SIV infection results in detrimental phenotypic and functional alterations of the naïve and memory B cell compartments that are initiated during acute infection

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Submitted to the Graduate Faculty of

the school of medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

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Multiple B cell abnormalities have been described in humans infected with HIV. These abnormalities include hypergammaglobulinaemia, diminished B cell response to mitogenic stimuli, lymphoma and a depletion of the memory B cell population. There is also evidence to suggest that B cells in HIV infected patients are functionally impaired. The initial antibