^+$ T lymphocyte depletion results in a greater increase in SIV viremia in controllers as compared to progressors. The 15 SIV-infected RMs that were part of this study

included ten normal progressors, with viral loads prior to $CD8^+$ T lymphocyte depletion ranging between 22,200 and 17,200,000 SIV-RNA copies/ml of plasma, and five controllers, with viral loads prior to $CD8^+$ T lymphocyte depletion ranging between 50-1,300 SIV-RNA copies/ml of plasma (Figure 2A). Consistent with previous studies, treatment with the CD8 depleting antibody resulted in an increase in viral loads in both groups of animals, while, as expected, no change in the level of viremia was observed in the three animals that were mock-depleted. Interestingly, the increase in viral load was more pronounced in the group of controllers than in the progressor RMs, with an average fold increase of 154 in progressors and 5,018 in controllers. In addition, as shown in Figure 2B, we found a significant inverse correlation between baseline viremia and the fold change in viremia post-CD8 depletion ($p=0.0004$). Overall, these data are consistent with the established hypothesis that $CD8^+$ T lymphocytes exhibit better suppression of virus replication in controller SIV-infected RMs as compared to progressors.

$CD8^+$ T lymphocyte depletion results in a homogenous increase in virus production within lymphoid tissues. While several studies have shown that $CD8^+$ T lymphocyte depletion in SIV-infected RMs results in increased virus replication (6-9), very little data are available with respect to the specific effect of this treatment in different anatomic sites. In this study, we examined the levels of SIV-RNA in five different tissues at necropsy, including mesenteric LNs, superficial LNs, lamina propria of the rectum, lymphoid aggregates in the rectum, and the spleen. As these tissues could not be collected longitudinally in the animals, we used the three mock-depleted animals as controls. As shown in Fig. 2C, the levels of SIV-RNA were consistently higher in CD8 depleted

progressor RMs compared to mock-depleted animals, and this effect was most pronounced in the spleen, mesenteric LN, and lymphoid aggregates of the rectum. Controller RMs also exhibited a trend towards higher levels of virus replication compared to mock-depleted animals, but this difference was only significant in the spleen. Interestingly, the levels of SIV-RNA post-CD8 depletion were similar between controller and progressor RMs in lamina propria of the rectum, while higher levels of virus replication was seen at all other sites examined in progressors compared to controllers. Of note, SIV-RNA was found in both follicles and paracortex (i.e., T cell area) in the lymph nodes of $CD8^+$ T lymphocyte depleted controllers RMs (data not shown), thus consistent with two recent studies linking the controller status with a compartmentalization of SIV in the lymph node follicles (206, 207). Overall, these histological data suggest that $CD8^+$ T lymphocyte depletion is followed by a diffuse increase of SIV replication in several lymphoid organs and tissues.

T-bet expression in $CD8^+$ T cells is the best correlate of the level of viral load increase post depletion. Relatively little is known regarding the specific feature(s) of total and/or SIV-specific $CD8^+$ T cell responses that best correlate with the level of viral rebound after CD8 depletion. To address this issue we examined a large number of $CD8^+$ T cell features in our cohort of SIV-infected progressor and controller RMs and investigated any potential correlation with the observed changes in viremia post-depletion in blood and tissues Specifically, we measured the level of Gag-specific cells by tetramers staining in blood, lymph nodes, and rectum; levels of cells producing IFNg, TNF, IL-2, or MIP1b after Gag-peptide stimulation; and the level of $CD8^+$ T cells expressing Ki-67, T-bet or

Eomesodermin). As shown in Figure 3, the only significant correlation that we observed was between the levels of T-bet expression in $CD8^+$ T cells isolated from both lymph nodes and rectal biopsies before $CD8^+$ T lymphocyte depletion and the level of plasma viremia after depletion (Figure 3A,B). The list of $CD8^+$ T cell features examined before $CD8^+$ T lymphocyte depletion that did not correlate with the level of viremia post-depletion is shown in Figure 3E. Taken together, these data suggest a role for the expression of the transcription factor T-bet in $CD8^+$ T cells of SIV-infected RMs as a predictor of the increase in viremia that follows $CD8^+$ T lymphocyte depletion in these animals. Since the CD8-depleting antibody also leads to depletion of NK cells, we assessed expression of various immunological markers on $CD3^-CD8^+$ NK cells.We found that both T-bet expression on NK cells in lymph nodes and Ki-67 expression on NK cells derived from whole blood correlated with postdepletion viral load (Fig. 3C and D). No other correlations between markers of NK cell activation/differentiation and postdepletion viral load were found (Fig. 3F).

SIV-DNA in $CD4^+$ T_N, T_{CM}, and T_{EM} increases post-depletion in progressor RMs, but not in controllers. HIV/SIV replication occurs at different levels in specific $CD4^+$ T cell subsets (34, 35). Relatively little is known, however, about the role played by $CD8^+$ T lymphocytes in controlling virus replication in the subsets of naïve (T_N), central memory (T_{CM}), and effector memory (T_{EM}) $CD4^+$ T cells. To address this question we sorted $CD4^+$ T_N, T_{CM}, and T_{EM}, as determined based on the expression of CD28, CD95, and CD62L, before and after $CD8^+$ T lymphocyte depletion in the 15 SIV-infected RMs included in this study, and measured the level of cell-associated SIV-DNA by RT-PCR.

As shown in Figure 4A, we found that the levels of SIV-DNA increased almost uniformly in $CD4^+$ T_N, (9/10 animals), T_{CM} (10/10), and T_{EM} (10/10) sorted from progressors, while in controllers the levels of SIV-DNA increased in $CD4^+$,T_{EM} of 4/5 RMs, but only 2/5 and 1/5 RMs for the T_N and T_{CM} subsets, respectively. Of note, we found a significant correlation between SIV plasma viremia and the levels of SIV-DNA in $CD4^+$ T_{EM} before and after $CD8^+$ T lymphocyte depletion (Figure 4B,C), and between plasma viremia and SIV-DNA in $CD4^+$ T_{CM} after $CD8^+$ depletion (Figure 4E). However, no correlation was found between SIV viremia and SIV-DNA levels in $CD4^+$ T_{CM} before $CD8^+$ depletion (Figure 4D). Overall, these data are consistent with the possibility that $CD8^+$ T lymphocyte-mediated control of infection operates through different mechanisms in specific $CD4^+$ T cell subsets.

$CD8^+$ lymphocyte depletion is associated with higher increase of $CD4^+$ T cell activation in controllers than progressors. The increase in virus replication that follows $CD8^+$ lymphocyte depletion in SIV-infected RMs may be, at least in part, the results of increased levels of $CD4^+$ T cell activation that can be caused by factors such as the homeostatic response to $CD8^+$ T cell depletion, increase availability of proliferative cytokines, and reactivation of latent viruses such as CMV (208, 209). To address the potential role of $CD4^+$ T cell activation in determining the levels of virus replication before and after $CD8^+$ lymphocyte depletion, we measured the expression of the proliferation marker Ki-67 in $CD4^+$ T_N, T_{CM}, and T_{EM} isolated from the blood, lymph nodes, and rectal biopsies of our cohort of 15 SIV-infected RMs (Fig 5). As shown in Figure 5, we found that Ki-67 expression remained stable overall in $CD4^+$ T cells of progerssors (and, in fact, even decreased in LN-derived T_{CM}), thus consistent with the fact that, in these

animals, the levels of $CD4^+$ T activation was already very high prior to CD8 depletion. In contrast, the level of Ki67 expression increased in controllers, with a statistically significant difference for both T_{CM} and T_{EM} in peripheral blood, and a similar, albeit non-significant trend in lymph nodes and rectal biopsies. Overall, these data indicate the $CD8^+$ lymphocyte depletion is followed by a more pronounced increase in $CD4^+$ T cell activation in controllers SIV-infected RMs as compared to progressors.

Discussion

There are many lines of scientific evidence supporting the role of $CD8^+$ T lymphocytes in controlling virus replication in the setting of HIV or SIV infection. Perhaps the most convincing observation is that *in vivo* experimental depletion of $CD8^+$ T cells in SIV-infected macaques is consistently followed by a significant increase in the level of virus replication. While this observation has been confirmed in numerous studies (108, 200), there are several aspects of this phenomenon that remain relatively poorly understood. For example, there is a lack of evidence regarding (i) the cellular and anatomic origin of the increased virus replication, (ii) the contribution of cytolytic vs. non–cytolytic mechanisms of virus control (101, 110) (iii) the role played by $CD8^+$ T cells vs. $CD8^+$ NK cells, which are also depleted by this treatment, and (iv) the contribution of the enhanced activation of $CD4^+$ T cells that follows $CD8^+$ T lymphocyte depletion in promoting higher levels of virus replication (199, 210). The current study was aimed at providing some insights into these poorly understood aspects of the antiviral role of $CD8^+$ T lymphocytes during HIV/SIV infections.

The main findings of this study are that (i) $CD8^+$ T lymphocyte depletion was followed by an increase in virus replication that was more prominent in SIV-infected controller as compared to progressor RMs, and was relatively homogenous in various lymphoid organs and tissues, (ii) the levels of T-bet expression in $CD8^+$ T cells prior to depletion was the best predictor of the magnitude of the increase of virus replication post-depletion, (iii) the

levels of SIV-DNA after $CD8^+$ T lymphocyte depletion increased in all $CD4^+$ T cell subsets (i.e., T_N, T_{CM}, and T_{EM}) in progressors, but only in T_{EM} in controllers, with a decline of SIV-DNA in T_{CM} of 4 out of 5 animals, and (iv) $CD8^+$ T lymphocyte depletion was associated with a greater increase in $CD4^+$ T cell activation in controllers as compared to progressors. While some of these results are overall confirmatory of previously published work by us and others, several of the above described findings are novel and deserve particular attention, especially regarding the differential role of $CD8^+$ T lymphocytes in controller vs. progressor SIV-infected RMs.

The observation that the increase in viral load following $CD8^+$ T lymphocyte depletion is more pronounced when virus replication is lower prior to depletion is consistent with what was observed in two previous studies (108, 198). In this study, however, we report for the first time a strong inverse correlation between pre-depletion viremia and fold-change in viremia post-depletion. Overall these data are quite consistent with the hypothesis that $CD8^+$ T cells are key contributors to the suppression of virus replication that is observed in HIV/SIV-infected "elite controllers" (both humans and rhesus macaques) and is associated with specific MHC class I alleles (195, 211). Interestingly, a detailed histological analysis of several lymphoid tissues and organs revealed that the frequency of virus-producing cells (i.e., SIV-RNA positive by *in situ* hybridization) increases after $CD8^+$ T lymphocyte depletion in a relatively uniform fashion in all examined tissues, with virus found in both B- and T cell areas of lymph nodes in the CD8-depleted controllers. This latter observation is consistent with two recent studies showing a specific virus compartmentalization within lymph nodes of controller vs

progressor SIV-infected RMs (206, 207).

While numerous studies have linked $CD8^+$ T lymphocyte depletion with increased virus replication (107, 108, 197-200), it is not clear what particular function or phenotypic marker of $CD8^+$ T cells is best correlated with this effect. In this study we investigated potential correlations between a number of specific aspects of the $CD8^+$ T cells—both SIV-specific and as "bulk" population—prior to $CD8^+$ T lymphocyte depletion and the observed changes in viral load following depletion. We found that expression of the transcription factor T-bet was the only predictor of the levels of viral load after $CD8^+$ T cell depletion. This is a novel finding that is consistent with a possible role of T-bet as a transcription factor that promotes effector $CD8^+$ T cell functions, including cytotoxic activity and production of multiple cytokines (212, 213), represses the expression of the inhibitory receptor PD-1 which suppresses many $CD8^+$ T cell functions (214), and has been associated with *in vivo* control of virus replication in HIV-infected humans (215, 216). Further studies involving *in vivo* blockade of T-bet-expressing $CD8^+$ T lymphocytes would be needed to further delineate the role of T-bet in controlling virus replication in SIV-infected RMs, particularly with respect to whether the levels of T-bet expression in $CD8^+$ T cells represent a cause or a consequence of prevailing levels of virus replication.

An intriguing novel finding of this study is that the impact of $CD8^+$ T lymphocyte depletion on the relative proportion of SIV-DNA-positive cells within the memory subsets of $CD4^+$ T cells (T_{CM} and T_{EM}) appears to be different in SIV-infected progressor

versus controller RMs. As expected, CD8-depletion resulted in increased levels of viral DNA in all $CD4^+$ T cell compartments of progressors. However, in CD8-depleted controllers, the level of SIV-DNA increased only in T_{EM}, and in fact decreased in T_{CM} of 4 out of 5 animals. Remarkably, this decline of the frequency of SIV-infected $CD4^+$ T_{CM} occurred concomitantly with an average increase in plasma viremia ranging between 1 and 4 .5 logs. Importantly, this decline in SIV-DNA in $CD4^+$ T_{CM} does not appear to be due to trafficking of this cell subset into lymph nodes or the GI tract as the same decline was seen at these sites as well as peripheral blood. At this time we have not identified a mechanism for this surprising finding, but it is tempting to speculate that, in CD8-depleted SIV-infected controllers, a substantial proportion of SIV-infected T_{CM} differentiated to become T_{EM}, perhaps as a result of the increased $CD4^+$ T cell activation, or was killed by the reactivated virus in absence of $CD8^+$ T lymphocytes. This latter possibility would be consistent with the proposed hypothesis that $CD8^+$ T cells may suppress HIV/SIV replication through non-cytolytic mechanisms that act at the level of provirus transcription (217).

Several studies have shown that $CD8^+$ T lymphocyte depletion is followed by an increased fraction of activated/proliferating $CD4^+$ T cells (199, 210). While the relative contribution of this phenomenon to the observed increase in viremia after $CD8^+$ depletion remains unclear, two findings suggest that its role may in fact be minor. First, the increased level of $CD4^+$ T cell activation is observed for the most part at a time when the increase in virus replication has already occurred (200). Second, when the increase of $CD4^+$ T cell activation following $CD8^+$ T lymphocyte depletion is abrogated through

blockade of IL-15 signaling, the increase in SIV replication is still present (199). In the current study, we found that the increase of $CD4^+$ T cell activation, as measured by the proliferation marker Ki-67, was more pronounced in CD8-depleted controllers as compared to progressors, perhaps indicating that, in SIV-infected RMs with high virus replication prior to $CD8^+$ T lymphocyte depletion a maximal level of $CD4^+$ T cell activation was already present in the immune system of these animals.

Overall, the current set of results indicates that experimental depletion of $CD8^+$ T lymphocytes during SIV infection of macaques remains a valuable research tool to investigate the mechanisms by which these cells suppress virus replication *in vivo*. Specifically, we have identified a series of differences between SIV-infected progressor and controller RMs with respect to the impact of CD8 depletion on the virology and immunology of SIV infection that provides novel insights into the *in vivo* function of these cells. Ultimately, a better understanding of the mechanisms responsible for the $CD8^+$ T cell-mediated protection from HIV/SIV replication may result in interventions that most effectively harness these antiviral functions in the setting of prophylactic and therapeutic vaccines for HIV/AIDS.

Acknowledgments

This work was supported primarily by R01-AI090797 to GS. In addition, it was supported by the NIH/NCRR P51RR000165 and is currently supported by the Office of Research Infrastructure Programs / OD P51OD011132 to the Yerkes National Primate Research Center. The Authors also wish to thank the Emory Center for AIDS Research (CFAR) Virology core for their technical support. AC gratefully acknowledges partial support by the American Foundation for AIDS Research (grant 108905-56-RGRL) and by an NICHD Child Health Research Career Development Award (K12 HD072245).

Figures

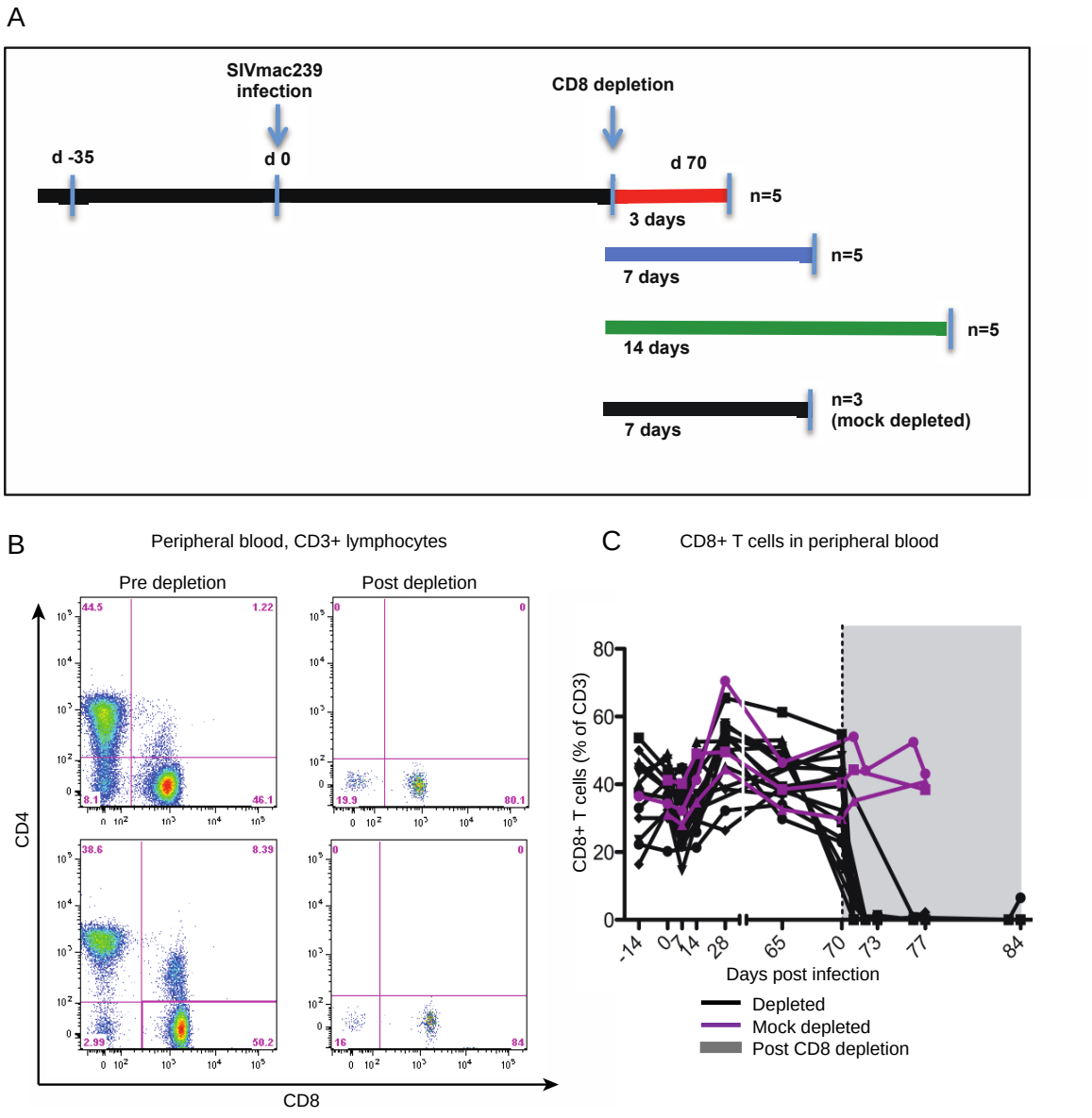


Figure 1. Experimental design and effective depletion of CD8$^+$ T lymphocytes. (A) Study design depicting timeline of SIV infection, antibody-mediated CD8 depletion and necropsy. Eighteen female rhesus macaque were intravenously infected with 3,000

$TCID_{50}$ of $SIVmac_{239}$. CD8 depleting antibody (M-T807R1) was administered at 70 days post infection to 15 RMs. Five CD8-deplet

ed animals each were necropsied 3, 7, and 14 days post depletion. Three RMs were mock depleted using primatized control IgG1 antibody at 70 days post infection and necropsied 7 days post mock-depletion.

(B) Representative flow cytometry plots of live $CD3^+$ T lymphocytes isolated from peripheral blood depicting $CD8^+$ T lymphocyte levels pre-depletion (5 days before depletion) and post-depletion (6 days after depletion).

(C) Longitudinal assessment of $CD8^+$ T cell frequencies (as a percentage of $CD3^+$ T cells) in peripheral blood for all experimentally depleted (black, n=15) and mock depleted (purple, n=3) animals at pre- and post-depleted (shaded grey) time points. Dotted black line indicates administration of depleting antibody.

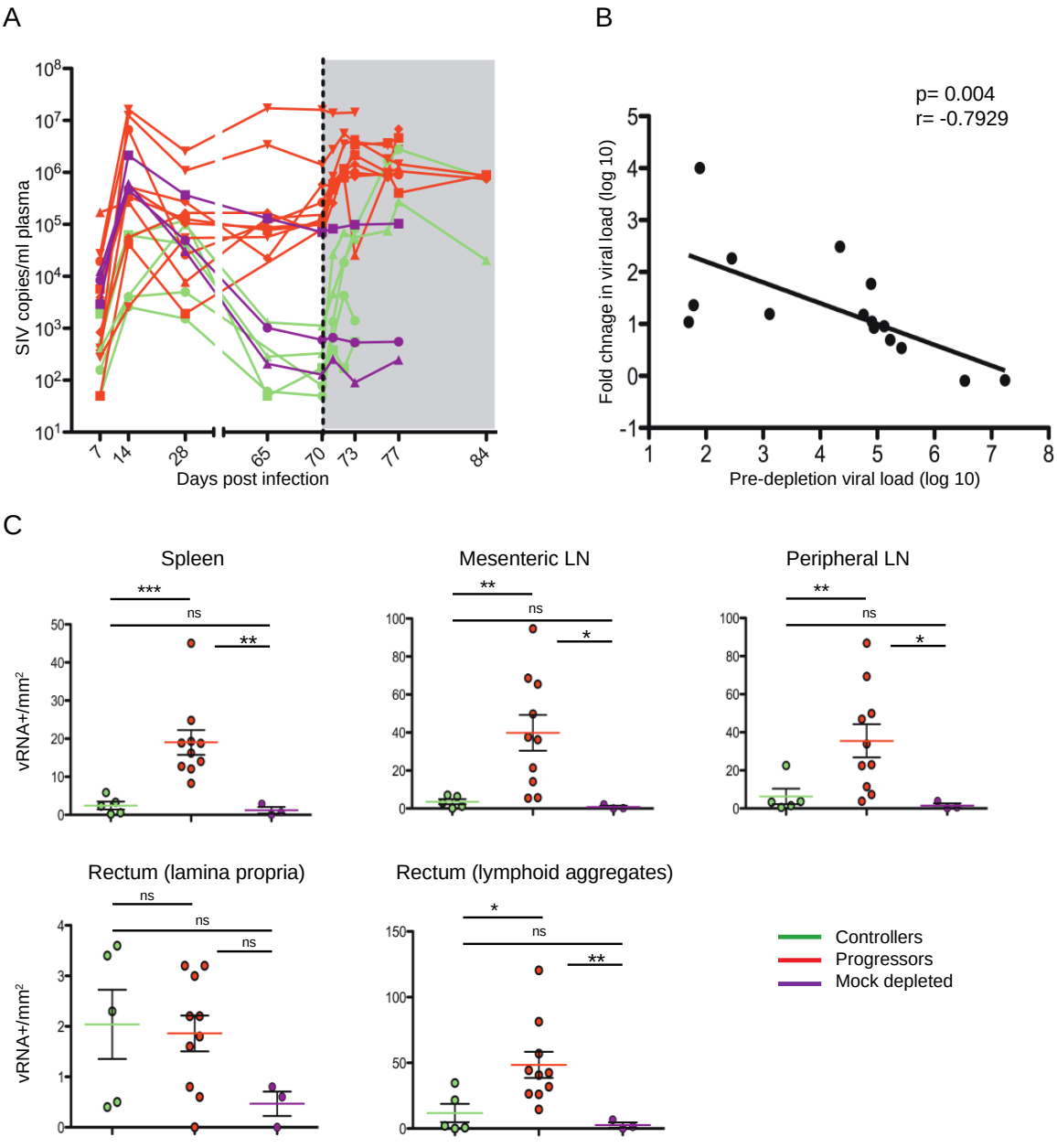
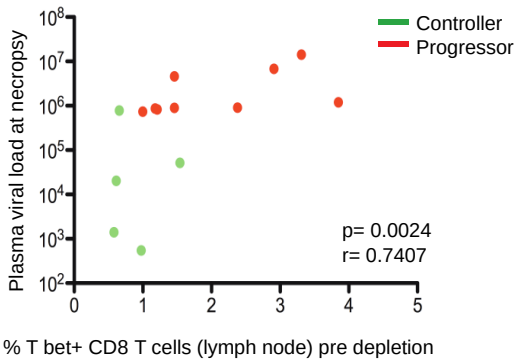
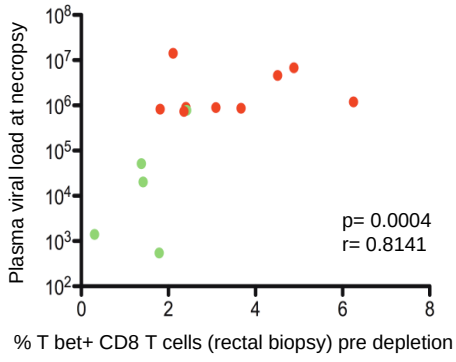

A
SIV copies/ml plasma
Days post infection
B
p= 0.004
r= -0.7929
Fold chnage in viral load (log 10)
Pre-depletion viral load (log 10)
C
Spleen
Mesenteric LN
Peripheral LN
Rectum (lamina propria)
Rectum (lymphoid aggregates)
vRNA+/mm²
Controllers
Progressors
Mock depleted

Figure 2. Plasma and tissue viral loads following CD8 T cell depletion. Plasma viral load measured longitudinally for each animal (green, controllers; red, progressors; purple, mock depleted). Dotted black line indicates administration of depleting antibody. Shaded grey area indicates post-depletion time points. (B) Correlation between fold change in viral load (viral load post-depletion / viral load pre-depletion) with pre-depletion viral load for all experimentally depleted animals. (C) Number of SIV-RNA^{+} cells within tissues of CD8-depleted controllers (green, n=5), progressors (red, n=10) and mock depleted (purple, n=3). Statistical analyses performed using Spearman rank correlation tests, Kruskal-Wallis one-way analysis of variance and Mann-Whitney U tests.

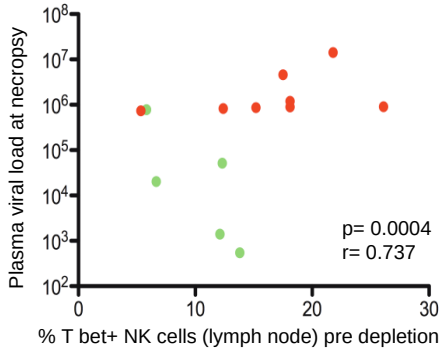
A



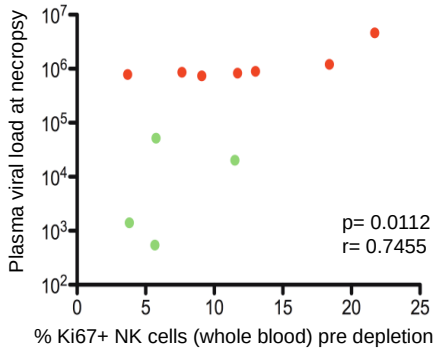
B



C



D



E

Levels of expression within CD8+ T-lymphocytes	Correlation to post depletion plasma viral load
Gag CM9 (Whole Blood)	Not significant
Eomesodermin (Whole Blood)	Not significant
Ki67 (Whole Blood)	Not significant
IFN-g in response to Gag peptide stimulation (Whole Blood)	Not significant
TNF-a in response to Gag peptide stimulation (Whole Blood)	Not significant
MIP-1a/b in response to Gag peptide stimulation (Whole Blood)	Not significant
IL-2 in response to Gag peptide stimulation (Whole Blood)	Not significant
Gag CM9 (Rectal Biopsy)	Not significant
Eomesodermin (Rectal Biopsy)	Not significant
Ki67 (Rectal Biopsy)	Not significant
Gag CM9 (Lymph Node)	Not significant
Eomesodermin (Lymph Node)	Not significant
Ki67 (Lymph Node)	Not significant

F

Levels of expression within CD8+ NK cells	Correlation to post depletion plasma viral load
Gag CM9 (Whole Blood)	Not significant
Eomesodermin (Whole Blood)	Not significant
Gag CM9 (Rectal Biopsy)	Not significant
Eomesodermin (Rectal Biopsy)	Not significant
Ki67 (Rectal Biopsy)	Not significant
Gag CM9 (Lymph Node)	Not significant
Eomesodermin (Lymph Node)	Not significant
Ki67 (Lymph Node)	Not significant

Figure on previous page.

Figure 3. T-bet expression in $CD8^+$ T cells is the best correlate of the level of viral load after CD8 depletion. Correlations between T-bet expressing $CD8^+$ T cells isolated pre-depletion from lymph nodes (A) and rectal biopsies (B) with plasma viral load at necropsy. (C) Levels of expression of several markers on $CD8^+$ T cells were assessed within peripheral blood, lymph node and rectal mucosa. None were found to correlate with plasma viral loads after $CD8^+$ T cell depletion. Controllers are shown in green, progressors in red. Spearman rank correlation tests were used to analyze all correlations.

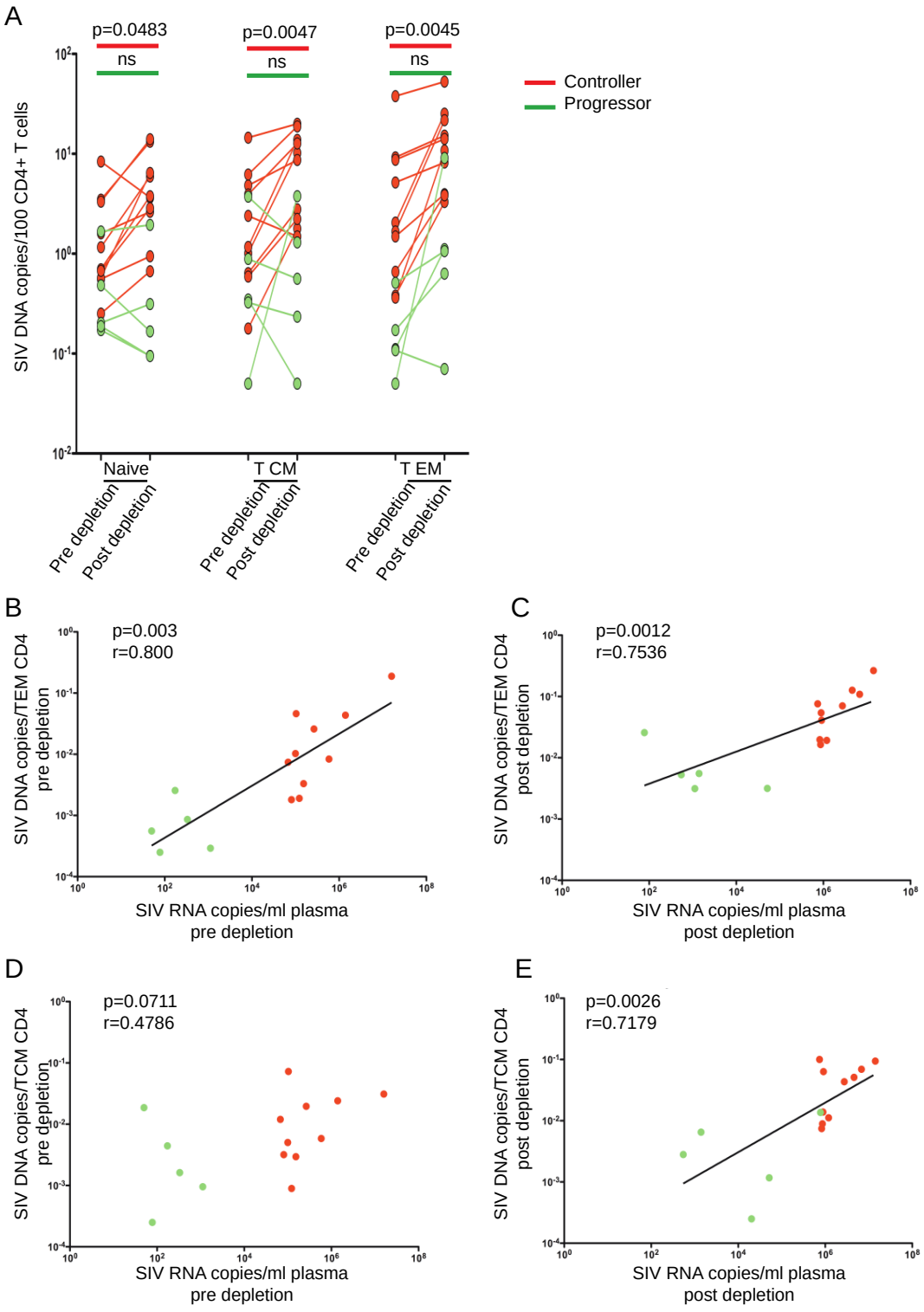

A
p=0.0483
ns
p=0.0047
ns
p=0.0045
ns
Controller
Progressor
SIV DNA copies/100 CD4+ T cells
Naive
T CM
T EM
Pre depletion
Post depletion
B
p=0.003
r=0.800
SIV DNA copies/TEM CD4 pre depletion
SIV RNA copies/ml plasma pre depletion
C
p=0.0012
r=0.7536
SIV DNA copies/TEM CD4 post depletion
SIV RNA copies/ml plasma post depletion
D
p=0.0711
r=0.4786
SIV DNA copies/TCM CD4 pre depletion
SIV RNA copies/ml plasma pre depletion
E
p=0.0026
r=0.7179
SIV DNA copies/TCM CD4 post depletion
SIV RNA copies/ml plasma post depletion

Figure on previous page.

Figure 4. SIV-DNA in CD4$^+$ T_N, T_{CM} and T_{EM} increases post-depletion in progressor but not controller RMs. (A) Comparison of cell-associated SIV-DNA levels in naïve, central memory (T_{CM}) and effector memory (T_{EM}) CD4$^+$ T cells of controllers (green, n=5) and progressors (red, n=10) pre- and post-depletion. (B) Correlations between plasma viral load and cell-associated DNA within T_{EM} (B, C) and T_{CM} (D,E) at pre-depletion and post-depletion time points. Statistical analyses were performed using Mann-Whitney U tests and Spearman rank correlation tests were used to analyze all correlations.

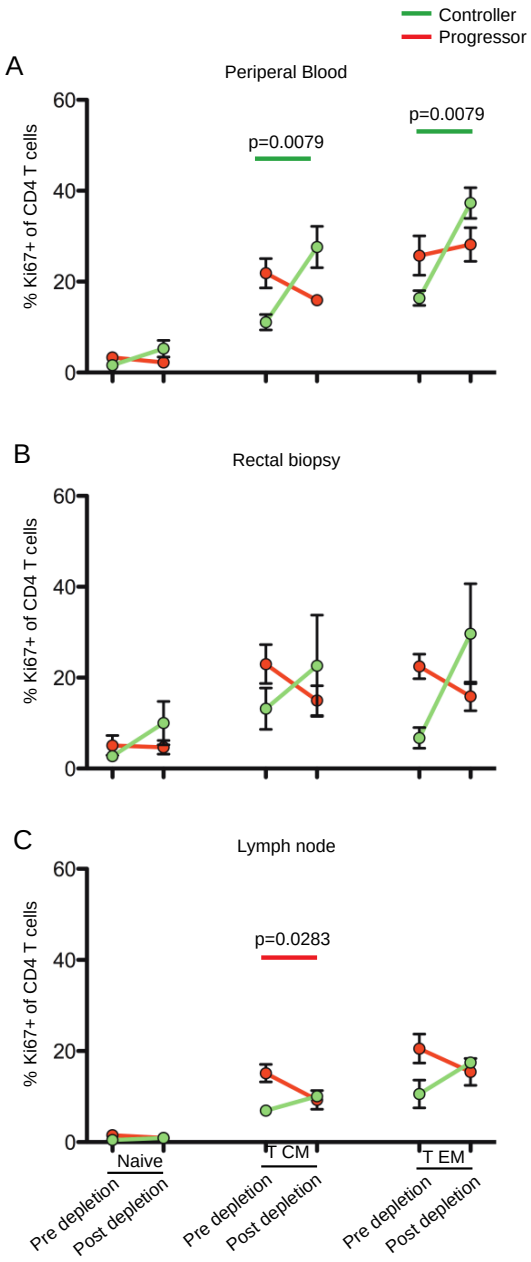


Figure 5. CD8$^+$ T lymphocyte depletion is associated with greater increase in CD4$^+$ T cell activation in controllers than in progressors. Comparative frequency of Ki-67$^+$ CD4$^+$ T cells within naïve, central memory and effector memory CD4$^+$ T cells pre- and post-depletion isolated from peripheral blood (A), rectal mucosa (B) and lymph nodes

(C) of progressors (red, n=10) and controllers (green, n=5). Statistical analyses were performed using Mann-Whitney U tests.

Chapter Three: Decreased T_{FR}/T_{FH} ratio in SIV-infected rhesus macaques may contribute to accumulation of T_{FH} cells in chronic infection[#]

Ankita Chowdhury[*], Perla Maria Estrada Del Rio[*†], Greg K Tharp[*], Ronald P Trible[*], Rama R Amara[*], Ann Chahroudi‡, Gustavo Reyes-Teran[†], Steven E. Bosinger[*], Guido Silvestri[*]

[*]Emory Vaccine Center and Yerkes National Primate Research Center, Emory University, Atlanta, GA

[†] Departamento de Investigación en Enfermedades Infecciosas, Instituto Nacional de Enfermedades Respiratorias, "Ismael Cosio Villegas". México D.F., México

‡ Department of Pediatrics, Emory University School of Medicine, Atlanta, GA

[#]Originally published in *The Journal of Immunology*. Chowdhury et al. 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. J. Immunology. 195(7):3237-47.

Available at: http://www.jimmunol.org/content/early/2015/08/20/jimmunol.1402701

Abstract

T follicular helper cells (T_{FH}) are critical for the development and maintenance of germinal centers (GC) and humoral immune responses. During chronic HIV/SIV infection T_{FH} accumulate, possibly as a result of antigen persistence. The HIV/SIV-associated T_{FH} expansion may also reflect lack of regulation by suppressive follicular regulatory $CD4^+$ T cells (T_{FR}). T_{FR} are natural regulatory T cells (T_{REG}) that migrate into the follicle and, similarly to T_{FH}, up-regulate CXCR5, Bcl-6, and PD1. Here we identified T_{FR} as $CD4^+CD25^+FoxP3^+CXCR5^+PD1^{hi}Bcl\text{-}6^+$ within lymph nodes of rhesus macaques (RM) and confirmed their localization within the GC by immunohistochemistry. RNA sequencing showed that T_{FR} exhibit a distinct transcriptional profile with shared features of both T_{FH} and T_{REG}, including intermediate expression of FoxP3, Bcl-6, PRDM1, IL-10, and IL-21. In healthy, SIV-uninfected RM, we observed a negative correlation between frequencies of T_{FR} and both T_{FH} and GC B-cells as well as levels of $CD4^+$ T cell proliferation. Following SIV infection, the T_{FR}/T_{FH} ratio was reduced with no change in the frequency of T_{REG} or T_{FR} within the total $CD4^+$ T cell pool. Finally, we examined whether higher levels of direct virus infection of T_{FR} were responsible for their relative depletion post-SIV infection. We found that T_{FH}, T_{FR} and T_{REG} sorted from SIV- infected RM harbor comparable levels of cell-associated viral DNA. Our data suggests that T_{FR} may contribute to the regulation and proliferation of T_{FH} and GC B-cells *in vivo* and that a decreased T_{FR}/T_{FH} ratio in chronic SIV infection may lead to unchecked expansion of both T_{FH} and GC B-cells.

Introduction

Several key findings over the past few years have energized efforts towards the development of an effective HIV vaccine, including the discovery and characterization of a number of broadly neutralizing antibodies (bnAbs) that develop in a subset of HIV-infected individuals. However, the mechanisms involved in shaping antibody responses to immunization with HIV antigens or natural HIV infection, including the generation of bnAbs remain incompletely understood (133). Importantly, HIV-Env-specific bnAbs develop at relatively late stages of HIV infection, and show peculiar genetic and molecular features, including a high level of divergence from germ line predecessors, which indicates that they are the products of extensive somatic hyper-mutation within germinal centers (GCs), as well as the presence of unusually long CDR3 regions (218). Perplexingly, there appear to be no direct or predictable routes to the development of these bnAbs from the germ line predecessors, and it remains unclear whether this process is driven by antigenic mutations and/or escape as opposed to specific intrinsic aspects of the B-cell or T-helper cell response (219). A better understanding of the mechanisms responsible for the development of bnAbs is crucial to harness this type of immunity for HIV prevention and therapy in humans.

T follicular helper cells (T_{FH}) are critical for the development and maintenance of GCs and competition for survival signals from T_{FH} via molecules such as CD40L and IL-21 is

thought to be a key mechanism of selection of high affinity B-cells (125). The regulation of T_{FH} frequency and function, is vital to the quality of the humoral immune response (139). While the presence of too few T_{FH} may lead to abortive GC formation and defective B-cell responses, an over-expansion is associated with the prevalence of autoantibodies (220, 221). It is possible that an expansion of T_{FH} also lowers the selection pressure on GC B-cells and leads to the emergence of low-affinity B-cells (222).

Several studies have shown that T_{FH} accumulate during the chronic stages of HIV/SIV infection. This accumulation occurs even though these cells support high levels of viral replication and represent an important component of the persistent virus reservoir under anti-retroviral therapy (136). The chronic expansion of T_{FH} in the case of HIV/SIV infection with persistent virus replication may be a direct result of antigenic persistence. As expected, HIV/SIV-associated expansion of T_{FH} is associated with dysregulation of B-cell responses with ineffective memory cell formation and hyper-gammaglobulinemia (137),(223). Whether and to what extent this T_{FH} expansion is also related to a deficit in the physiologic regulation of specific T_{FH} immune response within the lymph nodes remains unknown. However, this possibility would be consistent with the well-known observation that the chronic phase of pathogenic HIV/SIV infections is associated with a state of generalized immune activation that is resistant to the normal mechanisms of immune regulation.

Under normal circumstances, regulation of T_{FH} function is mediated at least in part by a recently described subset of $CD4^+$ T cells termed T follicular regulatory (T_{FR}) cells.

T_{FR} are thought to develop from thymic derived regulatory T cells (T_{REG}) that express lineage-associated markers such as FoxP3, CD25, as well as low levels of CD127. These T_{FR} migrate into the follicles of lymph nodes by virtue of their expression of CXCR5 (and down-modulation of CCR7) and, similarly to T_{FH}, express high levels of Bcl-6 and PD1 (224) (171). Of note, the role of T_{FR} in the immuno-pathogenesis of HIV/SIV infections is currently unknown, both in terms of ability to negatively regulate HIV-specific B-cell responses (including, potentially, the production of bnAbs) and to suppress local virus-induced immune activation. Indeed, none of the previous reports on T_{FH} dynamics in the context of SIV or HIV infection have distinguished between cells that either do or do not co-express T_{REG}-associated markers. Thus the T_{FR} subset within the broader $CXCR5^{+}Bcl\text{-}6^{+}PD1^{+}$ is not fully characterized in the setting of HIV/SIV infection.

In this study, we described and characterized phenotypically, histologically, and genomically the T_{FR} population that is found within the GCs of rhesus macaques (RM) and express markers associated with both T_{FH} and T_{REG} cells. The hypothesis that T_{FR} play a suppressive role *in vivo* is supported by the observation that their frequency is inversely correlated with both the levels of T_{FH} and GC B-cells and the percentage of proliferating $CD4^{+}$ T cells. In the setting of SIV infection, we found that T_{FR} show a slow *in vivo* proliferative response after the initial infection and exhibit only a small increase in their frequency within the total $CD4^{+}$ T cell pool during the chronic phase. In conjunction with the large expansion of T_{FH} observed following SIV infection, this phenomenon leads to a significantly decreased T_{FR}/T_{FH} ratio in the lymph nodes of chronically SIV-infected

RM. These data suggest that, during SIV infection, a lack of T_{FR} expansion may allow for a progressive accumulation of T_{FH} cells in the lymph nodes of chronically infected RM, thus indirectly contributing to the aberrant immune activation that characterizes this pathogenic infection.

Materials and Methods

Animals. The study involved a total of 40 Indian origin female rhesus macaques (RM) divided as follows: (i) Ten healthy, unvaccinated and SIV-uninfected RM; (ii) Ten healthy, SIV-immunized but SIV-uninfected RM; (iii) Eleven unvaccinated SIV-infected animals; and (iv) Nine vaccinated and SIV-infected RM. Animals were vaccinated with a SIVmac239 Gag-, Pol-, and Env-expressing DNA vaccine with inactivating mutations in proteases, half of which also co-expressed GM-CSF. These were followed by two boosts of a SIVmac239 Gag-, Pol-, and Env-expressing MVA vaccine as described previously (225). All infections were a result of SIVsmE660 intra-vaginal challenge at $2.06X10^4$ $TCID_{50}$ grown in RM peripheral blood mononuclear cells (PBMC). Lymph node biopsies were collected for measurement of a number of immunological parameters at day -35 prior to infection and days 14 and 168 after infection (i.e., acute and chronic phase, respectively). Spleen and lymph nodes were collected at necropsies performed at six months post infection. All animals were housed at Yerkes National Primate Center at Emory University and were cared for in accordance with National Institute of Health guidelines and following protocols approved by the Institutional Animal Care and Use Committee.

Tissue processing. Lymphocytes were isolated from freshly obtained lymph node and spleens by passing homogenized tissue through a 70-μm cell strainer and lysing blood cells with ACK Lysis buffer. Tissue collection was performed as previously described

(226). Cells to be later used for sorting were cryopreserved at -80 degrees C for a short term in FBS media containing 10% DMSO.

Immunophenotyping and flow cytometry. Multi-color flow cytometric analysis was performed on mononuclear cells isolated from blood and lymph nodes according to standard procedures using monoclonal antibodies directed against RM markers and human markers that also cross-react with the same markers in RM. For optimum staining of intra-cellular markers, permeabilization of cells using the eBioscience FoxP3-permeabalization buffer was performed as recommended by the manufacturers. Pre-determined optimal concentrations of the following antibodies and reagents were used: CD3-Alexa700 (clone SP34-2), CD4-Allophycocyanin-Cy7 (clone OKT-4), Bcl-6-PeTexasRed (clone K112-91), Ki67- FITC (clone B56), CCR5-PE (clone 3A9), CTLA4-BV421 (clone BNI3) from BD, CXCR5-PerCP eFlour 710 (clone MU5BEE), PD1-PeCy7 (clone J105), CD127-PeCy5 (clone eBio-RDR5) from eBioscience and CD20-BV650 or *PE-CF594* (clone 2H7), CD25-BV711 (clone BC96), Helios-FITC (clone 22F6) and FoxP3-Allophycocyanin (clone 150D) from Biolegend, and Live/Dead Fixable Aqua from Invitrogen. Flow cytometric data were acquired using LSRII flow cytometer using BD's FACS DiVA software. Acquired data were analyzed using Flow Jo version9.3.2 following the gating strategy described in Figure1. Further analyses were performed using PRISM (GraphPad) and Excel (Microsoft Office 2011) software.

Cell Sorting. Cryopreserved cells were thawed in a 37 degree C water bath and rested overnight for 8-10 hours and then stained for sorting. Splenocytes from 5 SIV-uninfected

and unvaccinated RM as well as 5 unvaccinated SIV-infected animals were used for sorting of T_{FH}, T_{FR}, and T_{REG}. Cell populations were sorted using FACS Aria II flow cytometer. Cells were first gated based on light scatter followed by positive gating on cells negative for Live/Dead Fixable Aqua and positive for CD3 and CD4. After collecting bulk $CD4^+$ cells the following three populations were collected: T_{FR} ($CXCR5^+PD1^{hi}CD127\text{-}CD25^+$), T_{FH} ($CXCR5^+PD1^{hi}CD127^{+/}\text{-}CD25\text{-}$) and T_{REG} ($CXCR5^{+/}\text{-}PD1^{lo/int}CD127\text{-}CD25^+$).

Immunohistochemistry and Confocal Microscopy. Immunohistochemstry was performed on 5-mm tissue sections mounted on glass-slides, which were deparaffinized and rehydrated with double-distilled H_2O. Antigen retrieval was performed in 1xDako Target Retrieval Solution (pH 6.0) in a pressure cooker heating slides to 122 degrees C for 30s. Slides were then rinsed in ddH_2O and incubated for 10 minutes using Dako Protein block. Slides were then incubated with rabbit anti-CD4(1:200), mouse anti-FoxP3 (1:100) and goat anti-PD1 (1:500) for 1 hour at room temperature. Next, slides were washed in TBS with 0.05% Tween-20. Slides were then incubated for an hour in the dark with secondary antibody cocktail containing donkey anti-rabbit Alexa 488 (1:500), donkey anti-mouse Alexa-594 (1:500) and donkey anti-goat Alexa 647 (1:500). After washing in TBS with 0.05% Tween-20, Prolong Gold with DAPI was applied to all the slides. Confocal microscope images were obtained using Olympus FV10i® Confocal Microscope with CellSens® 1.9 Digital Imaging software.

Quantitative PCR for SIV gag DNA. Cell-associated viral DNA was measured in sorted cell populations from RM lymph nodes by RT-PCR as previously described(200, 227, 228).

RNA-Seq Library Preparation. Total RNA was prepared using the QIAGEN RNEasy Micro Kit. Libraries were generated using the CLONTECH SMARTer HV kit, barcoding and sequencing primers were added using NexteraXT DNA kit. Libraries were validated by microelectrophoresis, quantified, pooled and clustered on Illumina TruSeq v3 flowcell. Clustered flowcell was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions.

RNA-Seq Data Analysis. RNA-Seq data were submitted to the GEO repository at the National Center for Biotechnology Information (NCBI). RNA-Seq data were aligned to a provisional assembly of Indian Macaca mulatta (MaSuRCA rhesus assembly v.7_20130927) using STAR version 2.3.0e (229) (230). Transcripts were annotated using the provisional UNMC annotation v7.6. Transcript assembly, abundance estimates, and differential expression analysis was performed using Cufflinks v2.1.1 and Cuffdiff (231). All samples had read counts > 12000000 and unique mapping percentages in the range of 63 – 76 %; no samples were excluded from the analysis for technical issues. Differentially expressed genes were defined by pair-wise comparison of each phenotype. Differential gene lists were uploaded to Ingenuity Pathway Analysis software (v1.0 Ingenuity Systems, http://www.ingenuity.com/) and pathways with significant enrichment by Fisher's Exact test and the Benjamini-Hochberg multiple testing

correction. Heat maps and other visualization were generated using Partek Genomics Suite v6.6. RNA-seq data is publically available at the GEO repositories (accession number: GSE69756, URL: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69756).

RT-PCR validation of RNA sequencing data. Total RNA was prepared using the QIAGEN RNEasy Micro Kit from sorted T_{FR}, T_{FH} and T_{REG} cells. RNA quantity was measured using Nanodrop analysis and reverse transcribed as previously described for RNA sequencing. Finally, 0.1 µl of cDNA was used for real time SYBR green PCR analysis using an ABI 7900 HT Real-time PCR instrument (Applied Biosystems). Primer sequences for PCR were GAPDH: Fwd5′- GCACCACCAACTGCTTAGCAC-3′, Rev 5′- TCTTCTGGGTGGCAGTGATG-3′. IL2RA: Fwd5′- GGCTTCATTTTCCCACGGT-3′, Rev 5′- GCAGCTGGCGGACCAA-3′. IL6R: Fwd5′- TTCGGCCGGACTGTTCTG-3′, Rev 5′- GCACCCCATCTCCGACG-3′. SLAMF6: Fwd5′- TGG AAC ATC TCT TGC CTT CAT AG-3′, Rev 5′- GTT GCT GAG TTT CAG GGA GTA G-3′. SAP/SH2D1A: Fwd5′- CTC TGC AGT ATC CAG TTG AGA AG-3′, Rev 5′- GGC TTT CAG GCA GAC ATC A-3′. XIAP: Fwd 5′- GAG GAA CCC TGC CAT GTA TAG-3′, Rev 5′- GTG TAG TAG AGT CCA GCA CTT G-3′; PRDM1: Fwd 5′- TGT GGT ATT GTC GGG ACT TTG-3′, Rev 5′- GCT TGA GAT TGC TCT GTG TTT G-3′; CCL20: Fwd 5′- GCA ACT TTG ACT GCT GTC TTC-3′, Rev 5′- CAG CAT TGA TGT CAC AGG TTT C-3′; PD1: Fwd 5′- TCCTTGGCCACTGGTGTTC-3′, Rev 5′- CTTCTCCTGAGGGAAGGAGC-3′; IL10: Fwd 5′- AAGACCCTCAGGCTGAGGCT-3′, Rev 5′- TCCACGGCCTTGCTCTTG-3′; IL21: Fwd 5′-

TGTGAATGACTTGGACCCTGAA-3′,

Rev 5′AAACAGGAAATAGCTGACCACTCA-3′. Relative RNA transcript levels were calculated normalized to primer efficiency and housekeeping gene RNA (GAPDH).

Statistical Analyses. Except for RNA sequencing data, all statistical analyses were conducted using GraphPad Prism 5.0. Comparisons of mean fluorescence intensity between cell populations in uninfected RM were made using Wilcoxon signed rank tests (Fig 2). Man-Whitney U tests were used to compare frequencies of populations in uninfected, acutely infected and chronically infected RM (Figure 4, 5). Spearman rank correlation tests were used to analyze all correlations (Figure 6). All p values less than 0.05 were defined as significant.

Results

T_{FR} are distinct from T_{FH} and T_{REG} and can be found within lymph node GCs in RM. Recent studies of GC T_{FH} have defined these cells based on their surface expression of the chemokine receptor CXCR5 and very high levels of the co-inhibitory receptor PD1 (137). However, a fraction of these canonically defined T_{FH} also express the lineage-specific T_{REG} marker FoxP3 and have been therefore defined as T_{FR} as proposed in (232-234). Here we identified $CD4^+$ T_{FR} by flow cytometry by their co-expression of CXCR5, PD1, FoxP3 and CD25 within lymph nodes of uninfected RM. The gating strategy used to define T_{FH}, T_{FR}, and T_{REG} throughout this study is shown in Figure 1A. Of note, the gating strategy for T_{REG} cells includes both $CXCR5^+$ and CXCR5- cells. To confirm the presence of T_{FR} within GCs we conducted an immuno-histochemistry (IHC) analysis. As shown in Figure 1B, single cells with nuclear expression for FoxP3 and surface expression of and PD1 were identified with GCs of uninfected RM. These T_{FR} can also be readily identified within GCs of SIV-infected RM (Fig 1C). Interestingly, several *bonafide* T_{REG}, identified by their expression of FoxP3 and but not PD1, are visible in the T cell zone just outside the GC (Fig 1C). Presumably, some of these T_{REG} migrate into the GC and up-regulate T_{FH}-like markers along their differentiation pathway to T_{FR}. Figure 1C also shows that, as expected, non-FoxP3 expressing "true" T_{FH} are also seen within GCs of the same animals.

T_{FR} express markers of both T_{FH} and T_{REG} differentiation. We next performed a comprehensive examination of the T_{FR} phenotype in healthy, SIV-uninfected RM. As shown in Figure 2, our analysis of relative mean fluorescence intensities (MFI) for T_{FR} markers confirmed that T_{FR} express FoxP3 and CD25 at comparable levels to T_{REG} (Figure 2A) and both CXCR5 and PD1 at comparable levels to T_{FH} (Figure 2B). We next examined in T_{FR} the expression patterns of a series of markers (i.e., CD127, CTLA4, Bcl-6, and Helios) that have been linked to either T_{FH} or T_{REG} phenotype and function (133, 235). CD127, the IL-7 receptor a-chain, is expressed at low levels on T_{REG} in humans (236), (237) (238). As expected, we find that T_{FR} express CD127 at lower levels than the bulk of $CD4^+$ T cells, and similar or even lower levels than those observed in T_{REG} and T_{FH} (Figure 2C). CTLA4 is a key negative T cell regulator that is constitutively expressed on T_{REG} and, upon ligation, induces down-modulation of cytokine production and inhibition of cell-cycle progression (235). Consistent with previous reports in murine models (224), we observed that T_{FR} express CTLA4 at a higher frequency and MFI than both T_{REG} and T_{FH} cell populations (Figure 2D). This is consistent with a putative role of T_{FR} as negative regulators of GC responses. Helios is a transcription factor expressed in thymus-derived natural T_{REG} cells (239). As shown in Figure 2F, T_{FR} express Helios at levels that are even higher than those observed in T_{REG} in terms of both frequency of positive cells and MFI, thus suggesting that T_{FR} originate from natural T_{REG} in RM as well as in mice.

Transcriptome analysis of T_{FR} reveals a distinct but overlapping transcriptional profile compared to T_{FH} and T_{REG}. To further define the functional features of T_{FR} in RM, we

next examined the transcriptional profiles of T_{FH}, T_{FR} and T_{REG} using RNA-Seq by Illumina technology. Splenocytes from five healthy, SIV-uninfected and unvaccinated RM were sorted into "bulk" $CD3^{+}CD4^{+}$ T cells, T_{REG}, T_{FH} and T_{FR} based on the following phenotypic markers: T_{FR} ($CXCR5^{+}PD1^{hi}CD127^{-}CD25^{+}$), T_{FH} ($CXCR5^{+}PD1^{hi}CD127^{+/-}CD25^{-}$) and T_{REG} ($CXCR5^{+/-}PD1^{lo/int}CD127^{-}CD25^{+}$). In mice, T_{FR} are derived from thymic T_{REG} precursors and acquire homing markers that allow them to traffic to GCs, while maintaining a transcriptome and suppressive function that most closely resembles T_{REG} (171, 224). To examine the transcriptional profile of T_{FR} relative to T_{REG} and T_{FH} in healthy, SIV-uninfected RM, we first performed principal component analysis (PCA) on a subset of the most highly expressed transcripts detected in T_{FH}, T_{REG} and T_{FR} (Figure 3A). The transcriptomes of each subset were clearly distinct and grouped by subset, with T_{REG} displaying the highest degree of intra-subset variability, and T_{FH} and T_{FR} subsets being more tightly distributed. We next compared the expression of several canonical T_{FH} and T_{REG} transcripts between the three subsets. As shown in Figure 3B-D, T_{FH}- and T_{REG}-related genes showed expression patterns that behaved as predicted with genes such as IL-10 expressed in T_{FR} and T_{REG} but absent in T_{FH}. Importantly, RNA sequencing data confirmed that T_{FR} share expression of T_{REG} signature transcripts such as FoxP3, GZMB, PRDM1 and IL2RA (Figure 3). However, we found that several other T_{REG}-specific transcripts were expressed at lower levels in T_{FR} than in T_{REG}, including TRAF6, CD74, CCL20 and IL1R1. Similar to previous studies in mice, T_{FR} also showed elevated expression of the prototypical T_{FH} genes CXCR5, PD1/PDCD1, BCL-6, CXCL13, and ICOS. In fact, T_{FR} demonstrated a peculiarly high expression of the T_{FH} and T_{REG}-specific genes Bcl-6 and FoxP3, respectively. Interestingly, for several genes

(SH2D1A/SAP, IL-21, CXCR5, IL-10) gene expression was higher in T_{FR} than either T_{REG} or T_{FH}, thus suggesting that the $CD25^{+}CXR5^{+}PD1^{hi}$ phenotype may represent a more transcriptionally active population than classical T_{REG} or T_{FH}. This latter set of RNA-sequencing data provides strong evidence that T_{FR} are indeed a distinct cell subset and that the somewhat hybrid transcriptional profile of T_{FR} is not simply due to the sample being a mixture of T_{REG} and T_{FH}. Of note, elevated expression of IL-10 in T_{FR} compared to T_{REG} has been previously reported in murine studies (171).

To then compare the profile of gene expression between T_{FR} with T_{REG} and T_{FH} subsets without using any *a priori* information, we defined T_{FH} and T_{REG} expression signatures by statistically contrasting RNA-sequencing data from T_{FH} and T_{REG} with bulk $CD4^{+}$ T cells. After exclusion of transcripts that had zero expression in any of the populations, a total of 88 genes made up the combined T_{FH} and T_{REG} signature of which 12 genes were T_{REG} related. Many, but not all, canonical T_{REG} and T_{FH} genes were also identified as significantly upregulated compared to bulk $CD4^{+}$ T cells. The lack of statistical significance for some prototypical transcripts is likely due to the presence T_{REG} and T_{FH} subsets within the bulk $CD4^{+}$ population used as a comparator sample. Nevertheless, we found that T_{FR} cells show similar levels of expression of T_{FH} signature genes such as Bcl-6, TIGIT, CD200, LAT and BATF (Figure 3C). T_{FR} cells also express mRNA for key T_{FH}-related genes that are important for B-cell help, including IL-21, SH2D1A, CD40L and CD84. One notable difference between our data in RM and previously published mouse studies is that we observed high expression of IL-21 in T_{FR}, suggesting that these cells have differential genomic and functional features in primates. As shown in

Supplementary Figure 2A-C, the expression patterns of IL-21, SH2D1A, SLAMF6, PD1, IL6R, CCL20, IL2RA, IL10, PRDM1, and XIAP were confirmed by RT-PCR quantification. In addiction, levels of protein expression of IL-21 by T_{FR} , T_{FH}, and T_{REG} were also measured by flow cytometry and further confirmed the pattern observed by RNA sequencing and RT-PCR (Supplementary Figure 2D).

SIV infection is associated with a decrease in the T_{FR}/T_{FH} ratio. The dynamics of T_{FR} in the setting of HIV or SIV infection have not been previously investigated, and in fact all published studies of T_{FH} dynamics during HIV/SIV infection used a definition of these cells that included T_{FR} as well. To study the kinetics of T_{FR}, T_{FH}, and T_{REG} following SIV infection of RM we measured the frequency of these cells within the lymph nodes prior to infection, 2 weeks post infection and 6 months post infection with SIVsmE660. The RM included in these kinetics analyses included both unvaccinated as well as animals that were challenged following immunization with a SIVmac239 Gag-, Pol-, and Env-expressing DNA vaccine (with or without GM-CSF) followed by two boosts of a SIV239 Gag-, Pol-, and Env-expressing MVA vaccine. As previously reported, we found a significant increase ($p<0.0001$) in frequency of T_{FH} at 24 weeks post infection (Figure 4A). Interestingly, the frequency of T_{FR} measured as percent of total $CD4^+$ T cells also showed a significant ($p=0.0001$) increase during chronic SIV infection (Figure 4A). However, when the frequency of T_{FR} is measured as percentage of total T_{FH}, we found that the T_{FR} decrease significantly at both the acute ($p=0.0385$) and chronic ($p=0.0016$) stages of SIV infection (Figure 4B). Accordingly, the overall ratio of T_{FR} to T_{FH} cells also decreased significantly ($p=0.0018$) at the week 24 post-infection time point as compared

to baseline (Figure 4C). The increase of both T_{FH} and T_{FR} as a percent of $CD4^+$ T cells after infection is likely the result of proliferation driven by antigen-persistence as well as virus-mediated depletion of other $CD4^+$ T cell subsets. However, the relative decrease in the frequency of T_{FR} when measured as percentage of T_{FH} suggests that the low frequency of these regulatory cells might contribute to the expansion and accumulation of T_{FH} in chronically SIV-infected RM. Of note, we found no significant changes in T_{REG} frequencies after SIV infection within the lymph nodes. To better define the kinetics of T_{FH} and T_{FR} during SIV infection we next measured the level of cell proliferation using the well-established marker Ki67. We observed that T_{FH} show a significant increase in proliferating cells during the acute ($p<0.0001$) phase and chronic ($p=0.0001$) phase of infection (Figure 4D). T_{FR} have a similar pattern of proliferation, with a significant increase in proliferating cells during the acute ($p<0.0001$) phase and chronic ($p=0.0376$) phase of infection (Figure 4D). In contrast, the level of Ki67 expression in T_{REG} remains relatively low throughout our analysis with a small significant increase ($p=0.0141$) during the chronic phase of infection (Figure 4D).

Similar levels of SIV infection of T_{FR} as compared to T_{FH} and T_{REG} despite higher CCR5 expression. Several studies have shown that, during HIV and SIV infection, T_{FH} are highly infected with the virus despite their relative increase within the total $CD4^+$ T cell pool (136). While the actual *in vivo* lifespan of T_{FH}, either infected or uninfected, remains unknown in the setting of HIV/SIV infection, the presence of a notable fraction of these cells expressing the proliferation marker Ki67 suggests that their number could be maintained through continual replenishing from precursors located outside the GC. To

measure the level of direct SIV infection of T_{FR}, T_{FH}, and T_{REG} we sorted these subpopulations from the lymph nodes of a subset of our studied animals and quantified the levels of total cell-associated SIV-DNA by RT-PCR. This analysis revealed that T_{FH}, T_{FR} and T_{REG} derived from chronically SIV-infected RM all harbor comparably high levels of cell-associated viral DNA (Figure 5A). Interestingly, the levels of SIV infection were similarly high between T_{FR} and T_{FH} even though the surface expression levels of the main SIV co-receptor CCR5 were significantly higher in T_{FR} as compared to T_{FH} (Figure 5B).

The frequency of T_{FR} is negatively correlated with the number and proliferation of both T_{FH} and GC B-cells. To further examine the relationship between T_{FR} and T_{FH} and GC B-cells we next performed a set of correlation analyses in the RM included in this study. In healthy uninfected RM, the frequency of T_{FR} (as fraction of the total T_{FH} pool) is negatively correlated with the percentages of T_{FH} (as fraction of total $CD4^+$ T cells) and GC B-cells (as fraction of total B-cells) (Figure 6A). In addition, we found that, in the same animals, the frequency of T_{FR} (as fraction of T_{FH}) is negatively correlated with the level of $CD4^+$ T cell proliferation as measured by Ki67 expression (Figure 6A).
We next performed the same correlation analyses in our cohort of SIV-infected RM. The SIV-infected RM included in these regression analyses included both unvaccinated as well as animals that were challenged following immunization. In the SIV- infected RM, similar to what was observed in uninfected animals, the frequency of T_{FR} (as fraction of T_{FH}) is negatively correlated with the percentages of both T_{FH} and GC B-cells (Figure 6B). However, the negative correlation between frequency of T_{FR} (as fraction of T_{FH}) and

the level of $CD4^+$ T cell proliferation as measured by Ki67 expression is not seen in SIV-infected RM (Figure 6B). The negative correlation between T_{FR} cells (as a frequency of T_{FH} cells) and both T_{FH} and GC B-cell frequencies is consistent with the hypothesis that T_{FR} cells play a role in regulating T_{FH} and GC responses under normal circumstances and in the setting of chronic SIV infection.

Comparative analysis of the T_{FR} transcriptome in SIV-infected and uninfected RM.

To further define the effect of SIV infection on T_{FR}, we next compared the transcription profiles of T_{FR} that were isolated from unvaccinated chronically SIV-infected and uninfected RM (Fig. 7). We performed RNA-Seq analysis and transcripts that were significantly differentially expressed in T_{FR} sorted from SIV infected vs. uninfected RM were analyzed by Ingenuity Pathway Analysis. Unsurprisingly, a large proportion of genes induced during SIV infection in T_{FR} (CD3G, FOS, CD4, ZAP70, PIK3CD, STAT3) were components of T cell proliferation, activation of T cell effector function, and co-stimulatory activation (data not shown). The enhanced T cell activation was consistent with our observation that T_{FR} cells express higher levels of the proliferation marker Ki67 compared to T_{REG} (Figure 4D). We also observed that several genes implicated in pathways regulating apoptosis or cell cycle control were perturbed in SIV-infected RMs. Of particular interest was the observation that the pro-apoptotic gene FASLG was >100-fold induced, while the anti-apoptotic regulator XIAP was significantly down-regulated. This latter finding was again validated by RT-PCR (Supplementary Figure 2B). Thus, while we observed a significant increase of the proliferation marker Ki67 in T_{FR} after SIV infection (Figure 4D), a pro-apoptotic shift of

gene expression may explain why only a modest increase in T_{FR} frequency was observed (Figure 4A).

T_{FH} require IL-6 signaling and STAT3 expression for differentiation and, once mature, produce several factors that support B-cell activation. Conversely, IL-2 receptor signaling drives STAT5 to activate Blimp1/PRDM1, which ultimately blocks T_{FH} differentiation (240). However, T_{FR} express both Blimp/PRDM1 and Bcl-6. Here we find that both IL-6 and IL-2 signaling genes are enriched in T_{FR} after SIV infection. However, several of these genes, such as MAPK1, are common to different cytokine signaling pathways, thus making it difficult to establish if SIV infection causes a shift in the T_{FR}/T_{FH} differentiation pressure. Interestingly, downstream signaling for IL-10, a regulatory cytokine produced by both T_{FR} and T_{REG}, is also enriched in T_{FR} post infection. Additionally, ICOS-ICOSL signaling was also enriched in T_{FR} post infection. These data suggest that T_{FR} may be engaged in similar T_{FH}-like cell-surface receptor-ligand interactions with B-cells. In addition to genes that were identified with differential expression without any *a priori* knowledge, we also examined genes with known function in T_{FR} and T_{REG}. After infection, T_{FR} cells show a significant increase in the expression of PD1, IL6R, SLAMF6 and CD84, i.e., all markers associated with T_{FH} differentiation and function (Figure 7B). We also found a significant decrease in STAT3 and IL2RA in T_{FR} after SIV infection and a non-significant decrease in Bcl-6 expression. Finally, as expected, we also observed several other changes in expression patterns of the T_{FH} and T_{FR} signature gene sets as we had previously determined

(Supplementary Figure 1). Overall, these data indicate a complex remodeling of gene expression in T_{FR} following SIV infection of RM.

Discussion

T_{FH} cells are critical to the development of the humoral response to infections, and their role in the setting of HIV and SIV infection (and vaccination) is the subject of intense investigation. However, some aspects of the complex T_{FH} response to HIV/SIV infection remain poorly understood, including (i) their role in promoting the development of broadly neutralizing HIV/SIV-specific antibodies, and (ii) their role in the immunopathogenesis of the infection. In particular, the mechanisms by which T_{FH} accumulate during the chronic stage of infection despite high levels of direct virus infection are unclear. Importantly, a series of recent studies have shown that T_{FH} include a subset of cells that are derived from thymic T_{REG} precursors, express the classical T_{REG} markers (i.e., FoxP3 and CD25 as well as low levels of CD127), and acquire T_{FH} markers (i.e., PD1, CXCR5, and Bcl-6) while migrating into the GC of lymph nodes, where they are thought to act as regulators of the host humoral immune response. To the best of our knowledge this study-- together with the independently generated set of data that are included in the accompanying manuscript by the group of Dr. Franchini and Dr. Vaccari -- represents the first description of the main features of T_{FR} in a non-human primate species. In this work we also investigated the dynamics of this cell subset during SIV infection of rhesus macaques (RM).

The main findings of the current study are the following: (i) T_{FR} show distinct yet overlapping phenotype as compared to T_{FH} and T_{REG} based on a combination of flow cytometric, histological, and transcriptional analyses; (ii) in healthy, SIV-uninfected RM,

the frequencies of T_{FR} are negatively correlated with the levels of both T_{FH} and GC B-cells; (iii) following SIV infection, the T_{FR}/T_{FH} ratio is reduced; and (iv) T_{FR} sorted from SIV-infected RM harbor comparable levels of cell-associated viral DNA as compared to T_{FH} and T_{REG}. Collectively, these data indicate that while T_{FR} closely resemble T_{FH} in several biological aspects, they are also clearly distinguishable from this cell subset in terms of both their immunophenotype and transcriptional profile. It is therefore important that, in future studies of T_{FH}, a distinction be made between T_{FR} and true, "non-T_{FR}" T_{FH} to fully take into account the complexity of the different $CD4^+$ T cell subsets that are present in the GC of lymph nodes.

The observation that T_{FR} express proteins typically expressed by T_{FH}, such as CD40L, as well as proteins typically expressed by T_{REG}, like IL-10 and CTLA4, is consistent with studies in mice showing that T_{FR} are thymic-derived T regulatory (nT_{REG}) cells that migrate into the follicle and, in a manner similar to T_{FH}, up-regulate CXCR5, Bcl-6 and PD1 in a B-cell dependent manner (234). The production of IL-21 by T_{FR} cells is an intriguing new finding and suggests that T_{FR} play a more complex role in RMs than has been described in mice. In addition, PCA suggests that the transcriptional profile of T_{FR} tend to be more similar to T_{FH} than T_{REG}. Further studies are required to fully investigate the functional role played by T_{FR} in RMs and, specifically, in the context of SIV infection. The finding that over 90% of T_{FR} express Helios as measured by flow cytometry is also consistent with the nT_{REG} origin of these cells. Importantly, these immunophenotypic and RNA sequencing data were complemented by histological analyses showing that T_{FR} are found within GCs of both uninfected and infected RM.

While $CD4^+FoxP3^+$ T_{REG} were found in abundance outside the GC, $CD4^+PD1^+$ T_{FH} and $CD4^+PD1^+FoxP3^+$ T_{FR} were both only seen within the GC. The hypothesis that T_{FR} play an immune regulatory role *in vivo* is supported by the observation that their frequency is inversely correlated with both the levels of T_{FH} and GC B-cells. These data are consistent with mouse studies indicating that (i) T_{FR} suppress T_{FH} *in vitro* and prevent the outgrowth of non-antigen specific B-cells (171), and that (ii) T_{FR} may inhibit antibody production without an effect on T cell activation, thus indicating an ability to directly regulate B-cells(241).

In the setting of *in vivo* SIV infection, we found that T_{FR} exhibited only a small increase in their frequency within the total $CD4^+$ T cell pool. In conjunction with the large expansion of T_{FH} that is consistently observed following SIV infection, the minor expansion of T_{FR} leads to a significantly decreased T_{FR}/T_{FH} ratio in the lymph nodes of chronically SIV-infected RM. We confirmed this trend in the ratio of T_{FR}/T_{FH} cells after SIV infection by quantifying the number of T_{FH} and T_{FR} cells by immunohistochemistry (Supplementary Table 1). While the current set of results does not allow us to determine whether and to what extent the kinetics of T_{FH} and T_{FR} during SIV infection are mechanistically linked, it is conceivable that the limited T_{FR} expansion facilitates progressive accumulation of T_{FH} in chronically SIV-infected RM, thus indirectly contributing to the aberrant immune activation that characterizes this pathogenic infection. On the other hand, it is also possible that the strong proliferation of T_{FH} and associated increase in PD1 expression following SIV infection hampers the development or differentiation of T_{FR} as suggested (241).

Comparison of the transcriptional profiles of T_{FR} cells pre and post-SIV infection showed a significant up-regulation of transcripts typically expressed by activated T_{REG} including FOSB, FABP5, USP2 and USP13 (data not shown) (242), thus suggesting that T_{FR} may be involved in the generalized immune activation associated with pathogenic SIV infection. Interestingly, we also observed a down regulation of several T_{FH} and T_{REG} signature genes as established by our own algorithm. This somewhat unexpected observation indicates that SIV infection has a complex effect on the *in vivo* phenotype and function of T_{FR}. A better understanding of the overall contribution of T_{FR} to the immunopathogenesis of AIDS, in terms of causing either the virus-associated B-cell dysfunction or the changes in the lymph node architecture and function, will require further *in vitro* and *in vivo* investigation of the suppressive effect by these T_{FR} on the function of either T_{FH} or GC B-cells.

While $CD4^+$ T cells are the main target for HIV and SIV infection, substantial difference exist between various $CD4^+$ T cell subsets in terms of their relative levels of direct virus infection *in vivo* (35, 243, 244). In this study we tested the possibility that the decrease in the T_{FR}/T_{FH} ratio observed during SIV infection of RM was associated with higher level of virus infection in T_{FR} as compared to T_{FH}. However, our comparative analysis of the cell-associated viral burdens in sorted T_{FR}, T_{FH}, and T_{REG} revealed similar levels of SIV-DNA in the three $CD4^+$ T cell subsets, even though T_{FR} exhibited higher levels of the SIV co-receptor CCR5 as compared to the other two subsets.

In summary, the presented data provide the first comprehensive description of T_{FR} in healthy, uninfected RM, as well as the first examination of the kinetics of these cells in the setting of pathogenic SIV infection. These results support the hypothesis that these cells play an important immune regulatory role *in vivo*, and that a relative decline of the T_{FR}/T_{FH} ratio may be involved in establishing a state of chronic immune activation in lymph nodes during pathogenic HIV and SIV infection.

Acknowledgements

We would like to thank Dr. Barbara Cervasi and Dr. Kiran Gill at the Flow Cytometry Core at Emory Vaccine Center and Dr. Prachi Sharma and Dr. Deepa Kodandera at the Molecular Pathology Core Lab. We would also like to thank the animal care and veterinary staff at the Yerkes National Primate Research Center.

Figures

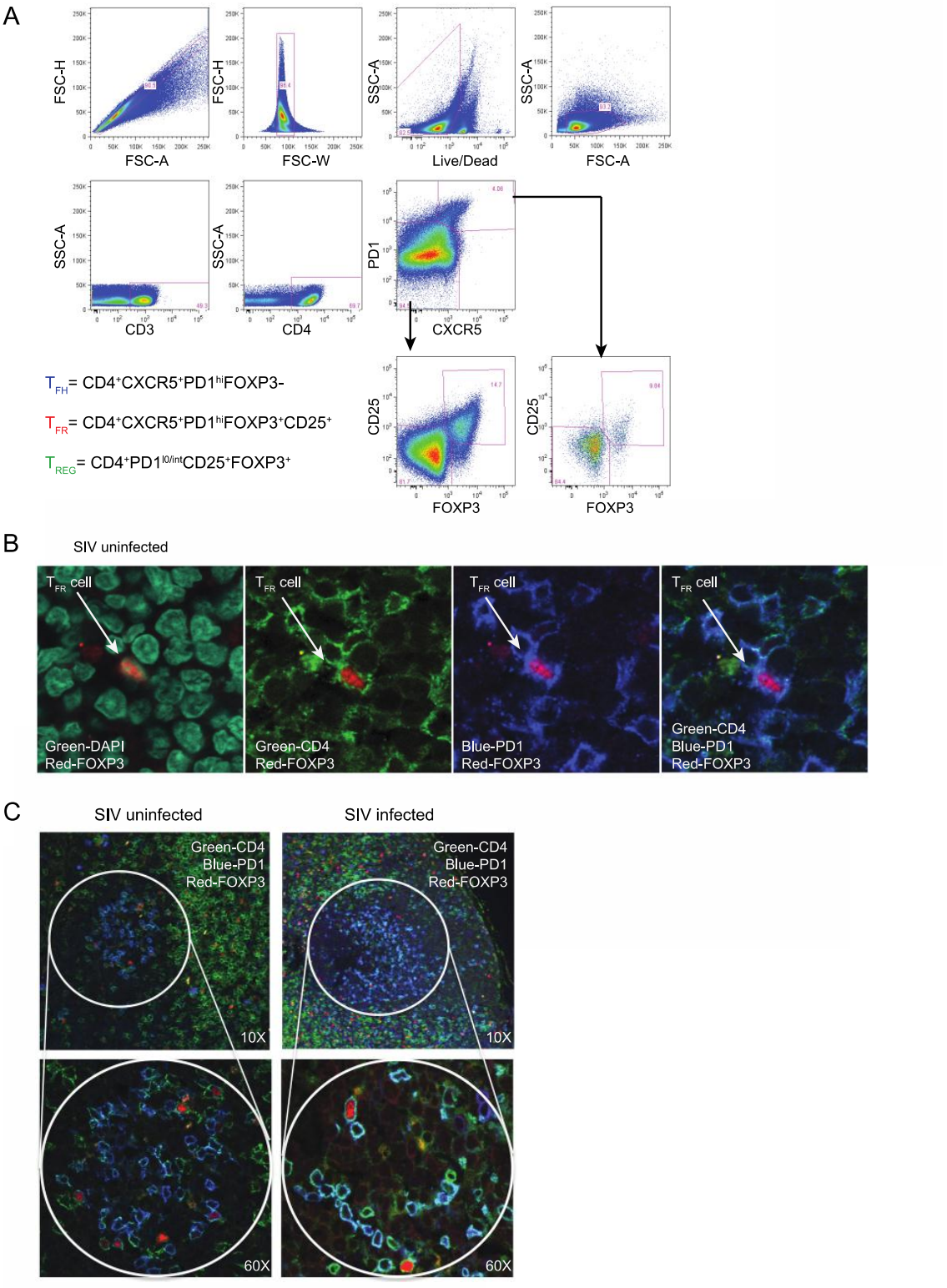

A
FSC-H
FSC-A
FSC-H
FSC-W
SSC-A
Live/Dead
SSC-A
FSC-A
SSC-A
CD3
SSC-A
CD4
PD1
CXCR5
T_{FH}= CD4⁺CXCR5⁺PD1^{hi}FOXP3-
T_{FR}= CD4⁺CXCR5⁺PD1^{hi}FOXP3⁺CD25⁺
T_{REG}= CD4⁺PD1$^{lo/int}$CD25⁺FOXP3⁺
CD25
FOXP3
CD25
FOXP3
B
SIV uninfected
T_{FR} cell
Green-DAPI
Red-FOXP3
T_{FR} cell
Green-CD4
Red-FOXP3
T_{FR} cell
Blue-PD1
Red-FOXP3
T_{FR} cell
Green-CD4
Blue-PD1
Red-FOXP3
C
SIV uninfected
SIV infected
Green-CD4
Blue-PD1
Red-FOXP3
10X
Green-CD4
Blue-PD1
Red-FOXP3
10X
60X
60X

Figure on previous page.

Figure 1. T_{FR} can be defined by flow cytometry and identified by confocal microscopy within the germinal centers of RM. (A) Representative flow cytometry plot of lymphocytes from lymph nodes of untreated uninfected RM showing the gating strategy used to define T_{FR}, T_{FH} and T_{REG} cell populations. (B) Representative confocal microscope image showing a single T_{FR} within the lymph node of an uninfected RM. The first image shows staining for DAPI (green) and FoxP3 (red). The second image shows the same section with CD4 (green) and FoxP3 (red), the third PD1 (blue) and FoxP3 (red) and the last image CD4 (green), PD1 (blue) and FoxP3 (red). (C) Representative images of lymph node biopsies from SIV uninfected and acutely infected RM showing cells stained with CD4 (green), FoxP3 (red) and PD1 (blue) within the GC regions.

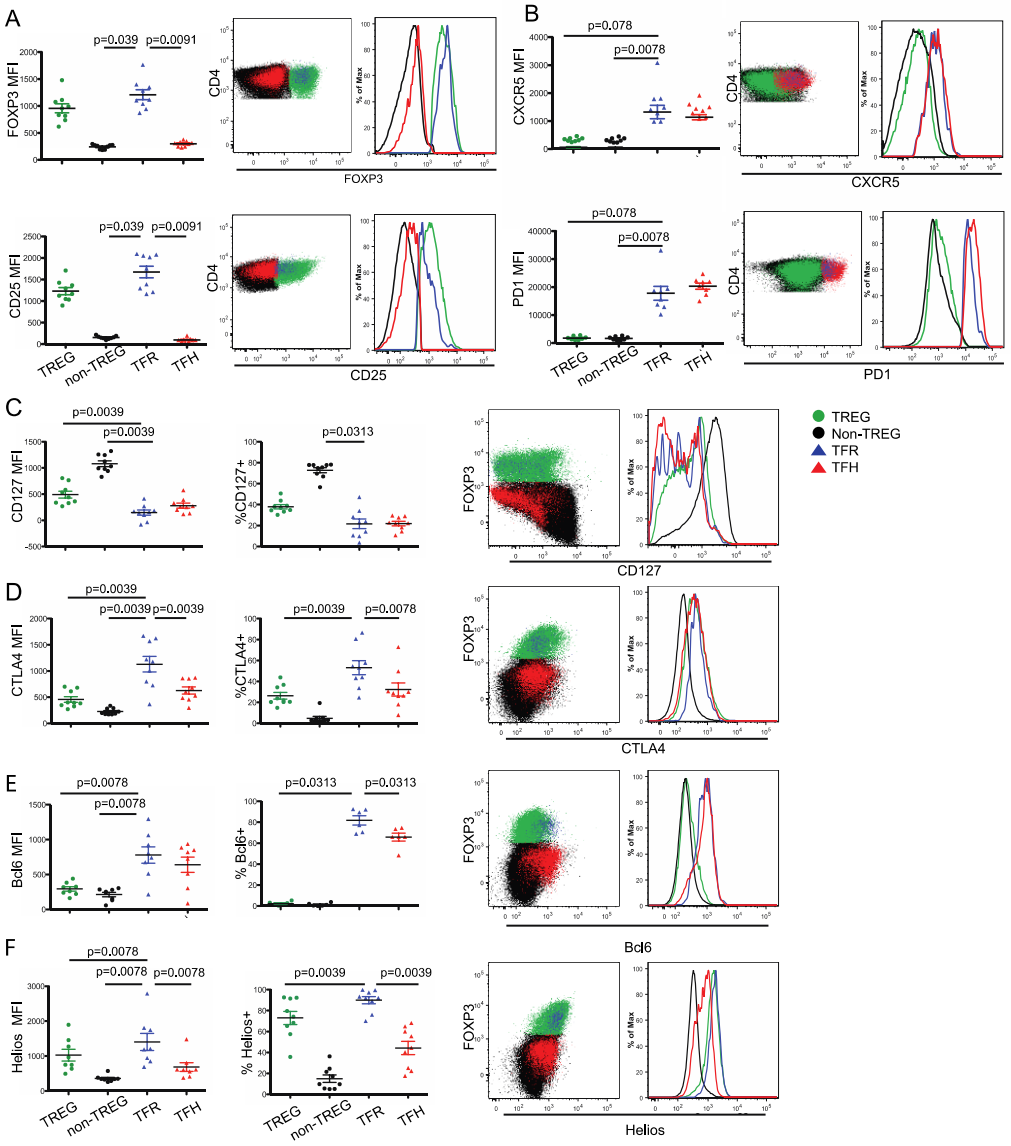


Figure 2. T_{FR} share immunophenotypical features of both T_{FH} and T_{REG} populations. Mean fluorescence intensity, percent positive, representative flow cytometry plots and histograms (Panels A, B, C, D, E, F) for expression of various immunophenotypical markers (i.e., FoxP3, CD25, CXCR5, PD-1, CD127, CTLA4, Bcl-6 and Helios) among T_{REG}, non- T_{REG}, T_{FR} and T_{FH} populations from LN of healthy, unvaccinated and

uninfected RM. Non-T_{REG} here are defined as all $CD4^{+}$CD25-FoxP3- T cells. Significance was determined by Wilcoxon signed rank tests.

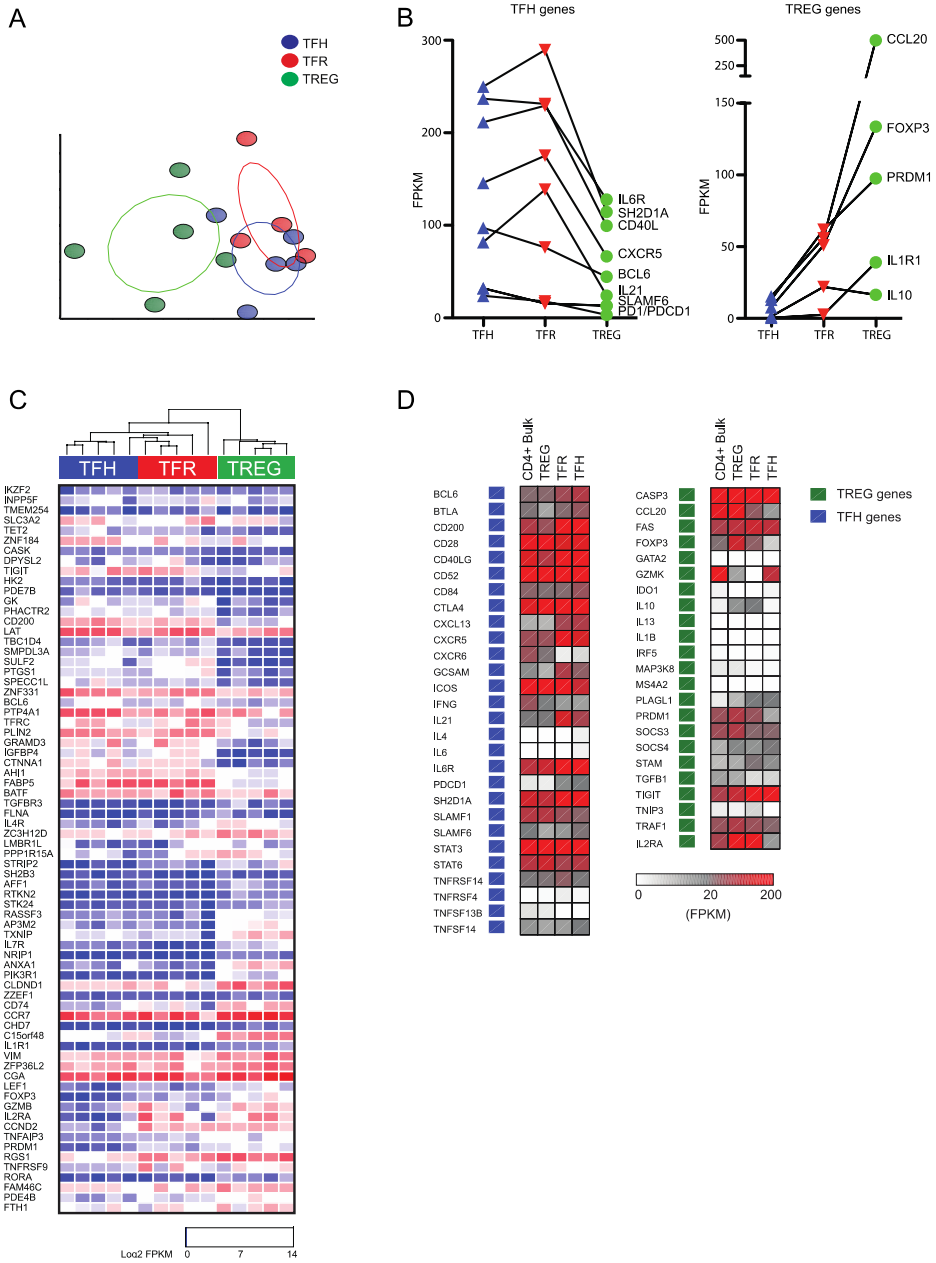


Figure 3. RNA expression patterns confirm that T_{FR} share T_{FH} and T_{REG} like phenotype. (A) Principal components analysis of RNA transcripts from five healthy, SIV-uninfected RM. Each circle represents the transcriptome of a sorted population of

T_{FH} (blue), T_{REG} (green), or T_{FR} (red) from a single animal. (B) Expression in FPKM of select T_{FH} and T_{REG} genes in sorted populations from uninfected RM. (C) Heat map of log 2 transformed gene expression of transcripts in FPKM. Transcripts represent T_{FH} and T_{REG} signature genes. The T_{FH} gene signature was defined as transcripts that were significantly differentially expressed in sorted T_{FH} compared to bulk $CD4^+$ T cells. T_{REG} gene signature was defined as genes that were significantly differentially expressed in sorted T_{REG} compared to bulk $CD4^+$ T cells. (D) Expression pattern of key T_{FH} and T_{REG} genes in sorted bulk $CD4^+$ T cell, T_{FH}, T_{FR} and T_{REG} populations.

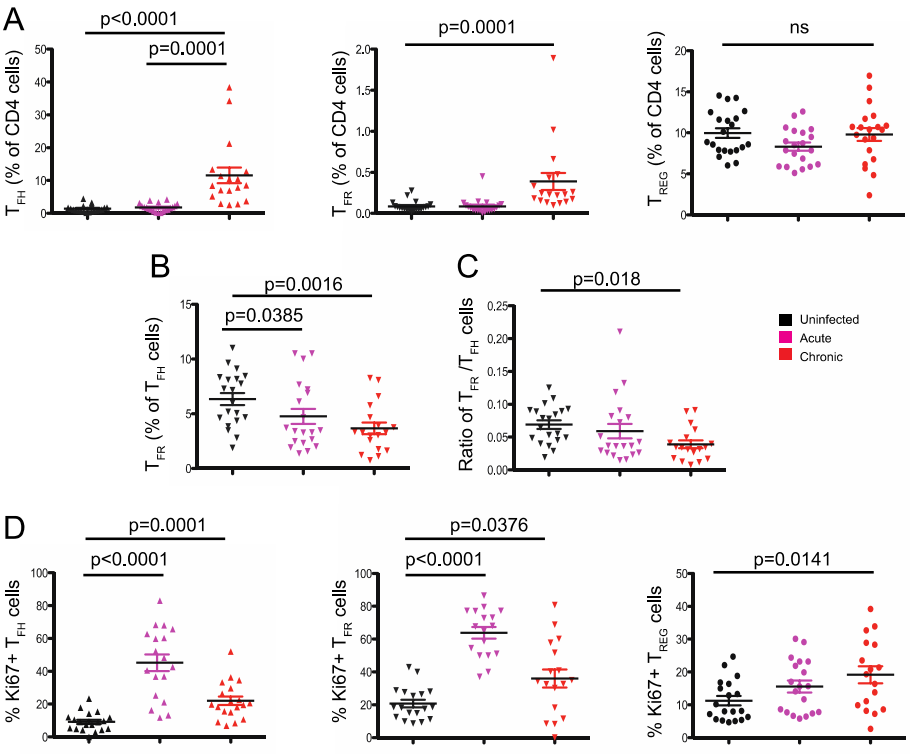


Figure 4. **Kinetics of T_{FR}, T_{FH} and T_{REG} after SIV infection.** (A) Frequency of T_{FH}, T_{FR} and T_{REG} as percentage of the total $CD4^+$ T cell population within lymph nodes of uninfected, acutely (week 2) SIV-infected and chronically (week 24) SIV-infected RM. (B) Frequency of T_{FR} as a percent of T_{FH} within the lymph nodes of the same animals. (C) Ratio of frequencies of T_{FR} to the frequency of T_{FH} (both calculated as a percent of the total $CD4^+$ T cell population). (D) Percent of proliferating, $Ki67^+$, T_{FH}, T_{FR} and T_{REG} within the lymph nodes of uninfected, acutely SIV-infected and chronically SIV-infected RM. Statistical analyses were performed using Mann-Whitney U tests.

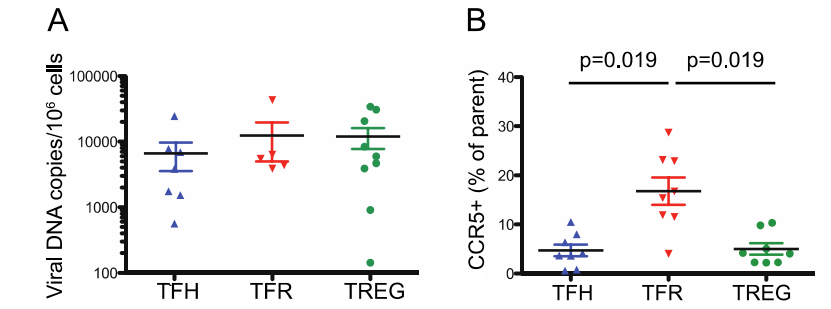


Figure 5. Comparable levels of SIV infection in T_{FH}, T_{FR} and T_{REG} isolated from the spleen of chronically SIV-infected RM. (A) Viral DNA copies per million sorted T_{FH}, T_{FR} and T_{REG} from spleens of unvaccinated chronically SIV-infected RM. (B) Percent of CCR5-expressing cells among T_{FH}, T_{FR} and T_{REG} in unvaccinated SIV-infected RM. Statistical analyses were performed using Mann-Whitney U tests.

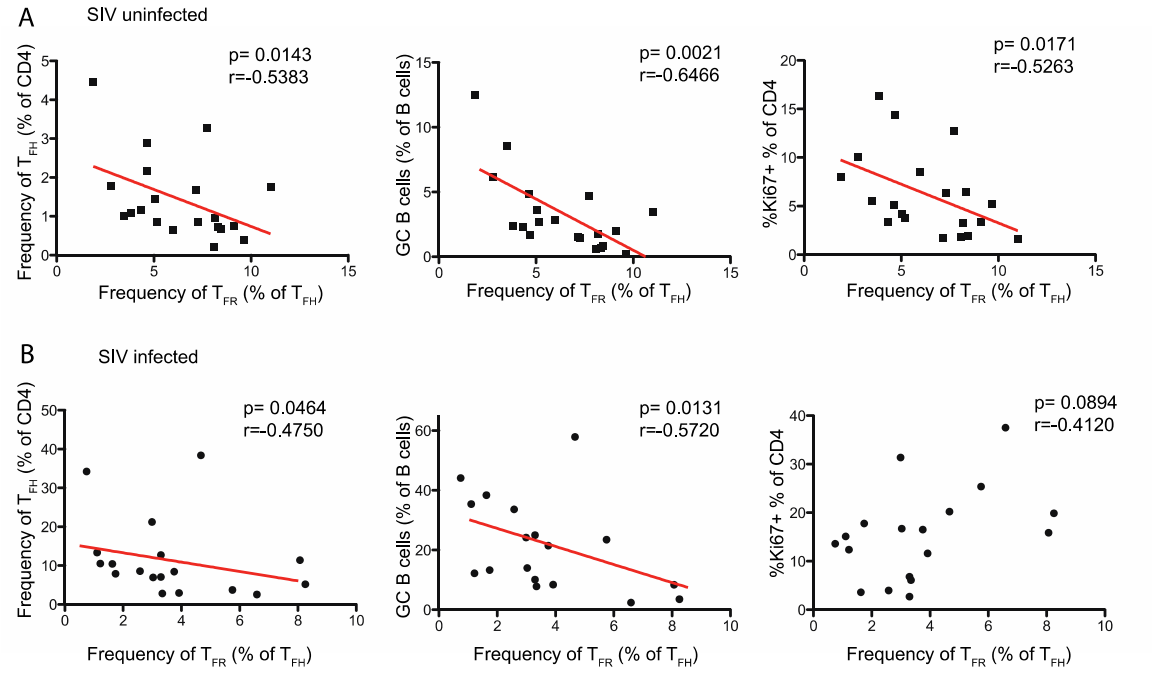


Figure 6. T_{FR} frequencies negatively correlate with T_{FH} and GC B-cell frequencies in the lymph nodes of RM. Correlations between the frequencies of T_{FR} (calculated as frequency of T_{FH}) and the frequencies of T_{FH} calculated as percent of total $CD4^+$ T cells (left) and GC B-cells calculated as percent of total B-cells (center) and proliferating (i.e., $Ki67^+$) $CD4^+$ T cells (right) within the lymph nodes of SIV-uninfected (A) and chronically SIV-infected (B) RM. Statistical analyses were performed using Spearman rank correlation tests.

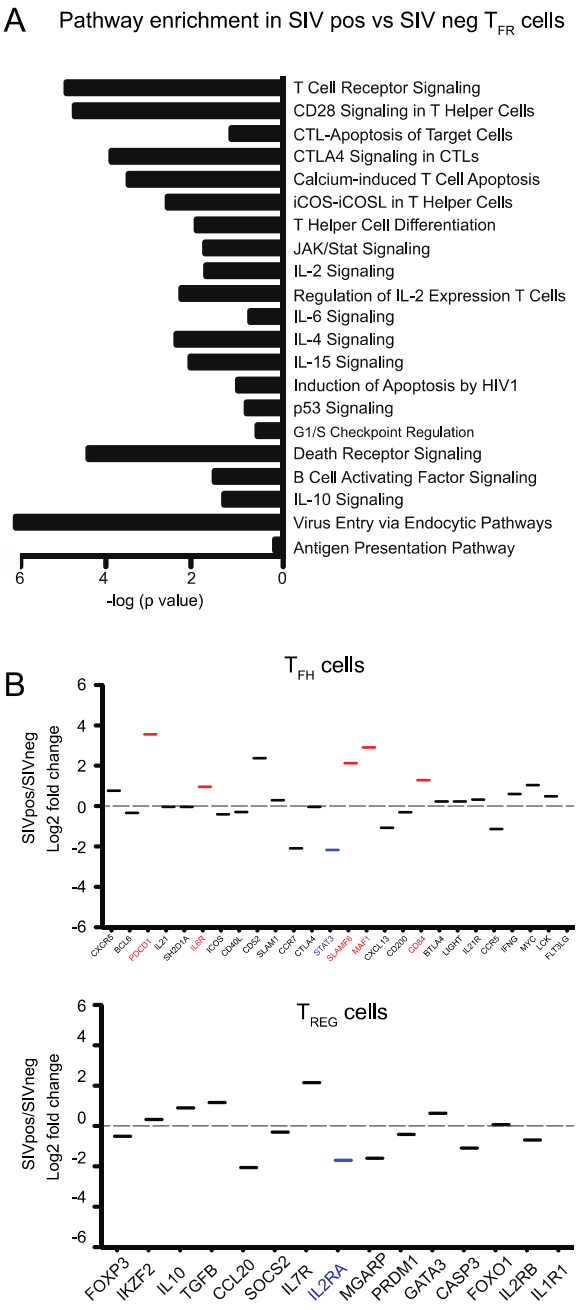
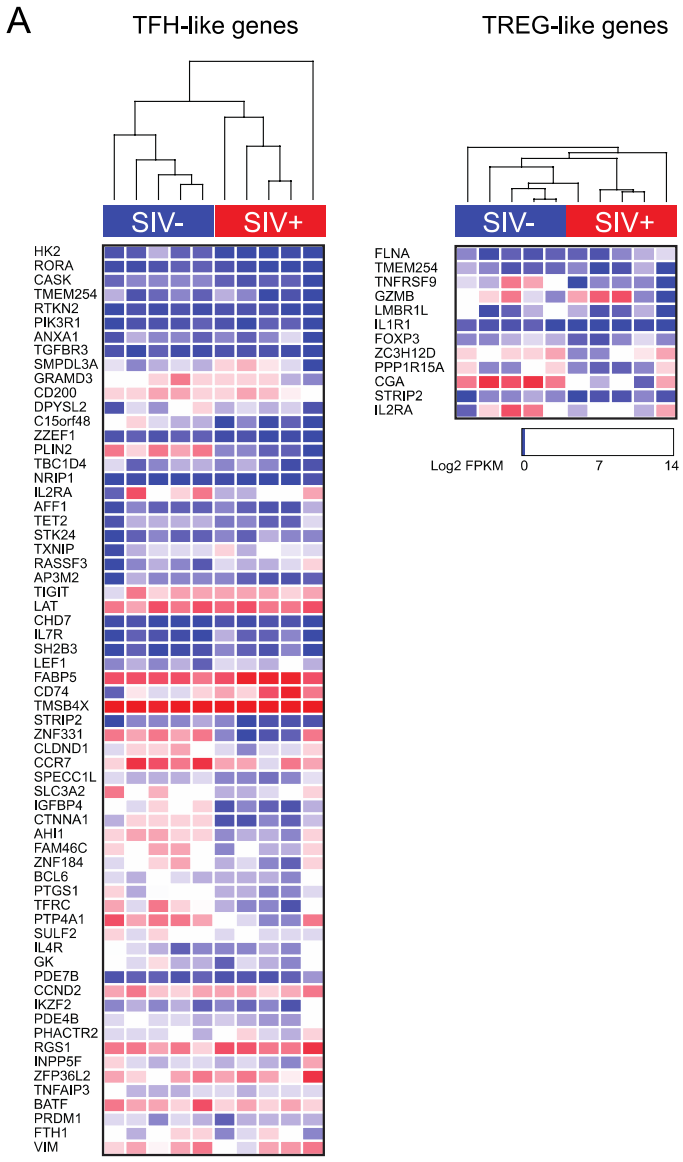


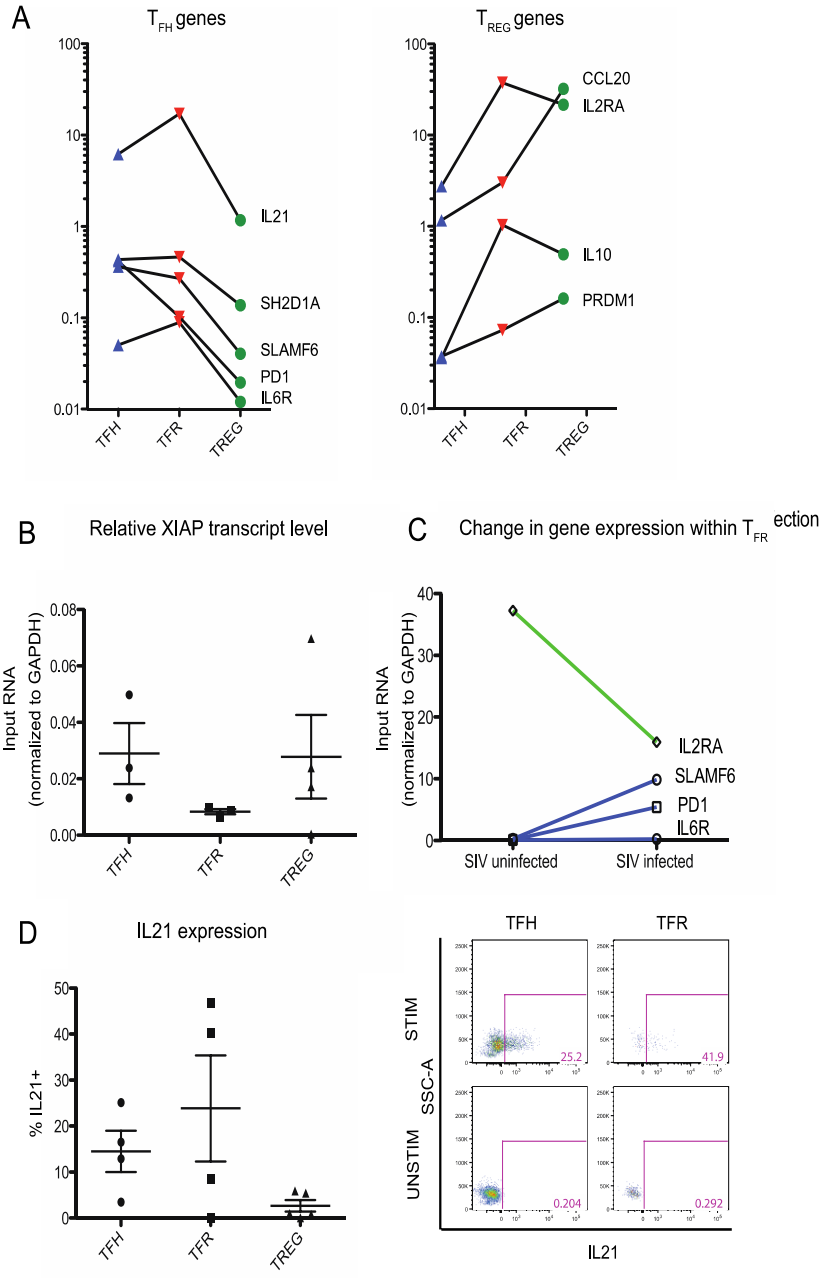
Figure 7. RNA expression within T_{FR} cells in uninfected and infected RM.

(A) Enrichment of gene pathways in T_{FR} derived from the lymph nodes of unvaccinated

SIV infected RM as compared to T_{FR} from SIV-uninfected animals as determined by Ingenuity Pathway Analysis. (B) Log 2 fold change of expression of T_{FH} and T_{REG} related gene transcripts in T_{FR} sorted from unvaccinated SIV-infected RM and SIV-uninfected RM. Significantly upregulated genes are in red and significantly down-regulated genes are in blue.



Supplementary Figure 1. **RNA expression within T_{FR} in uninfected and SIV infected RM.** (A) Heat map of log 2 transformed gene expression of transcripts in FPKM between T_{FR} cells from SIV uninfected and infected RM.



Supplementary Figure 2. PCR and flow cytometry validation of RNA-sequencing data.
Relative RNA transcript levels of key T_{FH} and T_{REG} genes (A) and XIAP (B) in T_{FH}, T_{FR} and T_{REG} populations from five SIV uninfected RM measured by RT-PCR. (C) Change in expression of genes post SIV-infection in sorted T_{FR} cells from 5 infected and 5 uninfected RM. All genes were normalized to the housekeeping gene, GAPDH. (D) Levels of IL21 expression as measured by low cytometry following PMA/ionomycin stimulatoion in T_{FH}, T_{FR} and T_{REG} populations and represetative FACS plot derived from LN of SIV uninfected RM. For this analysis T_{FR} cells were defined as $CXCR5^{+}PD1^{hi}CD127^{-}CD25^{+}$ cells.

Supplementary Table 1. Quantification of T_{FH} and T_{FR} cells by immunohistochemistry.

Animal	Timepoint	Number of TFH	Number of TFR	TFR/TFH Ratio
RM1	Uninfected	43	4	0.093
	Uninfected	72	6	0.083
RM2	Uninfected	57	6	0.105
	Uninfected	38	2	0.052
RM3	Acute Infection	67	6	0.089
	Acute Infection	47	5	0.106
RM4	Acute Infection	78	9	0.115
	Acute Infection	60	7	0.116
RM5	Chronic Infection	139	4	0.028
	Chronic Infection	95	4	0.042
RM6	Chronic Infection	66	3	0.045
	Chronic Infection	89	5	0.056

Supplementary Table 1. Quantification of T_{FH} and T_{FR} cells by immunohistochemistry.
Number of T_{FH} and T_{FR} cells counted per unit area within individual representative germinal centers of RM. T_{FH} cells were identified as $CD4^+PD1^+$ cell and T_{FR} cells were identified as $CD4^+PD1^+Foxp3^+$ cells.

Chapter Four: Identification of single celled lymphocytes with surface expression of T and B cell markers within primate lymph nodes.

Ankita Chowdhury[*], Perla Maria Estrada Del Rio[*†], Morgan Reuter[#], Ronald P Trible[*], Ann Chahroudi‡, Gustavo Reyes-Teran[†], Michael Betts[#], Steven E. Bosinger[*], Guido Silvestri[*]

[*]Emory Vaccine Center and Yerkes National Primate Research Center, Emory University, Atlanta, GA

[†] Departamento de Investigación en Enfermedades Infecciosas, Instituto Nacional de Enfermedades Respiratorias, "Ismael Cosio Villegas". México D.F., México

‡ Department of Pediatrics, Emory University School of Medicine, Atlanta, GA

[#]Institute for Immunology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104; Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

Abstract

$CD4^+$ T follicular helper cells (T_{FH}) play a critical role in the development of class-switched and affinity-matured antibodies by supporting germinal center (GC) B-cell responses. Studies of passive antibody transfer in macaques demonstrated that virus-specific neutralizing antibodies prevent Simian-Human Immunodeficiency Virus (SHIV) acquisition. However, these antibodies are only rarely produced during natural infection. Defining novel aspects of the interaction between T_{FH} and GC B-cells may improve our understanding of HIV immunology and pathogenesis.

This study describes a novel lymphocyte $CD3^+CD20^+$ 'double positive' population in lymph nodes and spleen of healthy macaques. Image stream and immunohistochemistry experiments confirmed co-expression of these two molecules on the same cell, and flow cytometric and microarray analysis demonstrated that a significant subset of these "double positive" cells have a T_{FH} phenotype (i.e., $CD4^+PD1^{bright}CXCR5^+ICOS^+Bcl\text{-}6^+$), function (IL-$21^+$IL-17-IL-$2^{+/-}$IFN-$g^-$), and profile of gene expression. In addition to CD20, DP cells also express B-cell markers such as CD79, CD21, and surface immunoglobulins. Importantly, expression of CD40L upon brief *in vitro* stimulation identifies DP cells that are T_{FH} in origin vs. those of B-cell lineage. We propose that (i) $CD3^+CD20^+$ cells arise as a result of membrane exchange in active germinal centers after high-affinity contact between T_{FH} and GC B-cells, in a process defined as trogocytosis, and (ii) the $CD3^+CD20^+$ phenotype may identify T_{FH} and GC B-cells that have recently undergone high affinity interactions during acute HIV and SIV infections.

Introduction

Vaccine design efforts in the field of HIV are currently focused on the design of immunogens that can elicit broadly neutralizing antibodies (94, 245, 246). While the appearance of bnAbs is rare and individuals that develop bnAbs do not have reduced viral loads, there is clear evidence that passive administration of bnAbs can prevent acquisition as well control replication of SHIV in rhesus macaque (RM) suggesting that pre-existence of bnAbs in immunized individuals may be able to protect individuals from infection (90, 247-251).

Analysis of their sequence and crystal structures has shown that bnAbs are highly divergent from their germline ancestors and have conserved targets of neutralization (i.e. CD4 binding-site, membrane-proximal region, N-linked glycans)(78, 252, 253). All class-switched and high-affinity antibodies are a result of the germinal center (GC) reaction (254). T follicular helper (T_{FH}) cells, a key cell-type found within GCs, play and important role in B cell selection and survival (125, 255). Several studies have shown that interaction between GC B and T_{FH} cells through numerous surface receptors such as ICOSL-ICOS, CD40-CD40L, SLAM-SAP are essential for the optimal development and survival of both these cell types (125, 132).

The selection mechanisms involved in the GC are poorly understood though it has been suggested that competition for T cell help is the main selection mechanism (120, 123, 256). Studies in mice have shown that B cells within a single GC are clonally restricted

whereas T_{FH} cells within a single GC are not (122). T_{FH} cells of varied specificities are equally distributed amongst GCs and appear to move freely between these GCs. On the other hand, while GC B cells are known to cycle between the light and dark zones of the GC they rarely leave the GC until they are fully differentiated as plasma cells or memory B cells. Two-photon imaging studies have shown that T_{FH} cells are motile and make several contacts with various B cells in order to survey antigens displayed by the B cells (121-123). These points of contact between the GC B cells and GC T_{FH} cells are presumably of vital importance to the selection mechanisms of the GC.

Here we describe our findings on lymphocytes found within the lymph nodes of humans and RM that can be identified by their surface expression of both T and B cell markers. Such cells were present in all surveyed secondary lymphoid organs but were absent in peripheral blood. Extensive efforts were made to confirm that these cells were in fact single cells with viable nuclei to distinguish them from doublets or conjugates of dead cell membranes with live cells. Our data indicate these cells are enriched for T_{FH} markers and GC B cell markers particularly those associated with the MHC-TCR synapse. We suggest that these cells are a consequence of intimate interaction between GC B cells and T_{FH} cells resulting in membrane exchange between these cells. Membrane exchange or trogocytosis is a well-described phenomenon that has the ability to alter regulatory, co-stimulatory or migratory ability of the cells that participate in membrane-exchange (190, 257). Trogocytosis between GC B and T_{FH} cells may alter the survival of GC B cells or the ability of T_{FH} cells to provide survival signals and may be a significant contributing factor to selection of B cell clones within GCs. Cells that have undergone trogocytosis

have also been used to identify antigen specific cells and may be useful to identify T_{FH} or B cells that have undergone high affinity interactions (258).Therefore, these single cells with enriched expression of T_{FH} cell markers and several B cell markers warrant further investigation.

Materials and Methods

Ethics statement. All ethical issues related to this project were evaluated and approved by the Institutional Bioethics and Science Committee of the National Institute of Respiratory Diseases in Mexico City (code B33-10). Peripheral blood and lymphoid tissue samples were obtained from HIV negative and HIV positive people after written consent was obtained. Cervical lymph nodes from HIV negative or HIV positive people were obtained in the Center for Research of Infectious Diseases in México under local anesthesia.

Study subjects and samples. Twenty persons that had no active opportunistic infections by the time of sample collection were included in this study. Paired samples (blood and LN biopsy) were obtained from one HIV negative donor, 2 HIV^+ with cART, and 10 HIV^+ without cART. Seven lymph node biopsies (no blood sample) from 7 HIV^+ donors without cART were also obtained. For LNMC characterization, we obtained samples from individuals that had palpable LN in the cervical area and were biopsied for diagnostic purposes; none of the patients included in this study had opportunistic infections. The day of the LN biopsy, blood samples were also collected from the same individual to determine pVL and $CD4^+$ T cell count. Characteristics of study subjects are listed in Table 1.Fresh PBMCs were separated from peripheral blood by density gradient centrifugation using Ficoll-Hypaque (Accurate, Florida, USA). LNMC were obtained after biopsy processing. Briefly, biopsy samples were placed in Hanks medium (Lonza, Walkersville, MD) and immediately transferred to the laboratory for further processing.

Lymphoid tissue was cut in small pieces with a scalpel and placed into GentleMACS C tubes (Miltenyi Biotec, Germany) containing 5 ml of HBSS (Lonza Inc, USA). Cell suspension was obtained by disintegration of the tissue using a GentleMACS tissue dissociator (Miltenyi Biotech). After cell separation, PBMCs and LNMCs were cryopreserved in FBS containing 10% DMSO for posterior determinations.

Animals. The study involved a total of 14 Indian origin female rhesus macaques (RM). Peripheral blood draws and lymph node biopsies were collected for measurement of a number of immunological parameters. All animals were housed at Yerkes National Primate Center at Emory University and were cared for in accordance with National Institute of Health guidelines and following protocols approved by the Institutional Animal Care and Use Committee. RMs were infected i.v. with $1x10^3$ $TCID_{50}$ of SHIV-Ad8.

Tissue processing. Lymphocytes were isolated from freshly obtained lymph node and spleens by passing homogenized tissue through a 70-μm cell strainer and lysing blood cells with ACK Lysis buffer. Tissue collection was performed as previously described (226). Cells to be later used for sorting were cryopreserved for short term at -80 degrees C in FBS media containing 10% DMSO.

Immunophenotyping and flow cytometry. Multi-color flow cytometric analysis was performed on mononuclear cells isolated from blood and lymph nodes according to standard procedures using monoclonal antibodies directed against RM markers and

human markers that also cross-react with the same markers in RM. Pre-determined optimal concentrations of the following antibodies and reagents were used: CD3-Alexa700 (clone SP34-2), CD4-Allophycocyanin-Cy7 (clone OKT-4), Bcl-6-PeTexasRed (clone K112-91), Ki67- FITC (clone B56), CD21-Pac Blue (clone B ly4), HLADR-APC from BD, CXCR5-PerCP eFlour 710 (clone MU5BEE), PD1-PeCy7 (clone J105), CD86- PeCy5 (clone IT2.2) from eBioscience and CD20-BV650 or PE-CF594 (clone 2H7), CD79B-PE (clone HM47), CD40- PerCP Cy5.5 (clone 5C3) , ICOS-PeCy 7 (clone C3984.A), CD40L PacBlue (clone 24-31) from Biolegend, and Live/Dead Fixable Aqua from Invitrogen. Flow cytometric data were acquired using LSRII flow cytometer using BD's FACS DiVA software. Acquired data were analyzed using Flow Jo version 9.3.2 following the gating strategy described in Figure1. Further analyses were performed using PRISM (GraphPad) and Excel (Microsoft Office 2011) software.

Cell Sorting. Lymphocytes from lymph nodes of SIV-uninfected as well as were used for sorting of $CD3^{+}CD20^{+}CD40L^{+}$ and $CD3^{+}CD20^{+}CD40L$-. Cell populations were sorted using FACS Aria II flow cytometer. Cells were first gated based on light scatter followed by positive gating on cells negative for Live/Dead Fixable Aqua and positive for CD3 and CD20.

Immunohistochemistry and Confocal Microscopy. Immunohistochemstry was performed on 5-mm tissue sections mounted on glass-slides, which were deparaffinized and rehydrated with double-distilled H_2O. Antigen retrieval was performed in 1xDako Target Retrieval Solution (pH 6.0) in a pressure cooker heating slides to 122 degrees C for 30s.

Slides were then rinsed in ddH2O and incubated for 10 minutes using Dako Protein block. Slides were then incubated with mouse anti-CD3 (1:500) and rabbit anti-CD20 (1:500) for 1 hour at room temperature. Next, slides were washed in TBS with 0.05% Tween-20. Slides were then incubated for an hour in the dark with secondary antibody cocktail containing donkey anti-rabbit Alexa 488 (1:500), donkey anti-mouse Alexa-594 and (1:500). After washing in TBS with 0.05% Tween-20, Prolong Gold with DAPI was applied to all the slides. Confocal microscope images were obtained using Olympus FV10i® Confocal Microscope with CellSens® 1.9 Digital Imaging software.

Imaging flow cytometry. Lymphocytes were isolated from RM spleen, mLN, sLN, and whole blood. Cells were washed with flow cytometry buffer (PBS, 1% bovine serum albumin, 0.1% sodium azide) followed by incubation for 30 minutes with fluorphore-conjugated antibodies diluted in 50µl of flow cytometry buffer to detect surface protein expression. Cells were washed with flow cytometry buffer and treated with BD Cytofix/Cytoperm for 17 minutes. Cells were washed with BD Perm/Wash buffer and stained intracellularly with DAPI (4',6-Diamidino-2-Phenylindole Dihydrochloride, Life Technologies) followed by fixation with 1% paraformaldehyde.

Data was collected at 60x with an extended depth of field filter on an ImageStream^X imaging flow cytometer equipped with INSPIRE software (Amnis, EMD Millipore). Image analysis was performed using IDEAS software (Amnis, EMD Millipore). Expression of CD3 and CD20 was assessed on focused (brightfield gradient RMS), single (brightfield aspect ratio vs. area), live (DAPI aspect ratio vs. intensity) cells.

Statistical Analyses. Except for RNA sequencing data, all statistical analyses were conducted using GraphPad Prism 5.0. Man-Whitney U tests were used to compare frequencies of lymphocyte populations in RM and humans (Figure 1 and 5). Spearman rank correlation tests were used to analyze all correlations (Figure 2). All p values less than 0.05 were defined as significant.

Results

A distinct population of $CD3^+CD20^+$ cells can be found within secondary lymphoid organs of Rhesus Macaque. We identified a population of cells within secondary lymphoid organs of rhesus macaque that express both CD3 and CD20 on the cell surface (Fig 1A). These cells are found at varying frequencies within different secondary lymphoid compartments in uninfected RM with the highest frequencies in spleen and mesenteric lymph node and negligible frequency in peripheral blood (Fig 1B). In order to insure these 'double positive' $CD3^+CD20^+$ where single cells extensive and conservative singlet gates were drawn during flow cytometric analysis (Supplementary Fig 1). Additionally, EDTA dissociation was performed during processing of all lymphocytes to dissociate any cell-cell conjugates. To insure these $CD3^+CD20^+$ cells were viable we performed Annexin staining. Indeed, these cells did not have any higher levels of apoptosis than the $CD3^+$CD20- T cells or $CD20^+$CD3- B cells as indicated by the comparable levels of annexin staining (Supplementary Fig 1). An extensive analysis of lymphocytes derived from spleen of RM was performed using ImageStream technology to further confirm that these cells were indeed single cells with viable nuclei. The top panel in Figure 1C depicts cells with $CD3^+$ surface membranes with some CD20 expression while the bottom panel depicts cells that are likely B cell in origin with some CD3 expression on the surface. We hypothesized these 'double positive' cells arise as a consequence of intimate contact between T and B cells likely occurring within germinal centers (GCs) of secondary lymphoid organs. This hypothesis is supported by the

observation that these 'double positive' cells are found at significantly higher frequencies in lymph nodes and spleen than in peripheral blood. Immunohistochemistry analysis on lymph nodes of RM was used to visualize several $CD3^+CD20^+$ cells within lymph nodes of RM (Fig 1D). Further, T and B cells can be seen in intimate contact, which may be the initiation of membrane exchange. We found that inclusion of doublets, dead cells and no treatment with EDTA resulted in higher numbers of $CD3^+CD20^+$ cells (Supplementary Fig 1). Live or dying lymphocytes with fragments of membranes from other dying B cells attached to them are likely to account for the increased numbers of 'double positive' cells that are found as such.

$CD3^+CD20^+$ cells in secondary lymphoid organs have enriched expression of T_{FH} associated markers. We hypothesized that these 'double positive' cells appear as a result of membrane exchange, or trogocytosis, between T and B cells. Their location within GCs, as demonstrated by immunohistochemistry, supports this hypothesis, as GCs are the site of intimate and frequent contact between T and B cells- more specifically, T_{FH} cells interacting with GC B cells. T_{FH} cells play a vital role in development of GC B cells by providing survival and selection signals to them (ref). We wanted to determine whether $CD3^+CD20^+$ cells had any T_{FH} like qualities. T_{FH} cells are commonly identified by their expression of CD4, CXCR5 and high levels of PD1. Indeed, analysis of $CD3^+CD20^+$ revealed an enrichment of cells with a T_{FH} phenotype (Fig 2A). There is even a significant positive correlation between the T_{FH} cells within the typical $CD4^+$ T cell population and the T_{FH} cells within the $CD3^+CD20^+$ population (Fig 2B). In addition, T_{FH}

cells express high levels of ICOS and are positive for Bcl6 and CD40L expression. A higher frequency of $CD3^+CD20^+$ cells expressed CXCR5, PD1 and ICOS than in non-T_{FH} cells or bulk $CD4^+$ T cells (Fig 2C). Similarly, BCl6, which is a key transcriptional factor for both T_{FH} cells and GC B cells, is expressed on a higher frequency of $CD3^+CD20^+$ cells than non-T_{FH} cells or bulk $CD4^+$ T cells (Fig 2C). Upon *in vitro* stimulation, $CD4^+$ T cells almost universally up-regulate CD40L expression, an important co-receptor used to provide 'help' to B cells. Again, nearly half the $CD3^+CD20^+$ cells express CD40L, unlike $CD20^+$CD3- B cells (Fig 2C). Functionally, T_{FH} cells are defined by their production of IL-21. Further, unlike Th_{17} cells T_{FH} cells do not produce significant amounts of IL-17. We found that $CD3^+CD20^+$ have an almost identical cytokine profile as T_{FH} cells (Fig 2D). Following *in vitro* stimulation, on average, 20% of $CD3^+CD20^+$ cells produce IL-21 but no IL-17 compared to 30% of T_{FH} cells further demonstrating the enrichment of cells of T_{FH} phenotype with the $CD3^+CD20^+$ population. Of note, typical B cells produce no IL-21 or IL-17. This enrichment of cells with a T_{FH} characteristic within the $CD3^+CD20^+$ populations support the hypothesis that these cells arise as a consequence of trogocytosis within germinal centers.

$CD3^+CD20^+$ cells express several B cell surface markers associated with the TCR-MHC synaptic complex. Trogocytosis is the exchange of fractions of membrane and therefore often results in the uptake of multiple surface molecules (190). Trogocytosis is also usually initiated at the immunological synapse and has been demonstrated in T cell, B cells, dendritic cells and natural killer cells (187, 259-262). We therefore hypothesized that $CD3^+CD20^+$ cells would express multiple B cells molecules, especially molecules

involved in the immunological synapse between T_{FH} cells and GC B cells. We analyzed the levels of expression of B cell surface markers HLA DR, CD86, CD40, CD79, IgD and CD21 (Fig 3A). For each marker, a higher percentage of $CD3^+CD20^+$ cells express the marker than traditional $CD4^+$ T cells. Notably, conventional T_{FH} cells also have CD20 and CD21 expression at higher MFI than non-T_{FH} $CD4^+$ T cells (Fig 3A). Interestingly, a majority of $CD3^+CD20^+$ cells express HLA DR on their surface. Activated antigen-mature cells initiate trogocytosis and RM $CD4^+$ T cells are known to up-regulate HLA DR upon activation. As an MHC molecule, HLA DR is part of the immunological synapse between T and B cells which further making it a likely candidate to be exchanged upon trogocytosis. Conversely, CD79B, a part of the B cell receptor complex is expressed only on about 16% of the 'double positive' cells, perhaps because the BCR complex is distal to the synaptic complex between T and B cells. Similarly, IgD, a BCR molecule and expressed largely on naïve B cells is exchanged infrequently- only 17% of $CD3^+CD20^+$ cells express IgD on their surface.

CD40L surface expression identifies cells of T_{FH} origin within $CD3^+CD20^+$ population. *In vitro* stimulation of RM lymphocytes leads to an upregulation of CD40L on $CD4^+$ T cells, including T_{FH} cells, but not on $CD20^+$ B cells. We found that approximately 50% of $CD3^+CD20^+$ cells (Fig 4A) also upregulate CD40L following *in vitro* stimulation. We analyzed $CD3^+CD20^+$ cells that upregulate CD40L and found them to be more T_{FH}-like, expressing higher levels of CXCR5 and IL-21 than $CD3^+CD20^+$ that did not upregulate CD40L (Fig 4A,B). To further confirm the $CD4^+$ T cell and T_{FH} origin of $CD3^+CD20^+CD40L^+$ cells we performed a gene set enrichment experiment on

$CD3^+CD20^+CD40L^+$ cells with a published T_{FH} cell gene set and found a significant positive enrichment (Fig 4C). Thus, $CD3^+CD20^+$ cells are comprised of a mixture of B cells that have acquired T cell membrane molecules and T_{FH} cells that have acquired B cell membrane markers. Finally, these data show that CD40L upregulation, following *in vitro* stimulation, can be used to identify $CD3^+CD20^+$ cells of T cell origin.

$CD3^+CD20^+$ cells increase in frequency following SHIV infection. In order to more closely study the kinetics of $CD3^+CD20^+$ cells in acute SIV/SHIV infection, lymph node biopsies were taken at day 14 prior to infection and at days 7, 14 and 21 post infection from RMs infected i.v. with $1x10^3$ $TCID_{50}$ of SHIV-Ad8. Following SHIV infection there is a significant increase in the frequency of $CD3^+CD20^+$ cells at 7 and 21 days post infection (Figure 5). This is in agreement with the increased occurrence of trogocytosis by activated cells at sites of immune activation as well as the large increase in frequency of both T_{FH} and GC B cell numbers after infection (188).

$CD3^+CD20^+$ population can be found within human lymphoid organs and reflect phenotype of $CD3^+CD20^+$ population in RM. The macaque and human immune systems are extremely well conserved (35, 263, 264). Nevertheless, we wanted to confirm the presence of a $CD3^+CD20^+$ lymphocyte population in humans. Lymph nodes and PBMCs from HIV^+ patients were analyzed and $CD3^+CD20^+$ cells in humans were found to reflect all aspects of the macaque 'double positive' population (Fig 6). As in macaque, human peripheral blood contains negligible numbers of $CD3^+CD20^+$ cells (Fig 6A). Interestingly, patients undergoing combined anti-retroviral therapy (cART) and an

uninfected human had lower frequencies of $CD3^{+}CD20^{+}$ cells in LN compared to HIV^{+} untreated patients (Fig 6B).

Discussion

Germinal centers within lymph nodes are the site of proliferation and selection of high affinity B cells that develop into memory B cells and plasma cells (256). Within GCs, T and B cells are in close contact as B cells depend on survival signals from T_{FH} cells. GC B cells present antigen to CD4 T cells that results in the formation of an immunological synapse. While T and B cells are conjugated, membrane fragments, including MHC:peptide complexes, can be exchanged between T and B cells (257, 265, 266). Membrane exchange or trogocytosis between T and B cells following immunological synapse formations has been described previously (259, 262). In this paper we have shown that lymphocytes that express surface markers of both T and B cells can be found within secondary lymphoid organs of primates. We were able to confirm the anatomical location of these cells by performing immunohistochemistry on lymph node biopsies of RMs. Confocal microscopy images demonstrate conjugates of T and B cells inside the GC and several cells with marked transfer of membranes (Figure 1). We hypothesize that these 'double positive' cells arise as a consequence of the frequent contact that occurs between T_{FH} and GC B cells within germinal centers. These 'double positive' cells are most likely a mix of T_{FH} cells and B cells as indicated by their intermediate levels of expression of several key T_{FH} and GC B cell markers (Fig 2 and 3). Further, ImageStream analysis also revealed that 'double positive' cells are more often T cells rather than B cells (Figure 1). This is agreement with the fact that GCs are sites of pronounced B cell apoptosis as a result of undergoing active selection in response to infectious agents (267). We were also able to sort $CD3^{+}CD20^{+}$ cells that upregulate CD40L following *in vitro*

stimulation and gene set enrichment analysis revealed that these cells were of T cell origin and were enriched for a T_{FH} gene signature. Thus, in CD40L expression on the surface can be used to distinguish $CD3^{+}CD20^{+}$ cells of T cell origin from those of B cell origin. Finally, in both SIV infected macaques and untreated HIV infected humans we found a higher frequency of these $CD3^{+}CD20^{+}$ cells (Figure 5 and Figure 6B).

It is unclear what the consequence of such membrane exchange might be on these cells. It can be expected that the acquisition or loss of self-cell molecules may alter survival as well as function of these cells. Acquisition of co-stimulatory molecules may lead to continued activation and survival signaling (191, 268). With the acquisition of peptide:MHC molecules, T_{FH} cells may act at antigen presenting cells (269). Recent studies using two-photon imaging of germinal centers has shown that GC B cells are motile and form stable GC-T cell contacts. GC T cells, likely T_{FH} cells, were also seen to carry fragments of B cells on them several minutes after their interaction with GC B cells (122). Most contacts appeared to be of short duration with a small percentage of interactions lasting more than 10 minutes. GC T cells were seen picking up, carrying fragments from dead B cells, and continuing to interact with other B cells. TCR analysis would reveal whether these ‘double positive’ cells have a restricted TCR repertoire. T_{FH} cells that have undergone trogocytosis can therefore potentially be used to identify activated, antigen-specific and even high-affinity T_{FH} cells(122, 258). Analysis of these cells following immunization may facilitate SIV/SHIV immunization studies.

Table 1

Paired samples (PBMC and LNMC)											
	GENDER	AGE	pVL	Log pVL	CD4	% CD4	CD8	% CD8	CD4:CD8	cART	COMMENTS
G-FASS	M	36	NA	NA	970	39	522	21	1.86	NA	HIV neg
G101	M	35	40	1.6	102	10	547	53	0.186	YES	41 months with ART
G104	M	26	104	2.02	398	26	659	46	0.6	YES	1 month with ART
G102	M	49	352055	5.55	363	19	1208	63	0.3	NO	
G109	M	19	124405	5.09	539	4.37	436	30	1.23	NO	
G110	M	25	14105	4.15	606	24	1316	52	0.46	NO	
G-SES	M	44	213173	5.33	425	17	1407	57	0.3	No	
G115	F	33	926025	5.97	351	17	884	44	0.4	NO	
G 121	M	45	1763	3.25	774	31	1392	56	0.56	NO	
G90 JMQV	M	48	133854	5.13	415	10	3271	80	0.13	NO	
G91 BSB	F	18	12331	4.09	566	24	1308	55	0.43	NO	
G86 RPP	M	21	209518	5.32	462	19	1445	59	0.32	NO	
G126 CGGM	M	23	133607	5.13	507	28	891	50	0.57	NO	
LNMC only											
	GENDER	AGE	pVL	Log pVL	CD4	% CD4	CD8	% CD8	CD4:CD8	cART	COMMENTS
G-DJQP	M	29	61586	4.79	406	25	876	54	0.46	NO	
G-JGVM	M	21	35847	4.55	258	20	621	49	0.41	NO	
G-VABD	M	26	71621	4.86	841	30	1303	46	0.65	NO	
G-GEMD	M	43	4481	3.65	548	18	1848	60	0.3	NO	
G-ODMCC	M	52	25659	4.41	501	25	1042	52	0.48	NO	
G47 JISV	M	29	58481	4.77	423	18	1379	59	0.31	NO	
G60 OAAM	M	25	67764	4.83	721	21	1471	44	0.61	NO	

Figures

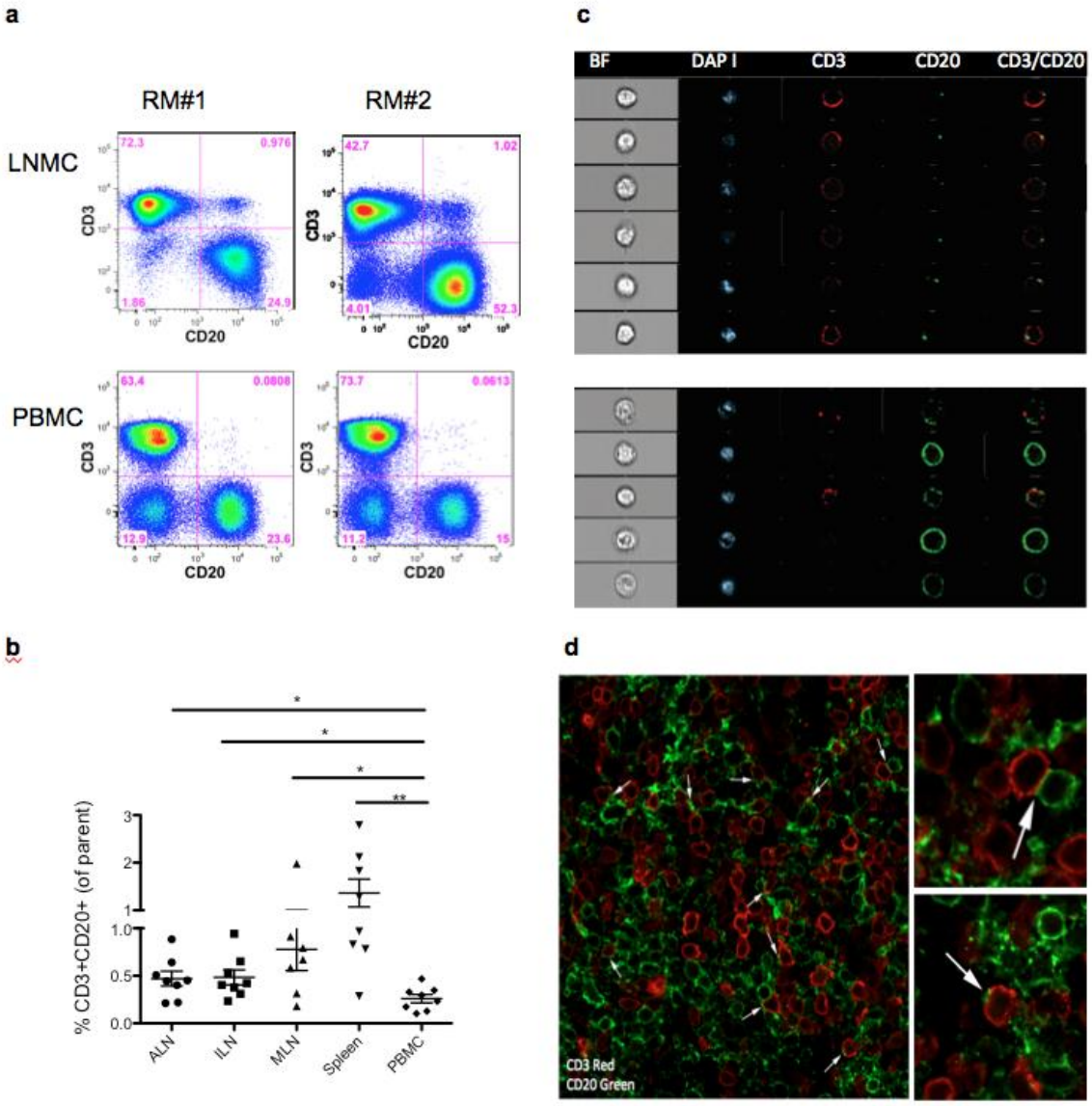


Figure 1. A distinct lymphocyte population identified by the expression of CD3 and CD20 on the cell surface is found in secondary lymphoid organs. (A) Representative flow cytometry plot showing live lymphocytes from rhesus macaque. $CD3^{+}CD20^{+}$ cells are found in lymph nodes but are absent in peripheral blood. (B) Tissue distribution of

$CD3^+CD20^+$ cells within various secondary lymphoid organs of RM. (C) ImageStream analysis of live lymphocytes derived from RM spleen. (D) Representative Immunohistochemistry image showing CD3 (red) and CD20 (green) cells within RM lymph nodes. White arrows indicate point to $CD3^+CD20^+$ cells. The top right panel depicts a T cell (red) in close contact with a B cell (green) and the bottom right panel shows a single T cell (red) with CD20 (green) on its surface.

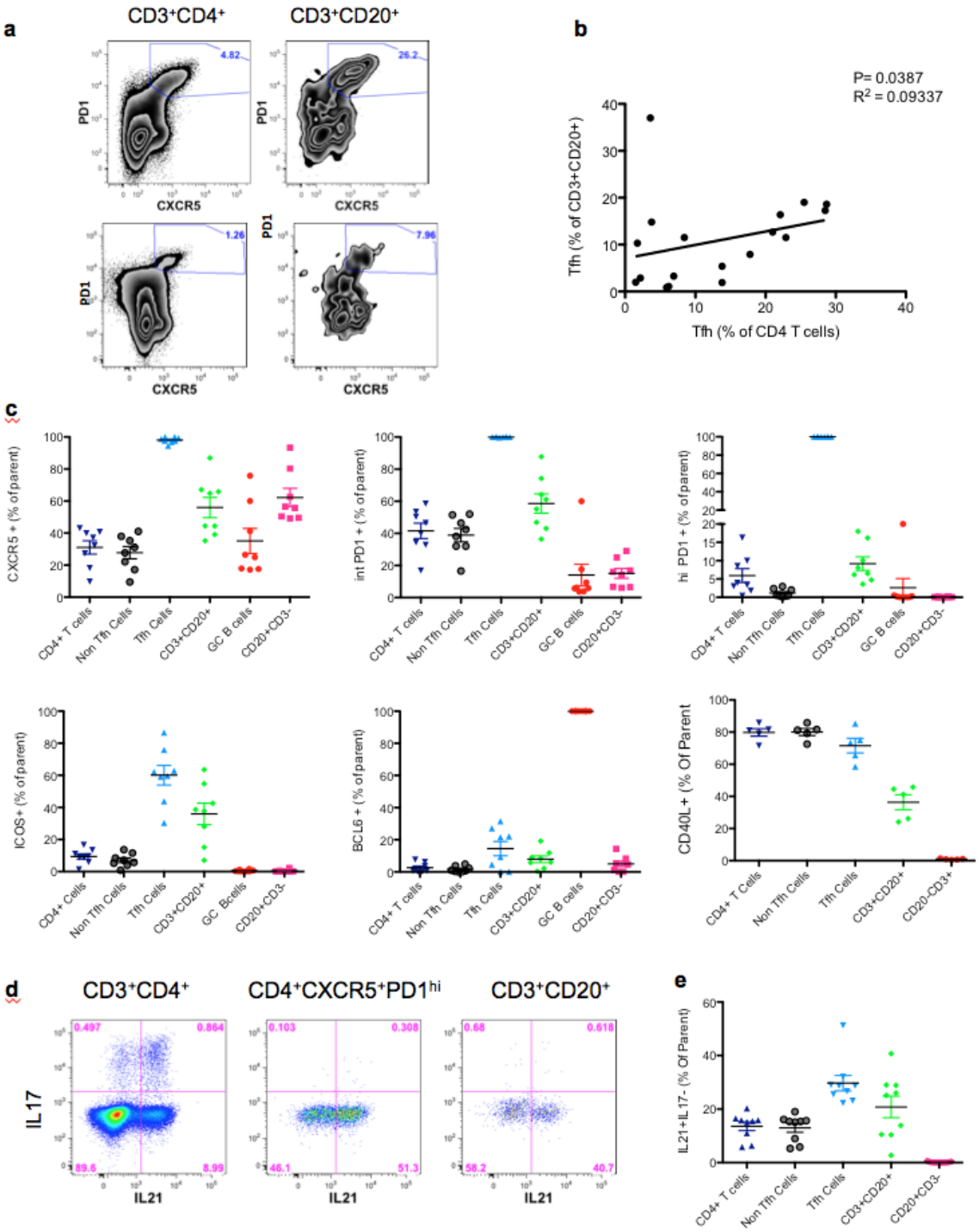

a
CD3+CD4+
CD3+CD20+
PD1
CXCR5
4.82
26.2
1.26
7.96
b
P= 0.0387
R2 = 0.09337
Tfh (% of CD3+CD20+)
Tfh (% of CD4 T cells)
c
CXCR5 + (% of parent)
int PD1 + (% of parent)
hi PD1 + (% of parent)
ICOS+ (% of parent)
BCL6 + (% of parent)
CD40L+ (% Of Parent
CD4+ T cells
Non Tfh Cells
Tfh Cells
CD3+CD20+
GC B cells
CD20+CD3-
d
CD3+CD4+
CD4+CXCR5+PD1hi
CD3+CD20+
IL17
IL21
0.497
0.864
89.6
8.99
0.103
0.308
46.1
51.3
0.68
0.618
58.2
40.7
e
IL21+IL17- (% Of Parent)

Figure on previous page.

Figure 2. $CD3^+CD20^+$ cells in secondary lymphoid organs have enriched expression of markers associated with T_{FH} cells. (A) A representative contour plots depicting PD1 and CXCR5 expression on lymph node derived lymphocytes. (B) Correlation between the frequency of T_{FH} cells within the $CD4^+$ T cell population and the frequency of T_{FH} within the $CD3^+CD20^+$ population. (C) Cumulative dot plots show relative expression of T_{FH} phenotype markers CXCR5, PD1, ICOS and BCl6 in $CD4^+$ T cells, Non T_{FH} $CD4^+$ T cells, T_{FH} cells, $CD3^+CD20^+$ cells, germinal center B cells and $CD20^+$CD3- B cells. (D) Representative flow cytometry plot (D) and cumulative dot plot (E) showing production of IL-21 and IL-17 by lymph node derived lymphocytes following 4 hour stimulation using PMA and ionomycin.

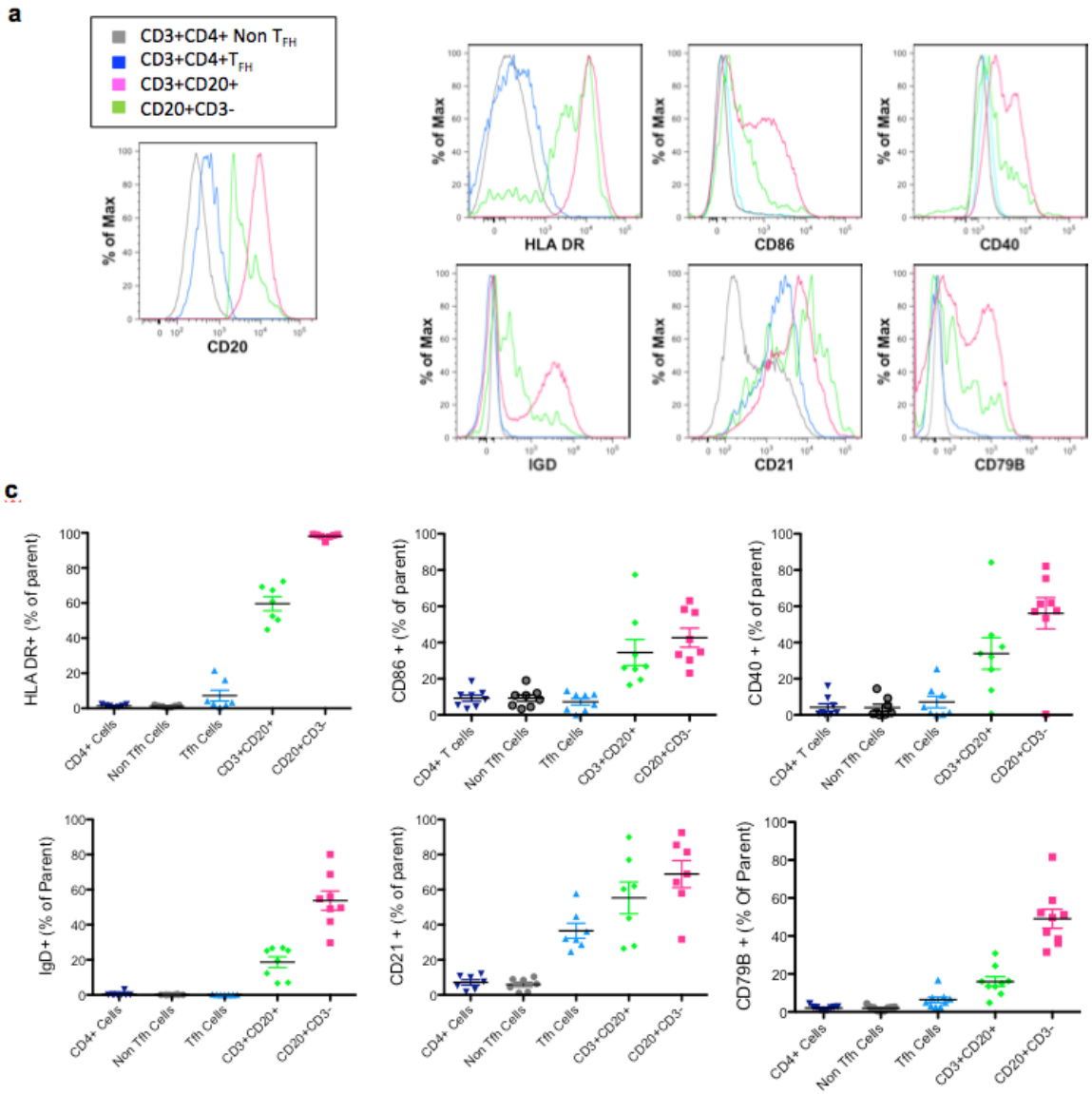


Figure 3. CD3^{+}CD20^{+} cells express several B cell surface markers associated with the TCR-MHC synaptic complex. (A, B) Histogram of relative CD20 expression on T_{FH} cells, Non- T_{FH} cells, CD3^{+}CD20^{+} cells and CD20^{+}CD3- B cells. (C) Representative cumulative dot plots showing expression levels of HLA-DR, CD79b, IgD, CD21, CD86 and CD40 on the same populations.

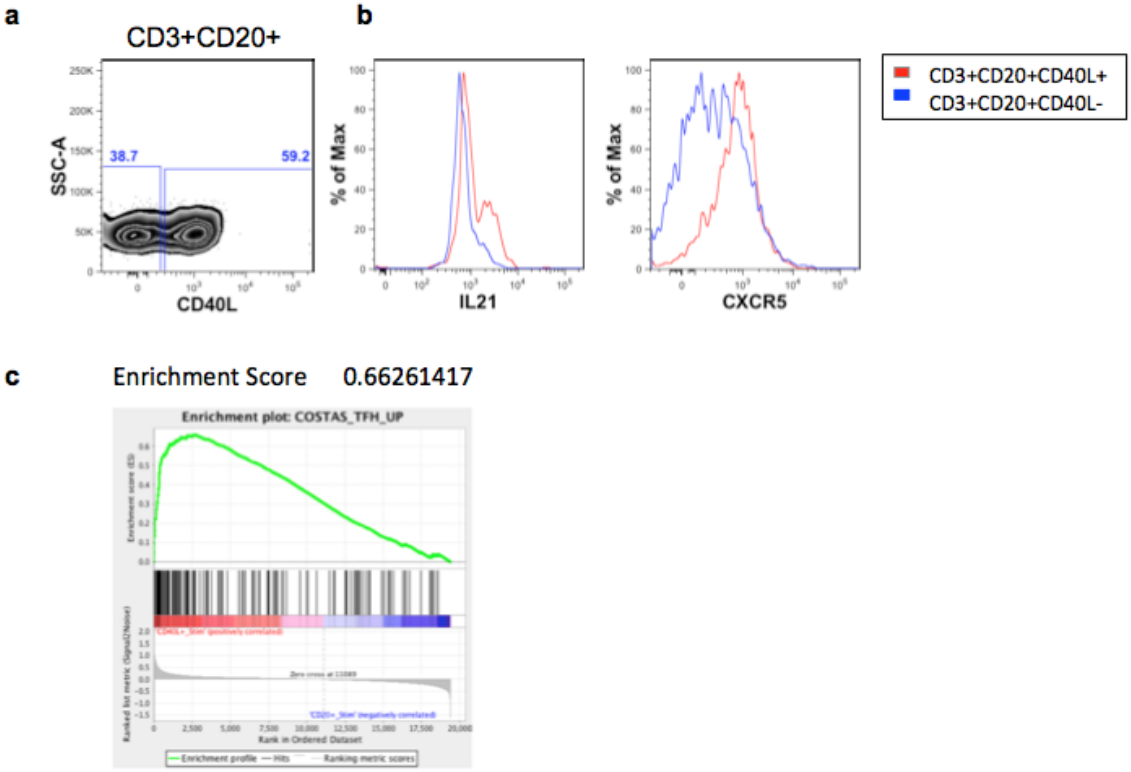


Figure 4. CD40L surface expression identifies cells of T_{FH} origin within $CD3^+CD20^+$ population. (A) Representative flow cytometry plot shows CD40L expression within $CD3^+CD20^+$ cells. (B) Representative histograms showing relative expression of T_{FH} markers CXCR5, and IL-21 on $CD40L^+$ and CD40L- $CD3^+CD20^+$ cells. (C) Gene enrichment plot of T_{FH} genes on $CD3^+CD20^+CD40L^+$ sorted population.

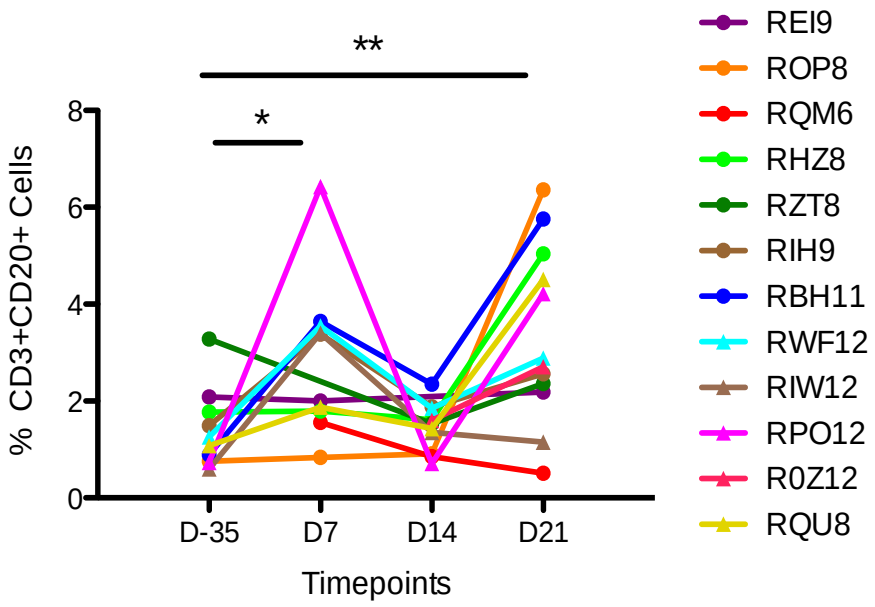


Figure 5. $CD3^{+}CD20^{+}$ cells increase in frequency following SHIV infection.

Frequency of CD3+CD20+ cells as a percent of parent populations within lymph nodes of SHIV infected RM.

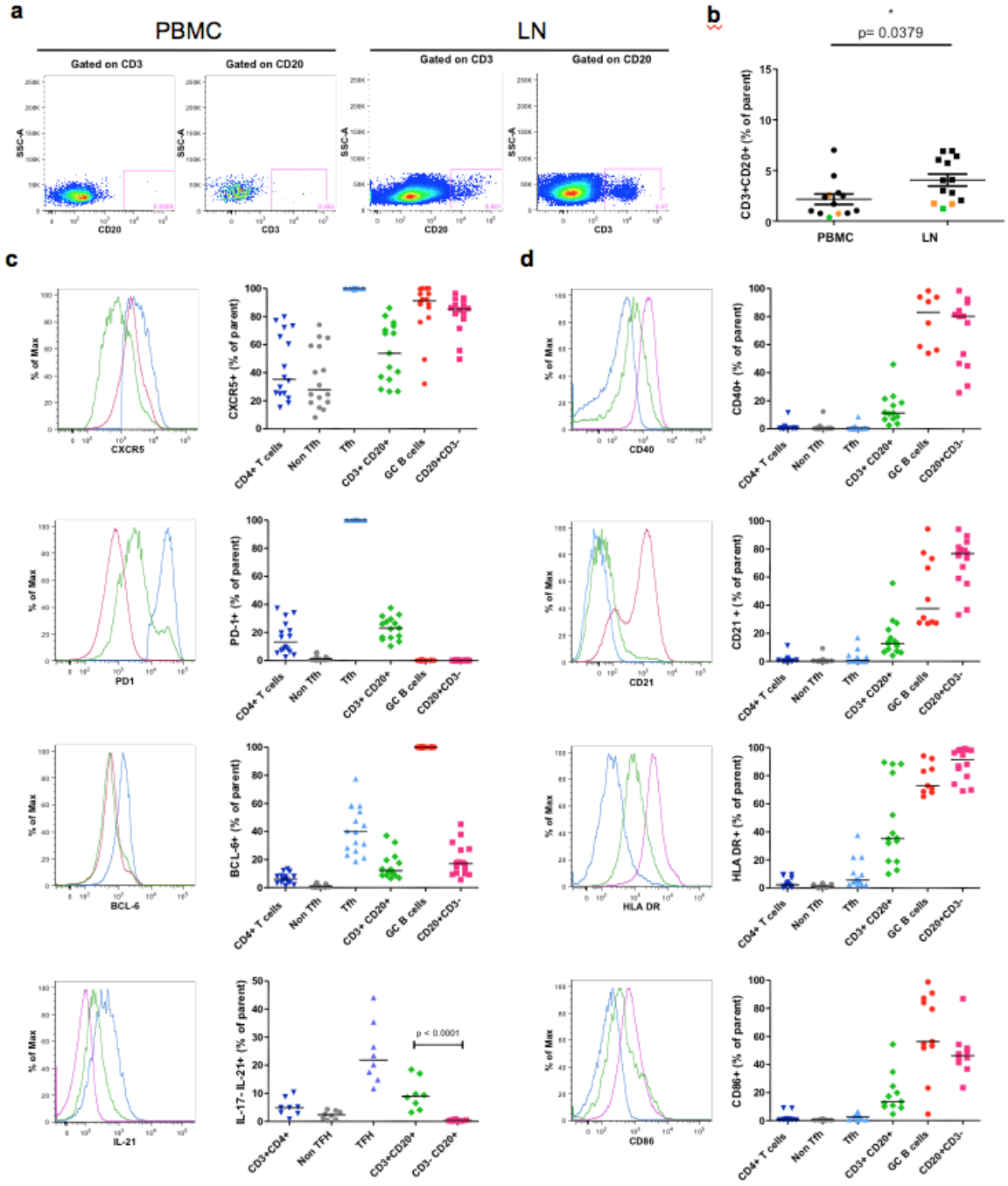
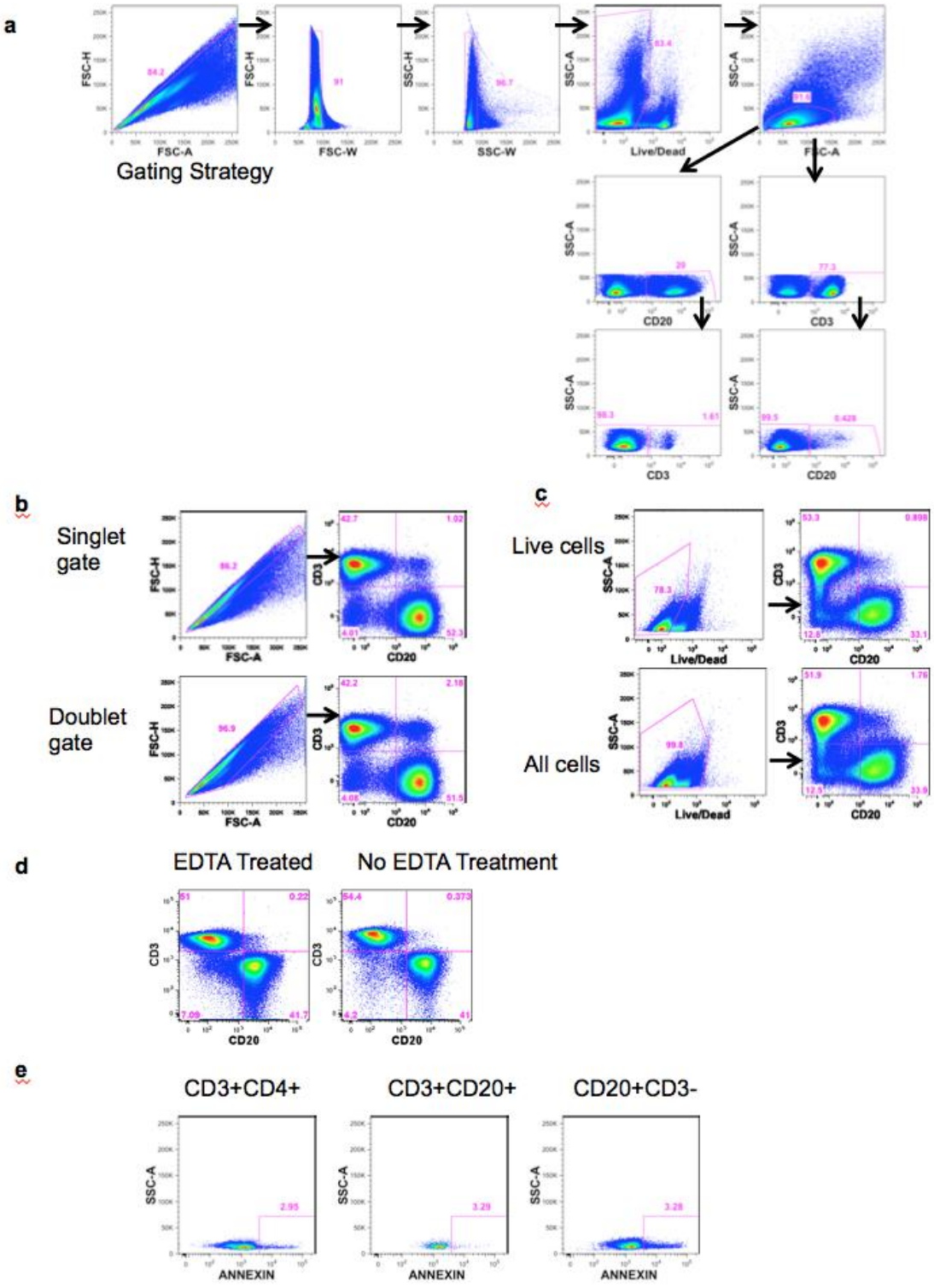
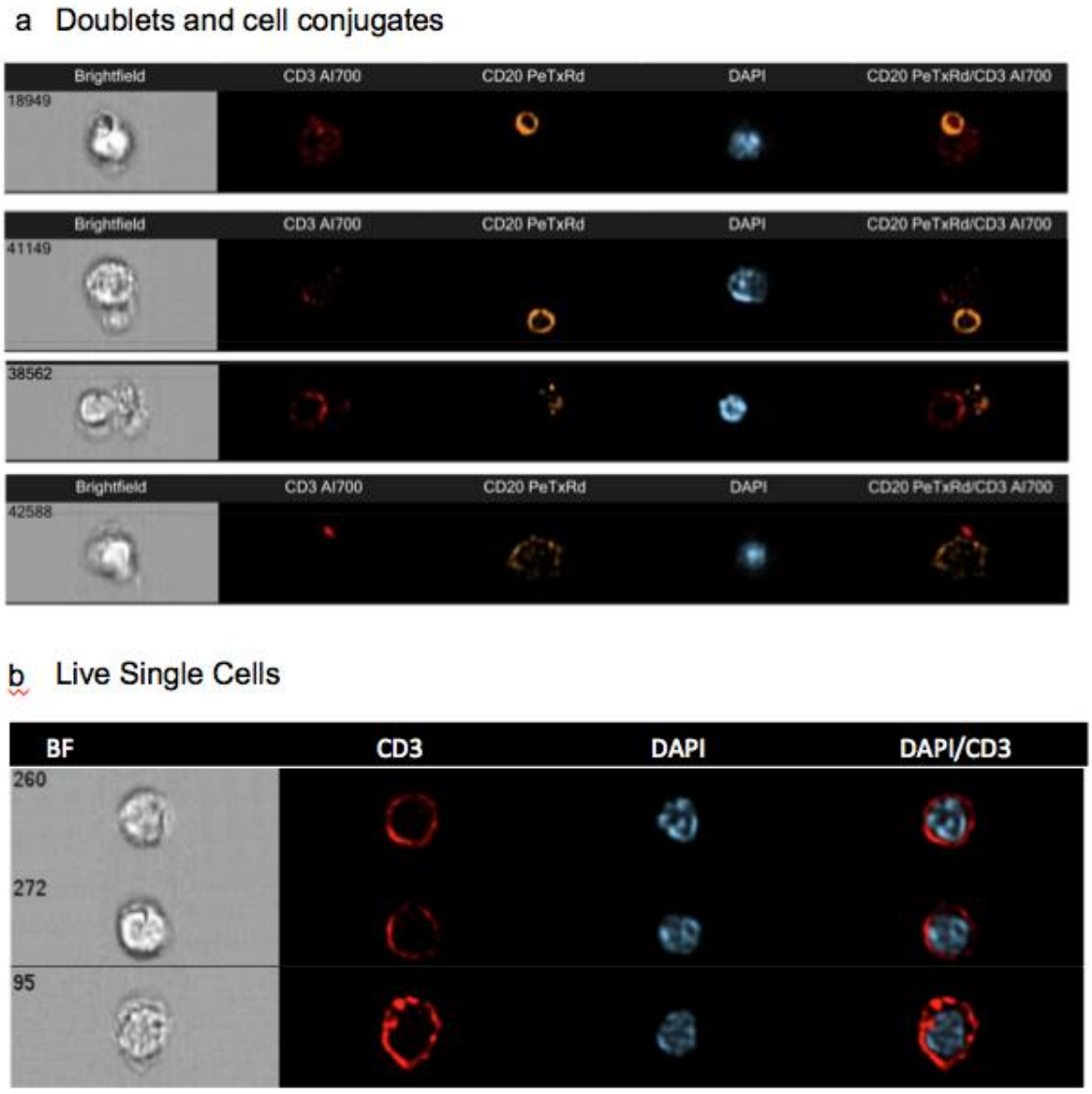

a
PBMC
LN
Gated on CD3
Gated on CD20
SSC-A
CD20
CD3
b
p= 0.0379
CD3+CD20+ (% of parent)
PBMC
LN
c
d
% of Max
CXCR5
CXCR5+ (% of parent)
PD1
PD-1+ (% of parent)
BCL-6
BCL-6+ (% of parent)
IL-21
IL-17- IL-21+ (% of parent)
p < 0.0001
CD40
CD40+ (% of parent)
CD21
CD21 + (% of parent)
HLA DR
HLA DR+ (% of parent)
CD86
CD86+ (% of parent)
CD4+ T cells
Non Tfh
Tfh
CD3+ CD20+
GC B cells
CD20+CD3-
CD3+CD4+
Non TFH
TFH
CD3+CD20+
CD3- CD20+

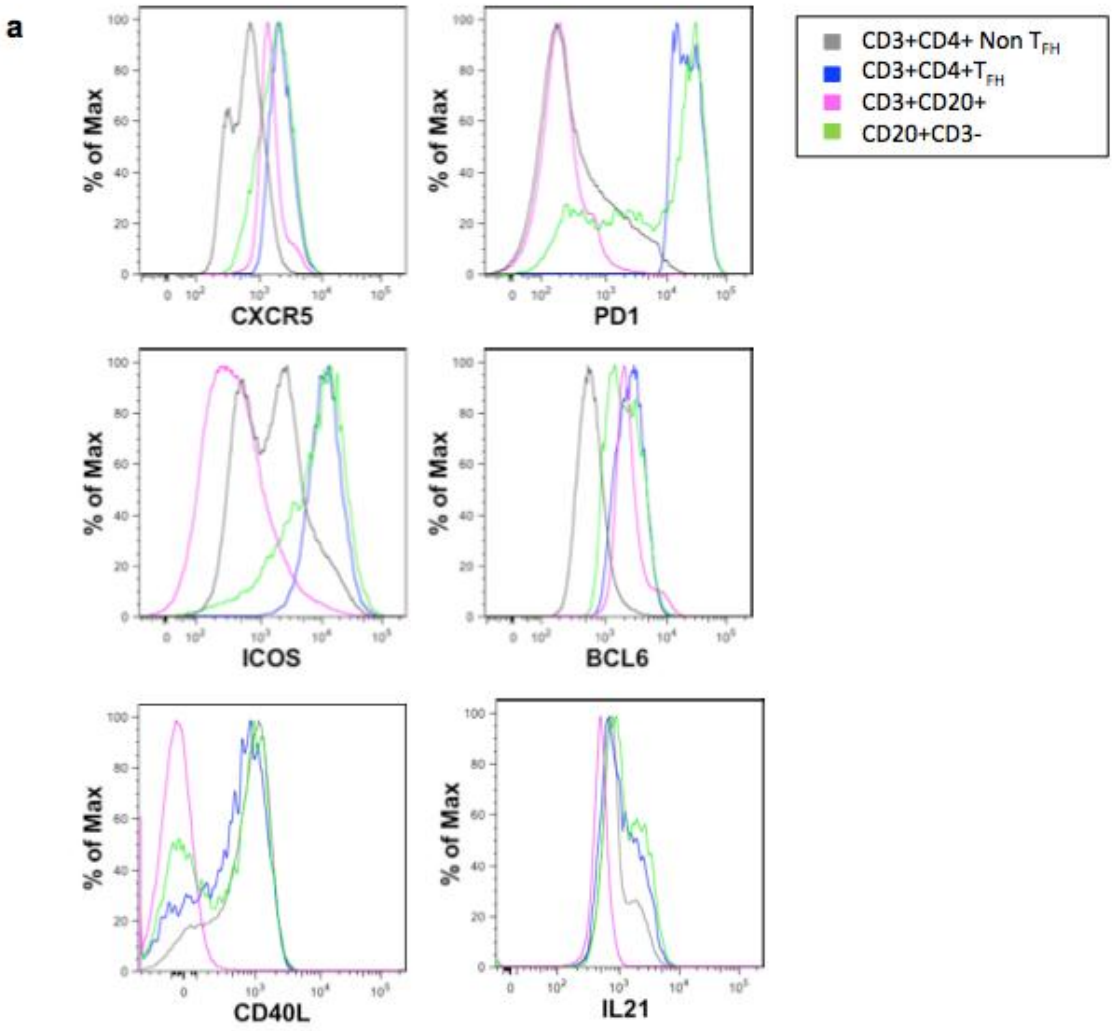
Figure 6. $CD3^+CD20^+$ population can be found within human lymphoid organs and reflect phenotype of $CD3^+CD20^+$ population in RM. (A) Representative flow cytometry plot (A) and scatter plot (B) showing frequency of $CD3^+CD20^+$ in HIV infected human lymphocytes derived from peripheral blood and lymph node. In the scatter plot, orange indicates lymphocytes from ART treated humans and green indicates lymphocytes from an HIV uninfected human. (C, D) Representative histograms and cumulative dot plots depicting relative levels of expression of T_{FH} and B cell markers on human lymph node derived lymphocyte populations.


a
FSC-H
FSC-A
FSC-W
SSC-W
SSC-A
Live/Dead
CD20
CD3
Gating Strategy
b
Singlet gate
Doublet gate
c
Live cells
All cells
d
EDTA Treated
No EDTA Treatment
e
CD3+CD4+
CD3+CD20+
CD20+CD3-
ANNEXIN

Supplementary Figure 1. Representative flow cytometry plot showing (A) gating strategy used to identify $CD3^{+}CD20^{+}$ cells, (B) singlet and doublet gating, (C) gating on live and dead cells, (D) EDTA treated and untreated lymphocytes. (E) Annexin staining on live singlet gated lymphocyte populations within lymph nodes of RM.



Supplementary Figure 2. Representative image of (A) doublets and cell-cell conjugates and (B) single cells with live nuclei within RM lymph nodes identified by ImageStream analysis.



Supplementary Figure 3. Histograms showing relative expression of T_{FH} surface markers CXCR5, PD1, ICOS, BCl6 and CD40L and cytokine production of IL-21 on T_{FH} cells, non-T_{FH} cells, $CD3^{+}CD20^{+}$ 'double positive' cells and $CD20^{+}CD3^{-}$ B cells.

Chapter Five: Discussion

Over the last three decades a global research effort has led to remarkable advancements in the understanding of HIV viral pathogenesis and expanded our understanding of human immunology. Nonetheless, HIV, with its beguiling diversity and ability to co-opt the immune system, has continually mounted significant challenges to researchers. Today, researchers are refocusing efforts to develop an efficient, safe and easily scalable strategy to finally stop the spread of HIV. Several aspects of the host immune response to HIV/SIV still need to be fully understood before this goal can be achieved.

Although, ultimately ineffective, the immune system mounts a robust response to HIV infection. In fact, the continued immune activation leads to much of the severe pathology seen in chronic infection. In my research here I have focused on studying three main aspects of the immune response I the context of SIV i) the contributions of CD8 mediated virus control, ii) regulation within germinal centers (GCs) and the cell types involved, and iii) a previously unknown occurrence of lymphocytes with surface expression of both T and B cell markers. These findings contribute to a better understanding of the complex immune dynamics in the context of SIV infection.

Using the model of antibody mediated CD8 depletion we examined the role of CD8 T cells in mediating viral control. Our first finding was that CD8 depletion led to a fold increase in viral load that was inversely correlated with pre-depletion viral load. In controllers we saw, on average, a 5000-fold increase in viral load while progressors only had an increase of around 150 fold. This data conclusively demonstrates that CD8 T cells play a vital role in controlling virus replication in controllers. Interestingly, after the initial explosion following depletion, viral loads in controllers showed a downward trend such that at necropsy (between 3 to 14 days after depletion) controllers once again had lower viral loads than progressors. This is likely due to the fact that antibody-mediated depletion of CD8s is only temporary and some CD8 T cells begin to reappear within a week after depletion.

T-bet regulates differentiation of CD8 T cells into short-lived effector cells and activates production of IFN-G and cytotoxic molecules (212, 270-272). Further, higher expression of T-bet is correlated with improved control of viral infection. Previous studies have reported that in HIV infected elite controllers, HIV specific CD8 T cells have high T-bet expression (216) while in chronic HIV patients, CD8 T cells have low levels of T-bet expression (214). We also found that the level of T-bet expression on pre-depletion CD8 T lymphocytes best predicts the post-CD8 depletion increase in viremia. However, we did not see any correlations between T-bet expression on SIV-specific cells and plasma viral load. It is somewhat surprising that we find controllers, with lower viral load to have lower CD8 T cells T-bet expression pre-depletion. It is possible that the high level of T-bet expression seen in progressors is a result of high viremia and indicates the activation

as a consequence of antigenic persistence. A large number of NK cells in macaques express CD8 and the CD8 depleting antibody therefore depletes them (200). In NK cells, T-bet regulates sphingosine-1 phosphate receptor 5 (SIP5) that regulates NK cell differentiation in the BM (273). Just as in CD8 T cells, T-bet and Eomes also play reciprocal roles in NK cell development and are important for the maintenance of two distinct lineages (274). We found that pre-depletion T-bet expression on NK cells in lymph nodes was also positively correlated with viral load at necropsy.

After depletion, we found an increase in viral DNA in all CD4 T cell subsets in progressor RMs. However, in the controllers we found a decrease in cell-associated viral DNA in central memory CD4 subset in 4 out of 5 controller RM. This suggests that in controllers, CD8 T cells extend different levels of control over different CD4 T cell subsets, potentially different transcriptional control. This could also suggest a change in the activation state or number of T_{CM} cells in controller RM in the face of increased viral load. Unsurprisingly, we also found an increase in activation status as measured by Ki-67 expression in all CD4 T cell subsets. However, the increase in Ki-67 expression was significantly higher in control RM than in progressor RM, concomitant with the higher fold increase in viral load seen in the controller RM. Presumably, progressors with higher levels of viremia pre-depletion already had high levels of activation and therefore only saw a modest increase in CD4 T cell activation post-depletion. Overall, these data are in agreement with previous reports suggesting CD8 T cells mediate significant control via non-cytolytic mechanisms.

In my second research project, I analyzed a subset of regulatory T cells found within GCs called T follicular regulatory (T_{FR}) cells. T_{FR} cells are a distinct population that can be identified within GCs of RM that in addition to expressing the key T_{FH} surface and inter cellular markers also express Foxp3 the master regulator of T_{REG} cells. Unfortunately, previous studies on T_{FH} cells have failed to distinguish between the T_{FR} and T_{FH} cells within the lymph node. On the subject of T_{REG} cells, studies often have contradictory results that are to a large extent due to the lack of consistency of markers used to define the population. In our study, we used both CD25 and Foxp3 to identify the regulatory populations- T_{REG} and T_{FR} cells. In depth flow cytometry phenotyping confirmed their low expression of CD127 and high expression of Helios, another transcriptional regulator, strengthening out identification of the populations (176). We sorted bulk CD4 T cells, T_{FH}, T_{REG} and T_{FR} populations and performed RNA sequencing to confirm their distinct yet overlapping phenotype of T_{FR} cells. Although they share a large number of surface markers with T_{FH} cells, studies in mice have shown that T_{FR} cells differentiate from T_{REG} cells.

Following SIV infection, we find an increase in the frequency of T_{FH} and T_{FR} cells within lymph nodes of SIVmac251 infected RM. However the relative increase in the numbers of T_{FH} cells is not matched by a similar increase in T_{FR} cell frequency resulting in a reduced T_{FR} to T_{FH} ratio in chronically infected RM. This reduced ratio may, in part, explain the high rate of proliferation seen in T_{FH} cells and the ultimate accumulation of T_{FH} cell s. Further, studies in mice have emphasized the importance of the T_{FR} to T_{FH} ration over just the number of T_{FH} cells in determining GC outcome (241).

We also looked at the level of infection within T_{FH}, T_{FR} and T_{REG} populations and found comparable high levels of SIV DNA. While T_{FH} cells are known to harbor high numbers of virus, the finding that T_{FR} and T_{REG} populations in lymph nodes also harbor similar levels of virus is surprising. Interestingly, we found that while both T_{FH} and T_{REG} cells have low surface expression of CCR5, a significantly higher frequency of T_{FR} cells express CCR5. Previous studies provided little consensus on the extent to which T_{REG} cells are susceptible to HIV infection and whether or not they serve as a reservoir of virus replication. Early *in vitro* studies showed that T_{REG} are highly susceptible to infection although these findings were not supported *in vivo*. In our data, we found that in both T_{FR} and T_{REG} within lymph node harbor high levels of virus suggesting that T_{FH} cells serve are not the only major reservoir of virus within lymph nodes in chronically infected RM.

We found increased levels of transcripts associated with immune activation in all three populations following SIV infection. These data emphasize the importance of regulation of immune activation and the apparent inability of T_{REG} and T_{FR} cells to regulate immune activation in chronic SIV infection. Studies in mice have demonstrated that T_{FR} cells are able to control the proliferation of T_{FH} cells and suppress the outgrowth of non-antigen specific B cells. While we were unable to perform *in vivo* experiments to demonstrate the regulatory capability of T_{FR} cells, we were able to find a negative correlation between the frequencies of T_{FR} cells and the frequencies of GC T_{FH} cells and GC B cells. We also found a negative correlation between the frequency of T_{FH} cells and proliferating CD4 T

cells within lymph nodes. These findings seem to support the regulatory role played by T_{FR} cells in the dynamic control of both T_{FH} and GC B cell populations.

T_{FR} cells may play a beneficial role by controlling T_{FH} proliferation and activation ultimately maintaining selective pressure on GC B cells and controlling virus replication. In addition to T_{FH} cells, T_{FR} cells may directly control proliferation and survival of GC B cells and may also impact the differentiation of GC B cells to plasma cells or memory B cells. The study of T_{FR} cells with special attention to their relative ratio to T_{FH} cells, in RM and other non-human primates is therefore essential to the through understanding of germinal center responses especially in the context of vaccine design and development.

My third research project focused on a previously unknown population of lymphocytes that have dual expression of T and B cell markers. The GC is the site of intense proliferation and is tightly packed with activated T and B cells. Activated T and B cells are capable of membrane exchange resulting in the swapping of intact proteins, known as trogocytosis. We discovered single, viable cells within lymph nodes of both non-human primates and humans that express T and B cells markers on their cell surface. This population was only found within secondary lymphoid organs and not in the peripheral blood. This likely reflects the high density of T and B cells within lymph nodes and the high number of interactions between these cells that occur at these sites. Confocal microscopy images further confirmed their localization within GCs. To ensure we were analyzing single, viable cells, we used stringent gating techniques and treated our samples with EDTA to dissociate any conjugates. We were able to confirm that these

cells were in fact single cells by ImageStream analysis. The GC is also the site for abundant B cell death and correspondingly, we did find several T cells with blebs of B cell membrane attached to them. We were careful to exclude any such conjugates from our analysis.

Trogocytosis is initiated at the immunological synapse (262). The immunological synapse between T and B cells consists of the TCR molecule, cognate peptide:MHC II complex and several accompanying co-stimulatory and adhesion molecules. Accordingly, we found $CD3^+CD20^+$ cells to express several B cell surface markers associated with the TCR-MHC synaptic complex. Interestingly, molecules distal from the synapse such as BCR and CD79 were rarely found on $CD3^+CD20^+$ cells. We also found that a large share of 'double positive' cells within lymph nodes is enriched for T_{FH} associated markers. In fact, T_{FH} cells are the ideal candidates to undergo trogocytosis. Intravital two-photon imaging has shown than T_{FH} cells are extremely motile and quickly sample several B cells before forming longer interactions with a few cognate B cells (121). It is possible that following such sustained, high affinity contacts, T_{FH} cells acquire some of the B cell membrane along with key B cell markers via membrane exchange. Both flow cytometry and ImageStream analysis revealed that $CD3^+CD20^+$ cells were in fact a mixed population with some cells of T cell origin and some of B cell origin. We sorted *in vitro* stimulated $CD3^+CD20^+$ cells based on CD40L expression and found that $CD3^+CD20^+CD40L^+$ cells were enriched for a T_{FH} gene signature. CD40L surface expression can therefore be used to differentiate cells of T_{FH} cell origin from those of B

cell origin. Similar $CD3^+CD20^+$ population can also be found within human lymphoid organs and reflect phenotype of $CD3^+CD20^+$ population in RM.

While it is clear that these single cells do capture and then present molecules from other cells, the stability and functional significance of this is as yet unknown. Further, we were unable to maintain these cells *in vitro* although this may be a result of our inability to replicate the lymph node environment and architecture *in vitro*. Lymphocytes that have undergone trogocytosis within GCs such as the ones we have identified may be an effective way to identify T and B cells that have recently undergone sustained interactions. By isolating or tracking these cells we may be able to learn more about the specificity and fate of B cells that have likely received survival signals from cognate T_{FH} cells. Further study of lymphocytes that have undergone trogocytosis, especially in the context of HIV/SIV infection may reveal additional means to identify antigen specific and activated cells at the site of immune activation or inflammation.

Conclusion

The research findings presented here provide strong evidence for the protective role played by CD8 T cells in efficiently suppressing virus. Recent efforts to understand the complex dynamics within germinal centers following infection or immunization have paid significant attention to T follicular helper cells. Here, I have provided a detailed analysis of T follicular regulatory cells within germinal centers and found evidence that supports their regulatory function. It is important that future studies on germinal center

responses distinguish between T follicular helper cells and T follicular regulatory cells. Finally, I have provided evidence of a lymphocyte population within lymph nodes of both humans and rhesus macaque that have surface expression of T and B cell markers. This population of cells may provide a simple technique to identify activated and strongly interacting cells following immunizations. In all, these finding contribute to the further understanding of the complex immune response to S/HIV.

References

1. Masur, H., M. A. Michelis, J. B. Greene, I. Onorato, R. A. Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles. 1981. An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *The New England journal of medicine* 305: 1431-1438.
2. Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *The New England journal of medicine* 305: 1425-1431.
3. Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220: 868-871.
4. Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 224: 497-500.
5. Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 225: 840-842.
6. Office of AIDS Research, N. I. o. H. 2015. FY 2016: NIH Trans-NIH Plan for HIV-related research U. S. D. o. H. a. H. Services, ed.
7. Hladik, F., and M. J. McElrath. 2008. Setting the stage: host invasion by HIV. *Nature reviews. Immunology* 8: 447-457.
8. BarreSinoussi, F. 1996. HIV as the cause of AIDS. *Lancet* 348: 31-35.
9. Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381: 661-666.
10. Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85: 1135-1148.
11. Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381: 667-673.

12. Berson, J. F., D. Long, B. J. Doranz, J. Rucker, F. R. Jirik, and R. W. Doms. 1996. A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. *Journal of virology* 70: 6288-6295.
13. Arien, K. K., G. Vanham, and E. J. Arts. 2007. Is HIV-1 evolving to a less virulent form in humans? *Nature reviews. Microbiology* 5: 141-151.
14. Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo. 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* 328: 543-547.
15. Hughes, A., and T. Corrah. 1990. Human immunodeficiency virus type 2 (HIV2). *Blood reviews* 4: 158-164.
16. Kanki, P. J., K. U. Travers, M. B. S, C. C. Hsieh, R. G. Marlink, N. A. Gueye, T. Siby, I. Thior, M. Hernandez-Avila, J. L. Sankale, and et al. 1994. Slower heterosexual spread of HIV-2 than HIV-1. *Lancet* 343: 943-946.
17. Zhu, T., B. T. Korber, A. J. Nahmias, E. Hooper, P. M. Sharp, and D. D. Ho. 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* 391: 594-597.
18. Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers. 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 228: 1201-1204.
19. Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* 397: 436-441.
20. Sharp, P. M., D. L. Robertson, and B. H. Hahn. 1995. Cross-species transmission and recombination of 'AIDS' viruses. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 349: 41-47.
21. Rambaut, A., D. Posada, K. A. Crandall, and E. C. Holmes. 2004. The causes and consequences of HIV evolution. *Nature reviews. Genetics* 5: 52-61.
22. Clapham, P. R., and R. A. Weiss. 1997. Immunodeficiency viruses. Spoilt for choice of co-receptors. *Nature* 388: 230-231.
23. Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* 362: 355-358.
24. Rowland-Jones, S. L. 2003. Timeline: AIDS pathogenesis: what have two decades of HIV research taught us? *Nature reviews. Immunology* 3: 343-348.
25. Chahroudi, A., S. E. Bosinger, T. H. Vanderford, M. Paiardini, and G. Silvestri. 2012. Natural SIV hosts: showing AIDS the door. *Science* 335: 1188-1193.
26. Letvin, N. L., M. D. Daniel, P. K. Sehgal, R. C. Desrosiers, R. D. Hunt, L. M. Waldron, J. J. MacKey, D. K. Schmidt, L. V. Chalifoux, and N. W. King. 1985. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* 230: 71-73.

27. Sodora, D. L., J. S. Allan, C. Apetrei, J. M. Brenchley, D. C. Douek, J. G. Else, J. D. Estes, B. H. Hahn, V. M. Hirsch, A. Kaur, F. Kirchhoff, M. Muller-Trutwin, I. Pandrea, J. E. Schmitz, and G. Silvestri. 2009. Toward an AIDS vaccine: lessons from natural simian immunodeficiency virus infections of African nonhuman primate hosts. *Nature medicine* 15: 861-865.
28. Barouch, D. H., K. E. Stephenson, E. N. Borducchi, K. Smith, K. Stanley, A. G. McNally, J. Liu, P. Abbink, L. F. Maxfield, M. S. Seaman, A. S. Dugast, G. Alter, M. Ferguson, W. Li, P. L. Earl, B. Moss, E. E. Giorgi, J. J. Szinger, L. A. Eller, E. A. Billings, M. Rao, S. Tovanabutra, E. Sanders-Buell, M. Weijtens, M. G. Pau, H. Schuitemaker, M. L. Robb, J. H. Kim, B. T. Korber, and N. L. Michael. 2013. Protective efficacy of a global HIV-1 mosaic vaccine against heterologous SHIV challenges in rhesus monkeys. *Cell* 155: 531-539.
29. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434: 1093-1097.
30. Okoye, A. A., and L. J. Picker. 2013. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. *Immunological reviews* 254: 54-64.
31. Okoye, A. A., M. Rohankhedkar, C. Abana, A. Pattenn, M. Reyes, C. Pexton, R. Lum, A. Sylwester, S. L. Planer, A. Legasse, B. S. Park, M. Piatak, Jr., J. D. Lifson, M. K. Axthelm, and L. J. Picker. 2012. Naive T cells are dispensable for memory CD4+ T cell homeostasis in progressive simian immunodeficiency virus infection. *The Journal of experimental medicine* 209: 641-651.
32. Zhu, J., H. Yamane, and W. E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annual review of immunology* 28: 445-489.
33. Okoye, A., M. Meier-Schellersheim, J. M. Brenchley, S. I. Hagen, J. M. Walker, M. Rohankhedkar, R. Lum, J. B. Edgar, S. L. Planer, A. Legasse, A. W. Sylwester, M. Piatak, Jr., J. D. Lifson, V. C. Maino, D. L. Sodora, D. C. Douek, M. K. Axthelm, Z. Grossman, and L. J. Picker. 2007. Progressive CD4+ central memory T cell decline results in CD4+ effector memory insufficiency and overt disease in chronic SIV infection. *The Journal of experimental medicine* 204: 2171-2185.
34. Paiardini, M., B. Cervasi, E. Reyes-Aviles, L. Micci, A. M. Ortiz, A. Chahroudi, C. Vinton, S. N. Gordon, S. E. Bosinger, N. Francella, P. L. Hallberg, E. Cramer, T. Schlub, M. L. Chan, N. E. Riddick, R. G. Collman, C. Apetrei, I. Pandrea, J. Else, J. Munch, F. Kirchhoff, M. P. Davenport, J. M. Brenchley, and G. Silvestri. 2011. Low levels of SIV infection in sooty mangabey central memory CD(4)(+) T cells are associated with limited CCR5 expression. *Nature medicine* 17: 830-836.
35. Brenchley, J. M., C. Vinton, B. Tabb, X. P. Hao, E. Connick, M. Paiardini, J. D. Lifson, G. Silvestri, and J. D. Estes. 2012. Differential infection patterns of CD4+ T cells and lymphoid tissue viral burden distinguish progressive and nonprogressive lentiviral infections. *Blood* 120: 4172-4181.
36. Schmitz, J. E., Z. M. Ma, E. A. Hagan, A. B. Wilks, K. L. Furr, C. H. Linde, R. C. Zahn, J. M. Brenchley, C. J. Miller, and S. R. Permar. 2012. Memory CD4(+) T lymphocytes in the gastrointestinal tract are a major source of cell-associated

simian immunodeficiency virus in chronic nonpathogenic infection of African green monkeys. *Journal of virology* 86: 11380-11385.

37. Descours, B., V. Avettand-Fenoel, C. Blanc, A. Samri, A. Melard, V. Supervie, I. Theodorou, G. Carcelain, C. Rouzioux, B. Autran, and A. A. C. S. Group. 2012. Immune responses driven by protective human leukocyte antigen alleles from long-term nonprogressors are associated with low HIV reservoir in central memory CD4 T cells. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 54: 1495-1503.
38. Saez-Cirion, A., C. Bacchus, L. Hocqueloux, V. Avettand-Fenoel, I. Girault, C. Lecuroux, V. Potard, P. Versmisse, A. Melard, T. Prazuck, B. Descours, J. Guergnon, J. P. Viard, F. Boufassa, O. Lambotte, C. Goujard, L. Meyer, D. Costagliola, A. Venet, G. Pancino, B. Autran, C. Rouzioux, and A. V. S. Group. 2013. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS pathogens* 9: e1003211.
39. Sodora, D. L., and G. Silvestri. Immune activation and AIDS pathogenesis.
40. Hunt, P. W., J. Brenchley, E. Sinclair, J. M. McCune, M. Roland, K. Page-Shafer, P. Hsue, B. Emu, M. Krone, H. Lampiris, D. Douek, J. N. Martin, and S. G. Deeks. 2008. Relationship between T cell activation and CD4(+) T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *Journal of Infectious Diseases* 197: 126-133.
41. Rotger, M., J. Dalmau, A. Rauch, P. McLaren, S. E. Bosinger, R. Martinez, N. G. Sandler, A. Roque, J. Liebner, M. Battegay, E. Bernasconi, P. Descombes, I. Erkizia, J. Fellay, B. Hirschel, J. M. Miro, E. Palou, M. Hoffmann, M. Massanella, J. Blanco, M. Woods, H. F. Gunthard, P. de Bakker, D. C. Douek, G. Silvestri, J. Martinez-Picado, and A. Telenti. 2011. Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. *The Journal of clinical investigation* 121: 2391-2400.
42. Begaud, E., L. Chartier, V. Marechal, J. Ipero, J. Leal, P. Versmisse, G. Breton, A. Fontanet, C. Capoulade-Metay, H. Fleury, F. Barre-Sinoussi, D. Scott-Algara, and G. Pancino. 2006. Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans. *Retrovirology* 3: 35.
43. Songok, E. M., M. Luo, B. Liang, P. McLaren, N. Kaefer, W. Apidi, G. Boucher, J. Kimani, C. Wachihi, R. Sekaly, K. Fowke, B. T. Ball, and F. A. Plummer. 2012. Microarray analysis of HIV resistant female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation state. *PloS one* 7: e30048.
44. Suy, A., P. Castro, M. Nomdedeu, F. Garcia, A. Lopez, E. Fumero, T. Gallart, L. Lopalco, O. Coll, J. M. Gatell, and M. Plana. 2007. Immunological profile of heterosexual highly HIV-exposed uninfected individuals: predominant role of CD4 and CD8 T-cell activation. *The Journal of infectious diseases* 196: 1191-1201.

45. Naranbhai, V., S. S. Abdool Karim, M. Altfeld, N. Samsunder, R. Durgiah, S. Sibeko, Q. Abdool Karim, W. H. Carr, and C. T. team. 2012. Innate immune activation enhances hiv acquisition in women, diminishing the effectiveness of tenofovir microbicide gel. *The Journal of infectious diseases* 206: 993-1001.
46. Tabb, B., D. R. Morcock, C. M. Trubey, O. A. Quinones, X. P. Hao, J. Smedley, R. Macallister, M. Piatak, L. D. Harris, M. Paiardini, G. Silvestri, J. M. Brenchley, W. G. Alvord, J. D. Lifson, and J. D. Estes. Microbial Translocation Across the GI Tract *.
47. Handley, S. A., L. B. Thackray, G. Zhao, R. Presti, A. D. Miller, L. Droit, P. Abbink, L. F. Maxfield, A. Kambal, E. Duan, K. Stanley, J. Kramer, S. C. Macri, S. R. Permar, J. E. Schmitz, K. Mansfield, J. M. Brenchley, R. S. Veazey, T. S. Stappenbeck, D. Wang, D. H. Barouch, and H. W. Virgin. 2012. Pathogenic simian immunodeficiency virus infection is associated with expansion of the enteric virome. *Cell* 151: 253-266.
48. Brenchley, J. M., and D. C. Douek. Pathogenic Simian Immunodeficiency Virus Infection Is Associated with Expansion of the Enteric Virome.
49. Yamamoto, T. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production.
50. Hong, J. J., P. K. Amancha, K. Rogers, A. A. Ansari, and F. Villinger. 2012. Spatial alterations between CD4(+) T follicular helper, B, and CD8(+) T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *Journal of immunology* 188: 3247-3256.
51. Zeng, M., A. J. Smith, S. W. Wietgrefe, P. J. Southern, T. W. Schacker, C. S. Reilly, J. D. Estes, G. F. Burton, G. Silvestri, J. D. Lifson, J. V. Carlis, and A. T. Haase. 2011. Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in HIV-1 and SIV infections. *The Journal of clinical investigation* 121: 998-1008.
52. Tabb, B., D. R. Morcock, C. M. Trubey, O. A. Quinones, X. P. Hao, J. Smedley, R. Macallister, M. Piatak, Jr., L. D. Harris, M. Paiardini, G. Silvestri, J. M. Brenchley, W. G. Alvord, J. D. Lifson, and J. D. Estes. 2013. Reduced inflammation and lymphoid tissue immunopathology in rhesus macaques receiving anti-tumor necrosis factor treatment during primary simian immunodeficiency virus infection. *The Journal of infectious diseases* 207: 880-892.
53. 2015. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. P. o. A. G. f. A. a. Adolescents, ed, Washington, DC.
54. Group, I. S. S. 2015. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. *The New England journal of medicine*.
55. Grinsztejn, B., M. C. Hosseinipour, H. J. Ribaudo, S. Swindells, J. Eron, Y. Q. Chen, L. Wang, S. S. Ou, M. Anderson, M. McCauley, T. Gamble, N. Kumarasamy, J. G. Hakim, J. Kumwenda, J. H. Pilotto, S. V. Godbole, S. Chariyalertsak, M. G. de Melo, K. H. Mayer, S. H. Eshleman, E. Piwowar-Manning, J. Makhema, L. A. Mills, R. Panchia, I. Sanne, J. Gallant, I. Hoffman, T.

E. Taha, K. Nielsen-Saines, D. Celentano, M. Essex, D. Havlir, M. S. Cohen, and H. A. S. Team. 2014. Effects of early versus delayed initiation of antiretroviral treatment on clinical outcomes of HIV-1 infection: results from the phase 3 HPTN 052 randomised controlled trial. *The Lancet. Infectious diseases* 14: 281-290.

56. Grant, R. M., J. R. Lama, P. L. Anderson, V. McMahan, A. Y. Liu, L. Vargas, P. Goicochea, M. Casapia, J. V. Guanira-Carranza, M. E. Ramirez-Cardich, O. Montoya-Herrera, T. Fernandez, V. G. Veloso, S. P. Buchbinder, S. Chariyalertsak, M. Schechter, L. G. Bekker, K. H. Mayer, E. G. Kallas, K. R. Amico, K. Mulligan, L. R. Bushman, R. J. Hance, C. Ganoza, P. Defechereux, B. Postle, F. Wang, J. J. McConnell, J. H. Zheng, J. Lee, J. F. Rooney, H. S. Jaffe, A. I. Martinez, D. N. Burns, D. V. Glidden, and T. iPrEx Study. 2010. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *The New England journal of medicine* 363: 2587-2599.
57. Baeten, J. M., D. Donnell, P. Ndase, N. R. Mugo, J. D. Campbell, J. Wangisi, J. W. Tappero, E. A. Bukusi, C. R. Cohen, E. Katabira, A. Ronald, E. Tumwesigye, E. Were, K. H. Fife, J. Kiarie, C. Farquhar, G. John-Stewart, A. Kakia, J. Odoyo, A. Mucunguzi, E. Nakku-Joloba, R. Twesigye, K. Ngure, C. Apaka, H. Tamooh, F. Gabona, A. Mujugira, D. Panteleeff, K. K. Thomas, L. Kidoguchi, M. Krows, J. Revall, S. Morrison, H. Haugen, M. Emmanuel-Ogier, L. Ondrejcek, R. W. Coombs, L. Frenkel, C. Hendrix, N. N. Bumpus, D. Bangsberg, J. E. Haberer, W. S. Stevens, J. R. Lingappa, C. Celum, and E. P. S. T. Partners Pr. 2012. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *The New England journal of medicine* 367: 399-410.
58. Choopanya, K., M. Martin, P. Suntharasamai, U. Sangkum, P. A. Mock, M. Leethochawalit, S. Chiamwongpaet, P. Kitisin, P. Natrujirote, S. Kittimunkong, R. Chuachoowong, R. J. Gvetadze, J. M. McNicholl, L. A. Paxton, M. E. Curlin, C. W. Hendrix, S. Vanichseni, and G. Bangkok Tenofovir Study. 2013. Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 381: 2083-2090.
59. Smith, D. K., L. A. Grohskopf, R. J. Black, J. D. Auerbach, F. Veronese, K. A. Struble, L. Cheever, M. Johnson, L. A. Paxton, I. M. Onorato, A. E. Greenberg, U. S. D. o. Health, and S. Human. 2005. Antiretroviral postexposure prophylaxis after sexual, injection-drug use, or other nonoccupational exposure to HIV in the United States: recommendations from the U.S. Department of Health and Human Services. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control* 54: 1-20.
60. Azzoni, L., A. S. Foulkes, E. Papasavvas, A. M. Mexas, K. M. Lynn, K. Mounzer, P. Tebas, J. M. Jacobson, I. Frank, M. P. Busch, S. G. Deeks, M. Carrington, U. O'Doherty, J. Kostman, and L. J. Montaner. Barriers to a cure for HIV: new ways to target and eradicate HIV-1 reservoirs.

61. Rajasuriar, R., E. Wright, and S. R. Lewin. 2015. Impact of antiretroviral therapy (ART) timing on chronic immune activation/inflammation and end-organ damage. *Current opinion in HIV and AIDS* 10: 35-42.
62. Hatano, H., V. Jain, P. W. Hunt, T. H. Lee, E. Sinclair, T. D. Do, R. Hoh, J. N. Martin, J. M. McCune, F. Hecht, M. P. Busch, and S. G. Deeks. 2013. Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells. *The Journal of infectious diseases* 208: 50-56.
63. Hunt, P. W. 2012. HIV and inflammation: mechanisms and consequences. *Current HIV/AIDS reports* 9: 139-147.
64. Katlama, C., S. G. Deeks, B. Autran, J. Martinez-Picado, J. van Lunzen, C. Rouzioux, M. Miller, S. Vella, J. E. Schmitz, J. Ahlers, D. D. Richman, and R. P. Sekaly. 2013. Barriers to a cure for HIV: new ways to target and eradicate HIV-1 reservoirs. *Lancet* 381: 2109-2117.
65. Chun, T. W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R. F. Siliciano. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nature medicine* 1: 1284-1290.
66. Finzi, D., J. Blankson, J. D. Siliciano, J. B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T. C. Quinn, R. E. Chaisson, E. Rosenberg, B. Walker, S. Gange, J. Gallant, and R. F. Siliciano. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nature medicine* 5: 512-517.
67. Whitney, J. B., A. L. Hill, S. Sanisetty, P. Penaloza-MacMaster, J. Liu, M. Shetty, L. Parenteau, C. Cabral, J. Shields, S. Blackmore, J. Y. Smith, A. L. Brinkman, L. E. Peter, S. I. Mathew, K. M. Smith, E. N. Borducchi, D. I. Rosenbloom, M. G. Lewis, J. Hattersley, B. Li, J. Hesselgesser, R. Geleziunas, M. L. Robb, J. H. Kim, N. L. Michael, and D. H. Barouch. 2014. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* 512: 74-77.
68. International, A. S. S. W. G. o. H. I. V. C., S. G. Deeks, B. Autran, B. Berkhout, M. Benkirane, S. Cairns, N. Chomont, T. W. Chun, M. Churchill, M. Di Mascio, C. Katlama, A. Lafeuillade, A. Landay, M. Lederman, S. R. Lewin, F. Maldarelli, D. Margolis, M. Markowitz, J. Martinez-Picado, J. I. Mullins, J. Mellors, S. Moreno, U. O'Doherty, S. Palmer, M. C. Penicaud, M. Peterlin, G. Poli, J. P. Routy, C. Rouzioux, G. Silvestri, M. Stevenson, A. Telenti, C. Van Lint, E. Verdin, A. Woolfrey, J. Zaia, and F. Barre-Sinoussi. 2012. Towards an HIV cure: a global scientific strategy. *Nature reviews. Immunology* 12: 607-614.
69. Cartwright, E. K., C. S. McGary, B. Cervasi, L. Micci, B. Lawson, S. T. C. Elliott, R. G. Collman, S. E. Bosinger, M. Paiardini, T. H. Vanderford, A. Chahroudi, and G. Silvestri. 2014. Divergent CD4+ T Memory Stem Cell Dynamics in Pathogenic and Nonpathogenic Simian Immunodeficiency Virus Infections. *The Journal of Immunology* 192: 4666-4673.
70. Chahroudi, A., G. Silvestri, and M. Lichterfeld. 2015. T memory stem cells and HIV: a long-term relationship. *Current HIV/AIDS reports* 12: 33-40.

71. Barouch, D. H., and S. G. Deeks. 2014. Immunologic strategies for HIV-1 remission and eradication. *Science* 345: 169-174.
72. Deng, K., M. Pertea, A. Rongvaux, L. Wang, C. M. Durand, G. Ghiaur, J. Lai, H. L. McHugh, H. Hao, H. Zhang, J. B. Margolick, C. Gurer, A. J. Murphy, D. M. Valenzuela, G. D. Yancopoulos, S. G. Deeks, T. Strowig, P. Kumar, J. D. Siliciano, S. L. Salzberg, R. A. Flavell, L. Shan, and R. F. Siliciano. 2015. Broad CTL response is required to clear latent HIV-1 due to dominance of escape mutations. *Nature* 517: 381-385.
73. Burton, D. R., R. Ahmed, D. H. Barouch, S. T. Butera, S. Crotty, A. Godzik, D. E. Kaufmann, M. J. McElrath, M. C. Nussenzweig, B. Pulendran, C. N. Scanlan, W. R. Schief, G. Silvestri, H. Streeck, B. D. Walker, L. M. Walker, A. B. Ward, I. A. Wilson, and R. Wyatt. 2012. A Blueprint for HIV Vaccine Discovery. *Cell host & microbe* 12: 396-407.
74. Fukazawa, Y., H. Park, M. J. Cameron, F. Lefebvre, R. Lum, N. Coombes, E. Mahyari, S. I. Hagen, J. Y. Bae, M. D. R. Iii, T. Swanson, A. W. Legasse, A. Sylwester, S. G. Hansen, A. T. Smith, P. Stafova, R. Shoemaker, Y. Li, K. Oswald, M. K. Axthelm, A. McDermott, G. Ferrari, D. C. Montefiori, P. T. Edlefsen, M. Piatak, J. D. Lifson, R. P. Sékaly, and L. J. Picker. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine.
75. Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-Johnson, N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J. Chiuchiolo, C. L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. Piatak, Jr., J. D. Lifson, and L. J. Picker. 2011. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* 473: 523-527.
76. Mudd, P. A., M. A. Martins, A. J. Ericsen, D. C. Tully, K. A. Power, A. T. Bean, S. M. Piaskowski, L. Duan, A. Seese, A. D. Gladden, K. L. Weisgrau, J. R. Furlott, Y. I. Kim, M. G. Veloso de Santana, E. Rakasz, S. Capuano, 3rd, N. A. Wilson, M. C. Bonaldo, R. Galler, D. B. Allison, M. Piatak, Jr., A. T. Haase, J. D. Lifson, T. M. Allen, and D. I. Watkins. 2012. Vaccine-induced CD8+ T cells control AIDS virus replication. *Nature* 491: 129-133.
77. Scheid, J. F., H. Mouquet, B. Ueberheide, R. Diskin, F. Klein, T. Y. Oliveira, J. Pietzsch, D. Fenyo, A. Abadir, K. Velinzon, A. Hurley, S. Myung, F. Boulad, P. Poignard, D. R. Burton, F. Pereyra, D. D. Ho, B. D. Walker, M. S. Seaman, P. J. Bjorkman, B. T. Chait, and M. C. Nussenzweig. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* 333: 1633-1637.
78. Wu, X., Z. Y. Yang, Y. Li, C. M. Hogerkorp, W. R. Schief, M. S. Seaman, T. Zhou, S. D. Schmidt, L. Wu, L. Xu, N. S. Longo, K. McKee, S. O'Dell, M. K. Louder, D. L. Wycuff, Y. Feng, M. Nason, N. Doria-Rose, M. Connors, P. D. Kwong, M. Roederer, R. T. Wyatt, G. J. Nabel, and J. R. Mascola. 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science* 329: 856-861.
79. Walker, L. M., M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J. P. Julien, S. K. Wang, A. Ramos, P. Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A.

Olsen, P. Phung, S. Fling, C. H. Wong, S. Phogat, T. Wrin, M. D. Simek, G. P. I. Protocol, W. C. Koff, I. A. Wilson, D. R. Burton, and P. Poignard. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477: 466-470.

80. McLellan, J. S., M. Pancera, C. Carrico, J. Gorman, J.-P. Julien, R. Khayat, R. Louder, R. Pejchal, M. Sastry, K. Dai, S. O'Dell, N. Patel, S. Shahzad-ul-Hussan, Y. Yang, B. Zhang, T. Zhou, J. Zhu, J. C. Boyington, G.-Y. Chuang, D. Diwanji, I. Georgiev, Y. Do Kwon, D. Lee, M. K. Louder, S. Moquin, S. D. Schmidt, Z.-Y. Yang, M. Bonsignori, J. A. Crump, S. H. Kapiga, N. E. Sam, B. F. Haynes, D. R. Burton, W. C. Koff, L. M. Walker, S. Phogat, R. Wyatt, J. Orwenyo, L.-X. Wang, J. Arthos, C. A. Bewley, J. R. Mascola, G. J. Nabel, W. R. Schief, A. B. Ward, I. A. Wilson, and P. D. Kwong. Rapid development of glycan-specific, broad, and potent anti-HIV-1 gp120 neutralizing antibodies in an R5 SIV/HIV chimeric virus infected macaque.
81. Pejchal, R., K. J. Doores, L. M. Walker, R. Khayat, P. S. Huang, S. K. Wang, R. L. Stanfield, J. P. Julien, A. Ramos, M. Crispin, R. Depetris, U. Katpally, A. Marozsan, A. Cupo, S. Maloveste, Y. Liu, R. McBride, Y. Ito, R. W. Sanders, C. Ogohara, J. C. Paulson, T. Feizi, C. N. Scanlan, C. H. Wong, J. P. Moore, W. C. Olson, A. B. Ward, P. Poignard, W. R. Schief, D. R. Burton, and I. A. Wilson. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9.
82. Walker, L. M., M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J.-P. Julien, S.-K. Wang, A. Ramos, P.-Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A. Olsen, P. Phung, S. Fling, C.-H. Wong, S. Phogat, T. Wrin, M. D. Simek, P. G. Principal Investigators, W. C. Koff, I. A. Wilson, D. R. Burton, and P. Poignard. A Potent and Broad Neutralizing Antibody Recognizes and Penetrates the HIV Glycan Shield.
83. Huang, J., G. Ofek, L. Laub, M. K. Louder, N. A. Doria-Rose, N. S. Longo, H. Imamichi, R. T. Bailer, B. Chakrabarti, S. K. Sharma, S. M. Alam, T. Wang, Y. Yang, B. Zhang, S. A. Migueles, R. Wyatt, B. F. Haynes, P. D. Kwong, J. R. Mascola, and M. Connors. 2012. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. *Nature* 491: 406-412.
84. Gray, E. S., M. C. Madiga, T. Hermanus, P. L. Moore, C. K. Wibmer, N. L. Tumba, L. Werner, K. Mlisana, S. Sibeko, C. Williamson, S. S. Abdool Karim, L. Morris, and C. S. Team. 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. *Journal of virology* 85: 4828-4840.
85. Sather, D. N., J. Armann, L. K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, X. Yu, B. Wood, S. Self, S. Kalams, and L. Stamatatos. 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *Journal of virology* 83: 757-769.

86. Stamatatos, L., L. Morris, D. R. Burton, and J. R. Mascola. 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nature medicine* 15: 866-870.
87. Hoot, S., A. T. McGuire, K. W. Cohen, R. K. Strong, L. Hangartner, F. Klein, R. Diskin, J. F. Scheid, D. N. Sather, D. R. Burton, and L. Stamatatos. 2013. Recombinant HIV envelope proteins fail to engage germline versions of anti-CD4bs bNAbs. *PLoS pathogens* 9: e1003106.
88. Liao, H. X., R. Lynch, T. Zhou, F. Gao, S. M. Alam, S. D. Boyd, A. Z. Fire, K. M. Roskin, C. A. Schramm, Z. Zhang, J. Zhu, L. Shapiro, N. C. S. Program, J. C. Mullikin, S. Gnanakaran, P. Hraber, K. Wiehe, G. Kelsoe, G. Yang, S. M. Xia, D. C. Montefiori, R. Parks, K. E. Lloyd, R. M. Scearce, K. A. Soderberg, M. Cohen, G. Kamanga, M. K. Louder, L. M. Tran, Y. Chen, F. Cai, S. Chen, S. Moquin, X. Du, M. G. Joyce, S. Srivatsan, B. Zhang, A. Zheng, G. M. Shaw, B. H. Hahn, T. B. Kepler, B. T. Korber, P. D. Kwong, J. R. Mascola, and B. F. Haynes. 2013. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature* 496: 469-476.
89. Moore, P. L., E. S. Gray, C. K. Wibmer, J. N. Bhiman, M. Nonyane, D. J. Sheward, T. Hermanus, S. Bajimaya, N. L. Tumba, M. R. Abrahams, B. E. Lambson, N. Ranchobe, L. Ping, N. Ngandu, Q. Abdool Karim, S. S. Abdool Karim, R. I. Swanstrom, M. S. Seaman, C. Williamson, and L. Morris. 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. *Nature medicine* 18: 1688-1692.
90. Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx, and M. G. Lewis. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nature medicine* 6: 207-210.
91. Hessell, A. J., L. Hangartner, M. Hunter, C. E. Havenith, F. J. Beurskens, J. M. Bakker, C. M. Lanigan, G. Landucci, D. N. Forthal, P. W. Parren, P. A. Marx, and D. R. Burton. 2007. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* 449: 101-104.
92. Moldt, B., E. G. Rakasz, N. Schultz, P. Y. Chan-Hui, K. Swiderek, K. L. Weisgrau, S. M. Piaskowski, Z. Bergman, D. I. Watkins, P. Poignard, and D. R. Burton. 2012. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 109: 18921-18925.
93. Jardine, J., J. P. Julien, S. Menis, T. Ota, O. Kalyuzhniy, A. McGuire, D. Sok, P. S. Huang, S. MacPherson, M. Jones, T. Nieusma, J. Mathison, D. Baker, A. B. Ward, D. R. Burton, L. Stamatatos, D. Nemazee, I. A. Wilson, and W. R. Schief. 2013. Rational HIV immunogen design to target specific germline B cell receptors. *Science* 340: 711-716.
94. Jardine, J. G., T. Ota, D. Sok, M. Pauthner, D. W. Kulp, O. Kalyuzhniy, P. D. Skog, T. C. Thinnes, D. Bhullar, B. Briney, S. Menis, M. Jones, M. Kubitz, S. Spencer, Y.

Adachi, D. R. Burton, W. R. Schief, and D. Nemazee. 2015. Priming a broadly neutralizing antibody response to HIV-1 using a germline-targeting immunogen. *Science*.

95. Dosenovic, P., L. von Boehmer, A. Escolano, J. Jardine, N. T. Freund, A. D. Gitlin, A. T. McGuire, D. W. Kulp, T. Oliveira, L. Scharf, J. Pietzsch, M. D. Gray, A. Cupo, M. J. van Gils, K. H. Yao, C. Liu, A. Gazumyan, M. S. Seaman, P. J. Bjorkman, R. W. Sanders, J. P. Moore, L. Stamatatos, W. R. Schief, and M. C. Nussenzweig. 2015. Immunization for HIV-1 Broadly Neutralizing Antibodies in Human Ig Knockin Mice. *Cell* 161: 1505-1515.
96. Soghoian, D. Z., H. Jessen, M. Flanders, K. Sierra-Davidson, S. Cutler, T. Pertel, S. Ranasinghe, M. Lindqvist, I. Davis, K. Lane, J. Rychert, E. S. Rosenberg, A. Piechocka-Trocha, A. L. Brass, J. M. Brenchley, B. D. Walker, and H. Streeck. 2012. HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome. *Science translational medicine* 4: 123ra125.
97. Ortiz, A. M., N. R. Klatt, B. Li, Y. Yi, B. Tabb, X. P. Hao, L. Sternberg, B. Lawson, P. M. Carnathan, E. M. Cramer, J. C. Engram, D. M. Little, E. Ryzhova, F. Gonzalez-Scarano, M. Paiardini, A. A. Ansari, S. Ratcliffe, J. G. Else, J. M. Brenchley, R. G. Collman, J. D. Estes, C. A. Derdeyn, and G. Silvestri. HIV-Specific Cytolytic CD4 T Cell Responses During Acute HIV Infection Predict Disease Outcome.
98. Harty, J. T., A. R. Tvinnereim, and D. W. White. 2000. CD8+ T cell effector mechanisms in resistance to infection. *Annual review of immunology* 18: 275-308.
99. Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234: 1563-1566.
100. Walker, C. M., A. L. Erickson, F. C. Hsueh, and J. A. Levy. 1991. Inhibition of human immunodeficiency virus replication in acutely infected CD4+ cells by CD8+ cells involves a noncytotoxic mechanism. *Journal of virology* 65: 5921-5927.
101. Wong, J. K., M. C. Strain, R. Porrata, E. Reay, S. Sankaran-Walters, C. C. Ignacio, T. Russell, S. K. Pillai, D. J. Looney, and S. Dandekar. 2010. In vivo CD8+ T-cell suppression of siv viremia is not mediated by CTL clearance of productively infected cells. *PLoS pathogens* 6: e1000748.
102. Streeck, H., J. S. Jolin, Y. Qi, B. Yassine-Diab, R. C. Johnson, D. S. Kwon, M. M. Addo, C. Brumme, J. P. Routy, S. Little, H. K. Jessen, A. D. Kelleher, F. M. Hecht, R. P. Sekaly, E. S. Rosenberg, B. D. Walker, M. Carrington, and M. Altfeld. 2009. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *Journal of virology* 83: 7641-7648.
103. Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during

primary infection demonstrated by rapid selection of CTL escape virus. *Nature medicine* 3: 205-211.

104. Mudd, P. A., A. J. Ericsen, B. J. Burwitz, N. A. Wilson, D. H. O'Connor, A. L. Hughes, and D. I. Watkins. 2012. Escape from CD8(+) T cell responses in Mamu-B*00801(+) macaques differentiates progressors from elite controllers. *Journal of immunology* 188: 3364-3370.
105. Makedonas, G., and M. R. Betts. 2011. Living in a house of cards: re-evaluating CD8+ T-cell immune correlates against HIV. *Immunological reviews* 239: 109-124.
106. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107: 4781-4789.
107. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283: 857-860.
108. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 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.
109. DeVico, A. L., and R. C. Gallo. 2004. Control of HIV-1 infection by soluble factors of the immune response. *Nature reviews. Microbiology* 2: 401-413.
110. Seich Al Basatena, N. K., K. Chatzimichalis, F. Graw, S. D. Frost, R. R. Regoes, and B. Asquith. 2013. Can non-lytic CD8+ T cells drive HIV-1 escape? *PLoS pathogens* 9: e1003656.
111. MacLennan, I. C. 1994. Germinal centers. *Annual review of immunology* 12: 117-139.
112. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature* 354: 389-392.
113. Blink, E. J., A. Light, A. Kallies, S. L. Nutt, P. D. Hodgkin, and D. M. Tarlinton. 2005. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *The Journal of experimental medicine* 201: 545-554.
114. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.
115. Okada, T., M. J. Miller, I. Parker, M. F. Krummel, M. Neighbors, S. B. Hartley, A. O'Garra, M. D. Cahalan, and J. G. Cyster. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS biology* 3: e150.
116. Paus, D., T. G. Phan, T. D. Chan, S. Gardam, A. Basten, and R. Brink. 2006. Antigen recognition strength regulates the choice between extrafollicular

plasma cell and germinal center B cell differentiation. *The Journal of experimental medicine* 203: 1081-1091.

117. Kerfoot, S. M., G. Yaari, J. R. Patel, K. L. Johnson, D. G. Gonzalez, S. H. Kleinstein, and A. M. Haberman. 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* 34: 947-960.
118. Bannard, O., R. M. Horton, C. D. Allen, J. An, T. Nagasawa, and J. G. Cyster. 2013. Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. *Immunity* 39: 912-924.
119. Allen, C. D., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nature immunology* 5: 943-952.
120. Shulman, Z., A. D. Gitlin, J. S. Weinstein, B. Lainez, E. Esplugues, R. A. Flavell, J. E. Craft, and M. C. Nussenzweig. 2014. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* 345: 1058-1062.
121. Allen, C. D., T. Okada, H. L. Tang, and J. G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. *Science* 315: 528-531.
122. Shulman, Z., A. D. Gitlin, S. Targ, M. Jankovic, G. Pasqual, M. C. Nussenzweig, and G. D. Victora. 2013. T follicular helper cell dynamics in germinal centers. *Science* 341: 673-677.
123. Victora, G. D., T. A. Schwickert, D. R. Fooksman, A. O. Kamphorst, M. Meyer-Hermann, M. L. Dustin, and M. C. Nussenzweig. 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* 143: 592-605.
124. Schwickert, T. A., G. D. Victora, D. R. Fooksman, A. O. Kamphorst, M. R. Mugnier, A. D. Gitlin, M. L. Dustin, and M. C. Nussenzweig. 2011. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *The Journal of experimental medicine* 208: 1243-1252.
125. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annual review of immunology* 29: 621-663.
126. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006-1010.
127. Kitano, M., S. Moriyama, Y. Ando, M. Hikida, Y. Mori, T. Kurosaki, and T. Okada. 2011. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* 34: 961-972.
128. Ballesteros-Tato, A., and T. D. Randall. 2014. Priming of T follicular helper cells by dendritic cells. *Immunol Cell Biol* 92: 22-27.
129. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34: 932-946.

130. Baumjohann, D., S. Preite, A. Reboldi, F. Ronchi, K. M. Ansel, A. Lanzavecchia, and F. Sallusto. 2013. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* 38: 596-605.
131. Kroenke, M. A., D. Eto, M. Locci, M. Cho, T. Davidson, E. K. Haddad, and S. Crotty. 2012. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *Journal of immunology* 188: 3734-3744.
132. Cannons, J. L., H. Qi, K. T. Lu, M. Dutta, J. Gomez-Rodriguez, J. Cheng, E. K. Wakeland, R. N. Germain, and P. L. Schwartzberg. 2010. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* 32: 253-265.
133. Pissani, F., and H. Streeck. 2014. Emerging concepts on T follicular helper cell dynamics in HIV infection. *Trends in immunology* 35: 278-286.
134. Zotos, D., J. M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L. M. Corcoran, D. I. Godfrey, K. M. Toellner, M. J. Smyth, S. L. Nutt, and D. M. Tarlinton. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *The Journal of experimental medicine* 207: 365-378.
135. Petrovas, C., T. Yamamoto, M. Y. Gerner, K. L. Boswell, K. Wloka, E. C. Smith, D. R. Ambrozak, N. G. Sandler, K. J. Timmer, X. Sun, L. Pan, A. Poholek, S. S. Rao, J. M. Brenchley, S. M. Alam, G. D. Tomaras, M. Roederer, D. C. Douek, R. A. Seder, R. N. Germain, E. K. Haddad, and R. A. Koup. 2012. CD4 T follicular helper cell dynamics during SIV infection. *The Journal of clinical investigation* 122: 3281-3294.
136. Perreau, M., A. L. Savoye, E. De Crignis, J. M. Corpataux, R. Cubas, E. K. Haddad, L. De Leval, C. Graziosi, and G. Pantaleo. 2013. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *The Journal of experimental medicine* 210: 143-156.
137. Lindqvist, M., J. van Lunzen, D. Z. Soghoian, B. D. Kuhl, S. Ranasinghe, G. Kranias, M. D. Flanders, S. Cutler, N. Yudanin, M. I. Muller, I. Davis, D. Farber, P. Hartjen, F. Haag, G. Alter, J. Schulze zur Wiesch, and H. Streeck. 2012. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *The Journal of clinical investigation* 122: 3271-3280.
138. Cubas, R. A., J. C. Mudd, A. L. Savoye, M. Perreau, J. van Grevenynghe, T. Metcalf, E. Connick, A. Meditz, G. J. Freeman, G. Abesada-Terk, Jr., J. M. Jacobson, A. D. Brooks, S. Crotty, J. D. Estes, G. Pantaleo, M. M. Lederman, and E. K. Haddad. 2013. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nature medicine* 19: 494-499.
139. Pratama, A., and C. G. Vinuesa. 2014. Control of TFH cell numbers: why and how? *Immunol Cell Biol* 92: 40-48.
140. Vinuesa, C. G., M. C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K. M. Hill, D. Yu, H. Domaschenz, B. Whittle, T. Lambe, I. S. Roberts, R. R. Copley, J. I. Bell, R. J. Cornall, and C. C. Goodnow. 2005. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 435: 452-458.

141. Vogel, K. U., S. L. Edelmann, K. M. Jeltsch, A. Bertossi, K. Heger, G. A. Heinz, J. Zoller, S. C. Warth, K. P. Hoefig, C. Lohs, F. Neff, E. Kremmer, J. Schick, D. Repsilber, A. Geerlof, H. Blum, W. Wurst, M. Heikenwalder, M. Schmidt-Supprian, and V. Heissmeyer. 2013. Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* 38: 655-668.
142. He, J., L. M. Tsai, Y. A. Leong, X. Hu, C. S. Ma, N. Chevalier, X. Sun, K. Vandenberg, S. Rockman, Y. Ding, L. Zhu, W. Wei, C. Wang, A. Karnowski, G. T. Belz, J. R. Ghali, M. C. Cook, D. S. Riminton, A. Veillette, P. L. Schwartzberg, F. Mackay, R. Brink, S. G. Tangye, C. G. Vinuesa, C. R. Mackay, Z. Li, and D. Yu. 2013. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* 39: 770-781.
143. Simpson, N., P. A. Gatenby, A. Wilson, S. Malik, D. A. Fulcher, S. G. Tangye, H. Manku, T. J. Vyse, G. Roncador, G. A. Huttley, C. C. Goodnow, C. G. Vinuesa, and M. C. Cook. 2010. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis and rheumatism* 62: 234-244.
144. Ma, J., C. Zhu, B. Ma, J. Tian, S. E. Baidoo, C. Mao, W. Wu, J. Chen, J. Tong, M. Yang, Z. Jiao, H. Xu, L. Lu, and S. Wang. 2012. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clinical & developmental immunology* 2012: 827480.
145. Luo, C., Y. Li, W. Liu, H. Feng, H. Wang, X. Huang, L. Qiu, and J. Ouyang. 2013. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *Journal of neuroimmunology* 256: 55-61.
146. Pratama, A., and C. G. Vinuesa. 2013. Control of TFH cell numbers: why and how? *Immunology and Cell Biology* 92: 40-48.
147. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-787.
148. Goodnow, C. C., J. Sprent, B. Fazekas de St Groth, and C. G. Vinuesa. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* 435: 590-597.
149. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature genetics* 27: 20-21.
150. Wildin, R. S., F. Ramsdell, J. Peake, F. Faravelli, J. L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F. D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M. E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nature genetics* 27: 18-20.
151. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to

CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of experimental medicine* 198: 1875-1886.
152. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nature immunology* 6: 1219-1227.
153. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nature immunology* 6: 338-344.
154. Shevach, E. M. 2008. Immunology. Regulating suppression. *Science* 322: 202-203.
155. Tang, Q., and J. A. Bluestone. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nature immunology* 9: 239-244.
156. Takahashi, M., and A. Kimura. 2010. HLA and CTLA4 polymorphisms may confer a synergistic risk in the susceptibility to Graves' disease. *Journal of human genetics* 55: 323-326.
157. Takahashi, S., H. Kataoka, S. Hara, T. Yokosuka, K. Takase, S. Yamasaki, W. Kobayashi, Y. Saito, and T. Saito. 2005. In vivo overexpression of CTLA-4 suppresses lymphoproliferative diseases and thymic negative selection. *European journal of immunology* 35: 399-407.
158. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *The Journal of experimental medicine* 192: 303-310.
159. Krummel, M. F., and J. P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *The Journal of experimental medicine* 183: 2533-2540.
160. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of experimental medicine* 182: 459-465.
161. Wang, W., R. Lau, D. Yu, W. Zhu, A. Korman, and J. Weber. 2009. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4+ CD25(Hi) regulatory T cells. *Int Immunol* 21: 1065-1077.
162. Fife, B. T., K. E. Pauken, T. N. Eagar, T. Obu, J. Wu, Q. Tang, M. Azuma, M. F. Krummel, and J. A. Bluestone. 2009. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nature immunology* 10: 1185-1192.
163. Miyara, M., and S. Sakaguchi. 2007. Natural regulatory T cells: mechanisms of suppression. *Trends in molecular medicine* 13: 108-116.
164. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *Journal of immunology* 164: 183-190.
165. Barthlott, T., H. Moncrieffe, M. Veldhoen, C. J. Atkins, J. Christensen, A. O'Garra, and B. Stockinger. 2005. CD25+ CD4+ T cells compete with naive

CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *Int Immunol* 17: 279-288.
166. Chevalier, M. F., and L. Weiss. 2013. The split personality of regulatory T cells in HIV infection. *Blood* 121: 29-37.
167. Kinter, A., J. McNally, L. Riggin, R. Jackson, G. Roby, and A. S. Fauci. 2007. Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals. *Proceedings of the National Academy of Sciences of the United States of America* 104: 3390-3395.
168. Chase, A. J., H. C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano. 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *Journal of virology* 82: 8307-8315.
169. Macatangay, B. J., M. E. Szajnik, T. L. Whiteside, S. A. Riddler, and C. R. Rinaldo. 2010. Regulatory T cell suppression of Gag-specific CD8 T cell polyfunctional response after therapeutic vaccination of HIV-1-infected patients on ART. *PloS one* 5: e9852.
170. Chung, Y., S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal, Y.-H. Wang, H. Lim, J. M. Reynolds, X.-h. Zhou, H.-m. Fan, Z.-m. Liu, S. S. Neelapu, and C. Dong. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nature medicine* 17: 983-988.
171. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, S. Fagarasan, A. Liston, K. G. Smith, and C. G. Vinuesa. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. *Nature medicine* 17: 975-982.
172. Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim. 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *Journal of immunology* 175: 4180-4183.
173. Lim, H. W., P. Hillsamer, and C. H. Kim. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *The Journal of clinical investigation* 114: 1640-1649.
174. Sage, P. T., and A. H. Sharpe. 2015. T follicular regulatory cells in the regulation of B cell responses. *Trends in immunology* 36: 410-418.
175. Wollenberg, I., A. Agua-Doce, A. Hernandez, C. Almeida, V. G. Oliveira, J. Faro, and L. Graca. 2011. Regulation of the Germinal Center Reaction by Foxp3+ Follicular Regulatory T Cells. *The Journal of Immunology* 187: 4553-4560.
176. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, S. Fagarasan, A. Liston, K. G. C. Smith, and C. G. Vinuesa. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. *Nature medicine* 17: 975-982.
177. Lanzavecchia, A., G. Iezzi, and A. Viola. 1999. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell* 96: 1-4.
178. Kupfer, A., S. J. Singer, C. A. Janeway, Jr., and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct

interaction of helper T cells and antigen-presenting cells. *Proceedings of the National Academy of Sciences of the United States of America* 84: 5888-5892.
179. Dustin, M. L., and J. A. Cooper. 2000. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nature immunology* 1: 23-29.
180. Monks, C. R., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395: 82-86.
181. Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221-227.
182. Davis, D. M., and M. L. Dustin. 2004. What is the importance of the immunological synapse? *Trends in immunology* 25: 323-327.
183. Poo, W. J., L. Conrad, and C. A. Janeway, Jr. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature* 332: 378-380.
184. Wetzel, S. A., T. W. McKeithan, and D. C. Parker. 2002. Live-cell dynamics and the role of costimulation in immunological synapse formation. *Journal of immunology* 169: 6092-6101.
185. Sattentau, Q. 2008. Avoiding the void: cell-to-cell spread of human viruses. *Nature reviews. Microbiology* 6: 815-826.
186. Hudrisier, D., J. Riond, H. Mazarguil, J. E. Gairin, and E. Joly. 2001. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. *Journal of immunology* 166: 3645-3649.
187. Aucher, A., E. Magdeleine, E. Joly, and D. Hudrisier. 2008. Capture of plasma membrane fragments from target cells by trogocytosis requires signaling in T cells but not in B cells. *Blood* 111: 5621-5628.
188. Haastert, B., R. J. Mellanby, S. M. Anderton, and R. A. O'Connor. 2013. T cells at the site of autoimmune inflammation show increased potential for trogocytosis. *PloS one* 8: e81404.
189. Martinez-Martin, N., E. Fernandez-Arenas, S. Cemerski, P. Delgado, M. Turner, J. Heuser, D. J. Irvine, B. Huang, X. R. Bustelo, A. Shaw, and B. Alarcon. 2011. T cell receptor internalization from the immunological synapse is mediated by TC21 and RhoG GTPase-dependent phagocytosis. *Immunity* 35: 208-222.
190. Joly, E., and D. Hudrisier. 2003. What is trogocytosis and what is its purpose? *Nature immunology* 4: 815.
191. Osborne, D. G., and S. A. Wetzel. 2012. Trogocytosis results in sustained intracellular signaling in CD4(+) T cells. *Journal of immunology* 189: 4728-4739.
192. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 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.
193. Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 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.

194. Goulder, P. J., and D. I. Watkins. 2004. HIV and SIV CTL escape: implications for vaccine design. *Nature reviews. Immunology* 4: 630-640.
195. Carrington, M., and S. J. O'Brien. 2003. The influence of HLA genotype on AIDS. *Annual review of medicine* 54: 535-551.
196. Goulder, P. J., and D. I. Watkins. 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. *Nature reviews. Immunology* 8: 619-630.
197. Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane, and M. A. Martin. 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.
198. Lifson, J. D., J. L. Rossio, M. Piatak, Jr., T. Parks, L. Li, R. Kiser, V. Coalter, B. Fisher, B. M. Flynn, S. Czajak, V. M. Hirsch, K. A. Reimann, J. E. Schmitz, J. Ghrayeb, N. Bischofberger, M. A. Nowak, R. C. Desrosiers, and D. Wodarz. 2001. Role of CD8(+) lymphocytes in control of simian immunodeficiency virus infection and resistance to rechallenge after transient early antiretroviral treatment. *Journal of virology* 75: 10187-10199.
199. Okoye, A., H. Park, M. Rohankhedkar, L. Coyne-Johnson, R. Lum, J. M. Walker, S. L. Planer, A. W. Legasse, A. W. Sylwester, M. Piatak, Jr., J. D. Lifson, D. L. Sodora, F. Villinger, M. K. Axthelm, J. E. Schmitz, and L. J. Picker. 2009. Profound CD4+/CCR5+ T cell expansion is induced by CD8+ lymphocyte depletion but does not account for accelerated SIV pathogenesis. *The Journal of experimental medicine* 206: 1575-1588.
200. Klatt, N. R., E. Shudo, A. M. Ortiz, J. C. Engram, M. Paiardini, B. Lawson, M. D. Miller, J. Else, I. Pandrea, J. D. Estes, C. Apetrei, J. E. Schmitz, R. M. Ribeiro, A. S. Perelson, and G. Silvestri. 2010. CD8+ lymphocytes control viral replication in SIVmac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. *PLoS pathogens* 6: e1000747.
201. Elemans, M., N. K. Seich Al Basatena, N. R. Klatt, C. Gkekas, G. Silvestri, and B. Asquith. 2011. Why don't CD8+ T cells reduce the lifespan of SIV-infected cells in vivo? *PLoS computational biology* 7: e1002200.
202. Dunham, R., P. Pagliardini, S. Gordon, B. Sumpter, J. Engram, A. Moanna, M. Paiardini, J. N. Mandl, B. Lawson, S. Garg, H. M. McClure, Y. X. Xu, C. Ibegbu, K. Easley, N. Katz, I. Pandrea, C. Apetrei, D. L. Sodora, S. I. Staprans, M. B. Feinberg, and G. Silvestri. 2006. The AIDS resistance of naturally SIV-infected sooty mangabeys is independent of cellular immunity to the virus. *Blood* 108: 209-217.
203. Garber, D. A., G. Silvestri, A. P. Barry, A. Fedanov, N. Kozyr, H. McClure, D. C. Montefiori, C. P. Larsen, J. D. Altman, S. I. Staprans, and M. B. Feinberg. 2004. Blockade of T cell costimulation reveals interrelated actions of CD4+ and CD8+ T cells in control of SIV replication. *The Journal of clinical investigation* 113: 836-845.

204. Chahroudi, A., E. Cartwright, S. T. Lee, M. Mavigner, D. G. Carnathan, B. Lawson, P. M. Carnathan, T. Hashempoor, M. K. Murphy, T. Meeker, S. Ehnert, C. Souder, J. G. Else, J. Cohen, R. G. Collman, T. H. Vanderford, S. R. Permar, C. A. Derdeyn, F. Villinger, and G. Silvestri. 2014. Target cell availability, rather than breast milk factors, dictates mother-to-infant transmission of SIV in sooty mangabeys and rhesus macaques. *PLoS pathogens* 10: e1003958.
205. Micci, L., X. Alvarez, R. I. Iriele, A. M. Ortiz, E. S. Ryan, C. S. McGary, C. Deleage, B. B. McAtee, T. He, C. Apetrei, K. Easley, S. Pahwa, R. G. Collman, C. A. Derdeyn, M. P. Davenport, J. D. Estes, G. Silvestri, A. A. Lackner, and M. Paiardini. 2014. CD4 depletion in SIV-infected macaques results in macrophage and microglia infection with rapid turnover of infected cells. *PLoS pathogens* 10: e1004467.
206. Connick, E., J. M. Folkvord, K. T. Lind, E. G. Rakasz, B. Miles, N. A. Wilson, M. L. Santiago, K. Schmitt, E. B. Stephens, H. O. Kim, R. Wagstaff, S. Li, H. M. Abdelaal, N. Kemp, D. I. Watkins, S. MaWhinney, and P. J. Skinner. 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* 193: 5613-5625.
207. Fukazawa, Y., R. Lum, A. A. Okoye, H. Park, K. Matsuda, J. Y. Bae, S. I. Hagen, R. Shoemaker, C. Deleage, C. Lucero, D. Morcock, T. Swanson, A. W. Legasse, M. K. Axthelm, J. Hesselgesser, R. Geleziunas, V. M. Hirsch, P. T. Edlefsen, M. Piatak, Jr., J. D. Estes, J. D. Lifson, and L. J. Picker. 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nature medicine* 21: 132-139.
208. Singh, R. A., and M. A. Barry. 2004. Repertoire and immunofocusing of CD8 T cell responses generated by HIV-1 gag-pol and expression library immunization vaccines. *Journal of immunology* 173: 4387-4393.
209. Bosinger, S. E., S. P. Jochems, K. A. Folkner, T. L. Hayes, N. R. Klatt, and G. Silvestri. 2013. Transcriptional profiling of experimental CD8(+) lymphocyte depletion in rhesus macaques infected with simian immunodeficiency virus SIVmac239. *Journal of virology* 87: 433-443.
210. Mueller, Y. M., D. H. Do, J. D. Boyer, M. Kader, J. J. Mattapallil, M. G. Lewis, D. B. Weiner, and P. D. Katsikis. 2009. CD8+ cell depletion of SHIV89.6P-infected macaques induces CD4+ T cell proliferation that contributes to increased viral loads. *Journal of immunology* 183: 5006-5012.
211. Teixeira, S. L., N. B. de Sa, D. P. Campos, A. B. Coelho, M. L. Guimaraes, T. C. Leite, V. G. Veloso, and M. G. Morgado. 2014. Association of the HLA-B*52 allele with non-progression to AIDS in Brazilian HIV-1-infected individuals. *Genes and immunity* 15: 256-262.
212. Sullivan, B. M., A. Juedes, S. J. Szabo, M. von Herrath, and L. H. Glimcher. 2003. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proceedings of the National Academy of Sciences of the United States of America* 100: 15818-15823.

213. Jenner, R. G., M. J. Townsend, I. Jackson, K. Sun, R. D. Bouwman, R. A. Young, L. H. Glimcher, and G. M. Lord. 2009. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proceedings of the National Academy of Sciences of the United States of America* 106: 17876-17881.
214. Kao, C., K. J. Oestreich, M. A. Paley, A. Crawford, J. M. Angelosanto, M. A. Ali, A. M. Intlekofer, J. M. Boss, S. L. Reiner, A. S. Weinmann, and E. J. Wherry. 2011. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nature immunology* 12: 663-671.
215. Buggert, M., J. Tauriainen, T. Yamamoto, J. Frederiksen, M. A. Ivarsson, J. Michaelsson, O. Lund, B. Hejdeman, M. Jansson, A. Sonnerborg, R. A. Koup, M. R. Betts, and A. C. Karlsson. 2014. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS pathogens* 10: e1004251.
216. Hersperger, A. R., J. N. Martin, L. Y. Shin, P. M. Sheth, C. M. Kovacs, G. L. Cosma, G. Makedonas, F. Pereyra, B. D. Walker, R. Kaul, S. G. Deeks, and M. R. Betts. 2011. Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood* 117: 3799-3808.
217. Mackewicz, C. E., D. J. Blackbourn, and J. A. Levy. 1995. CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proceedings of the National Academy of Sciences of the United States of America* 92: 2308-2312.
218. Corti, D., and A. Lanzavecchia. 2013. Broadly neutralizing antiviral antibodies. *Annual review of immunology* 31: 705-742.
219. Murphy, M. K., L. Yue, R. Pan, S. Boliar, A. Sethi, J. Tian, K. Pfafferot, E. Karita, S. A. Allen, E. Cormier, P. A. Goepfert, P. Borrow, J. E. Robinson, S. Gnanakaran, E. Hunter, X. P. Kong, and C. A. Derdeyn. 2013. Viral escape from neutralizing antibodies in early subtype A HIV-1 infection drives an increase in autologous neutralization breadth. *PLoS pathogens* 9: e1003173.
220. Yang, X., J. Yang, Y. Chu, Y. Xue, D. Xuan, S. Zheng, and H. Zou. 2014. T follicular helper cells and regulatory B cells dynamics in systemic lupus erythematosus. *PloS one* 9: e88441.
221. Ma, C. S., and E. K. Deenick. 2014. Human T follicular helper (Tfh) cells and disease. *Immunology and cell biology* 92: 64-71.
222. Vinuesa, C. G. 2012. HIV and T follicular helper cells: a dangerous relationship. *The Journal of clinical investigation* 122: 3059-3062.
223. Petrovas, C., and R. A. Koup. 2014. T follicular helper cells and HIV/SIV-specific antibody responses. *Current opinion in HIV and AIDS* 9: 235-241.
224. Chung, Y., S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal, Y. H. Wang, H. Lim, J. M. Reynolds, X. H. Zhou, H. M. Fan, Z. M. Liu, S. S. Neelapu, and C. Dong. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nature medicine* 17: 983-988.

225. Lai, L., S. Kwa, P. A. Kozlowski, D. C. Montefiori, G. Ferrari, W. E. Johnson, V. Hirsch, F. Villinger, L. Chennareddi, P. L. Earl, B. Moss, R. R. Amara, and H. L. Robinson. 2011. Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. *The Journal of infectious diseases* 204: 164-173.
226. Ortiz, A. M., N. R. Klatt, B. Li, Y. Yi, B. Tabb, X. P. Hao, L. Sternberg, B. Lawson, P. M. Carnathan, E. M. Cramer, J. C. Engram, D. M. Little, E. Ryzhova, F. Gonzalez-Scarano, M. Paiardini, A. A. Ansari, S. Ratcliffe, J. G. Else, J. M. Brenchley, R. G. Collman, J. D. Estes, C. A. Derdeyn, and G. Silvestri. 2011. Depletion of CD4(+) T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. *The Journal of clinical investigation* 121: 4433-4445.
227. Cartwright, E. K., C. S. McGary, B. Cervasi, L. Micci, B. Lawson, S. T. Elliott, R. G. Collman, S. E. Bosinger, M. Paiardini, T. H. Vanderford, A. Chahroudi, and G. Silvestri. 2014. Divergent CD4+ T memory stem cell dynamics in pathogenic and nonpathogenic simian immunodeficiency virus infections. *Journal of immunology* 192: 4666-4673.
228. Vanderford, T. H., C. Slichter, K. A. Rogers, B. O. Lawson, R. Obaede, J. Else, F. Villinger, S. E. Bosinger, and G. Silvestri. 2012. Treatment of SIV-infected sooty mangabeys with a type-I IFN agonist results in decreased virus replication without inducing hyperimmune activation. *Blood* 119: 5750-5757.
229. Sandler, N. G., S. E. Bosinger, J. D. Estes, R. T. Zhu, G. K. Tharp, E. Boritz, D. Levin, S. Wijeyesinghe, K. N. Makamdop, G. Q. del Prete, B. J. Hill, J. K. Timmer, E. Reiss, G. Yarden, S. Darko, E. Contijoch, J. P. Todd, G. Silvestri, M. Nason, R. B. Norgren, Jr., B. F. Keele, S. Rao, J. A. Langer, J. D. Lifson, G. Schreiber, and D. C. Douek. 2014. Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. *Nature* 511: 601-605.
230. Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29: 15-21.
231. Trapnell, C., D. G. Hendrickson, M. Sauvageau, L. Goff, J. L. Rinn, and L. Pachter. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature biotechnology* 31: 46-53.
232. Vinuesa, C. G., and J. G. Cyster. 2011. How T cells earn the follicular rite of passage. *Immunity* 35: 671-680.
233. Alexander, C. M., L. T. Tygrett, A. W. Boyden, K. L. Wolniak, K. L. Legge, and T. J. Waldschmidt. 2011. T regulatory cells participate in the control of germinal centre reactions. *Immunology* 133: 452-468.
234. Wollenberg, I., A. Agua-Doce, A. Hernandez, C. Almeida, V. G. Oliveira, J. Faro, and L. Graca. 2011. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *Journal of immunology* 187: 4553-4560.

235. Wing, J. B., and S. Sakaguchi. 2014. Foxp3(+) T(reg) cells in humoral immunity. *International immunology* 26: 61-69.
236. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine* 203: 1701-1711.
237. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, A. Kelleher, and B. Fazekas de St Groth. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine* 203: 1693-1700.
238. Dunham, R. M., B. Cervasi, J. M. Brenchley, H. Albrecht, A. Weintrob, B. Sumpter, J. Engram, S. Gordon, N. R. Klatt, I. Frank, D. L. Sodora, D. C. Douek, M. Paiardini, and G. Silvestri. 2008. CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection. *Journal of immunology* 180: 5582-5592.
239. Getnet, D., J. F. Grosso, M. V. Goldberg, T. J. Harris, H. R. Yen, T. C. Bruno, N. M. Durham, E. L. Hipkiss, K. J. Pyle, S. Wada, F. Pan, D. M. Pardoll, and C. G. Drake. 2010. A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. *Molecular immunology* 47: 1595-1600.
240. Ray, J. P., H. D. Marshall, B. J. Laidlaw, M. M. Staron, S. M. Kaech, and J. Craft. 2014. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* 40: 367-377.
241. Sage, P. T., L. M. Francisco, C. V. Carman, and A. H. Sharpe. 2013. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nature immunology* 14: 152-161.
242. Birzele, F., T. Fauti, H. Stahl, M. C. Lenter, E. Simon, D. Knebel, A. Weith, T. Hildebrandt, and D. Mennerich. 2011. Next-generation insights into regulatory T cells: expression profiling and FoxP3 occupancy in Human. *Nucleic acids research* 39: 7946-7960.
243. Moreno-Fernandez, M. E., W. Zapata, J. T. Blackard, G. Franchini, and C. A. Chougnet. 2009. Human regulatory T cells are targets for human immunodeficiency Virus (HIV) infection, and their susceptibility differs depending on the HIV type 1 strain. *Journal of virology* 83: 12925-12933.
244. Brenchley, J. M., B. J. Hill, D. R. Ambrozak, D. A. Price, F. J. Guenaga, J. P. Casazza, J. Kuruppu, J. Yazdani, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup. 2004. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. *Journal of virology* 78: 1160-1168.
245. Kwong, P. D., J. R. Mascola, and G. J. Nabel. 2013. Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nature reviews. Immunology* 13: 693-701.

246. Haynes, B. F., and T. Bradley. 2015. Broadly Neutralizing Antibodies and the Development of Vaccines. *Jama* 313: 2419-2420.
247. Hessell, A. J., E. G. Rakasz, P. Poignard, L. Hangartner, G. Landucci, D. N. Forthal, W. C. Koff, D. I. Watkins, and D. R. Burton. 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. *PLoS pathogens* 5: e1000433.
248. Hessell, A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. Parren, P. A. Marx, and D. R. Burton. 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nature medicine* 15: 951-954.
249. Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx. 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *Journal of virology* 73: 4009-4018.
250. Wu, X., Z. Zhang, C. A. Schramm, M. G. Joyce, Y. D. Kwon, T. Zhou, Z. Sheng, B. Zhang, S. O'Dell, K. McKee, I. S. Georgiev, G. Y. Chuang, N. S. Longo, R. M. Lynch, K. O. Saunders, C. Soto, S. Srivatsan, Y. Yang, R. T. Bailer, M. K. Louder, N. C. S. Program, J. C. Mullikin, M. Connors, P. D. Kwong, J. R. Mascola, and L. Shapiro. 2015. Maturation and Diversity of the VRC01-Antibody Lineage over 15 Years of Chronic HIV-1 Infection. *Cell* 161: 470-485.
251. Burton, D. R., R. L. Stanfield, and I. A. Wilson. 2005. Antibody vs. HIV in a clash of evolutionary titans. *Proceedings of the National Academy of Sciences of the United States of America* 102: 14943-14948.
252. Pritchard, L. K., D. I. Spencer, L. Royle, C. Bonomelli, G. E. Seabright, A. J. Behrens, D. W. Kulp, S. Menis, S. A. Krumm, D. C. Dunlop, D. J. Crispin, T. A. Bowden, C. N. Scanlan, A. B. Ward, W. R. Schief, K. J. Doores, and M. Crispin. 2015. Glycan clustering stabilizes the mannose patch of HIV-1 and preserves vulnerability to broadly neutralizing antibodies. *Nature communications* 6: 7479.
253. Verkoczy, L., G. Kelsoe, M. A. Moody, and B. F. Haynes. 2011. Role of immune mechanisms in induction of HIV-1 broadly neutralizing antibodies. *Current opinion in immunology* 23: 383-390.
254. De Silva, N. S., and U. Klein. 2015. Dynamics of B cells in germinal centres. *Nature reviews. Immunology* 15: 137-148.
255. Ramiscal, R. R., and C. G. Vinuesa. 2013. T-cell subsets in the germinal center. *Immunological reviews* 252: 146-155.
256. Victora, G. D., and M. C. Nussenzweig. 2012. Germinal centers. *Annual review of immunology* 30: 429-457.
257. Daubeuf, S., A. Aucher, C. Bordier, A. Salles, L. Serre, G. Gaibelet, J. C. Faye, G. Favre, E. Joly, and D. Hudrisier. 2010. Preferential transfer of certain plasma membrane proteins onto T and B cells by trogocytosis. *PloS one* 5: e8716.

258. Daubeuf, S., A. L. Puaux, E. Joly, and D. Hudrisier. 2006. A simple trogocytosis-based method to detect, quantify, characterize and purify antigen-specific live lymphocytes by flow cytometry, via their capture of membrane fragments from antigen-presenting cells. *Nature protocols* 1: 2536-2542.
259. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature* 411: 489-494.
260. Herrera, O. B., D. Golshayan, R. Tibbott, F. Salcido Ochoa, M. J. James, F. M. Marelli-Berg, and R. I. Lechler. 2004. A novel pathway of alloantigen presentation by dendritic cells. *Journal of immunology* 173: 4828-4837.
261. Tabiasco, J., E. Espinosa, D. Hudrisier, E. Joly, J. J. Fournie, and A. Vercellone. 2002. Active trans-synaptic capture of membrane fragments by natural killer cells. *European journal of immunology* 32: 1502-1508.
262. Hudrisier, D., A. Aucher, A. L. Puaux, C. Bordier, and E. Joly. 2007. Capture of target cell membrane components via trogocytosis is triggered by a selected set of surface molecules on T or B cells. *Journal of immunology* 178: 3637-3647.
263. Messaoudi, I., R. Estep, B. Robinson, and S. W. Wong. 2011. Nonhuman primate models of human immunology. *Antioxidants & redox signaling* 14: 261-273.
264. Sundling, C., Y. Li, N. Huynh, C. Poulsen, R. Wilson, S. O'Dell, Y. Feng, J. R. Mascola, R. T. Wyatt, and G. B. Karlsson Hedestam. 2012. High-resolution definition of vaccine-elicited B cell responses against the HIV primary receptor binding site. *Science translational medicine* 4: 142ra196.
265. Wetzel, S. A., T. W. McKeithan, and D. C. Parker. 2005. Peptide-specific intercellular transfer of MHC class II to CD4+ T cells directly from the immunological synapse upon cellular dissociation. *Journal of immunology* 174: 80-89.
266. Patel, D. M., P. Y. Arnold, G. A. White, J. P. Nardella, and M. D. Mannie. 1999. Class II MHC/peptide complexes are released from APC and are acquired by T cell responders during specific antigen recognition. *Journal of immunology* 163: 5201-5210.
267. Liu, Y. J., D. E. Joshua, G. T. Williams, C. A. Smith, J. Gordon, and I. C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342: 929-931.
268. Zhou, J., Y. Tagaya, R. Tolouei-Semnani, J. Schlom, and H. Sabzevari. 2005. Physiological relevance of antigen presentasome (APS), an acquired MHC/costimulatory complex, in the sustained activation of CD4+ T cells in the absence of APCs. *Blood* 105: 3238-3246.
269. Tsang, J. Y., J. G. Chai, and R. Lechler. 2003. Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion? *Blood* 101: 2704-2710.
270. Takemoto, N., A. M. Intlekofer, J. T. Northrup, E. J. Wherry, and S. L. Reiner. 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin

expression during pathogen-induced CD8+ T cell differentiation. *Journal of immunology* 177: 7515-7519.

271. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292: 1907-1910.
272. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655-669.
273. Walzer, T., L. Chiossone, J. Chaix, A. Calver, C. Carozzo, L. Garrigue-Antar, Y. Jacques, M. Baratin, E. Tomasello, and E. Vivier. 2007. Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nature immunology* 8: 1337-1344.
274. Daussy, C., F. Faure, K. Mayol, S. Viel, G. Gasteiger, E. Charrier, J. Bienvenu, T. Henry, E. Debien, U. A. Hasan, J. Marvel, K. Yoh, S. Takahashi, I. Prinz, S. de Bernard, L. Buffat, and T. Walzer. 2014. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *The Journal of experimental medicine* 211: 563-577.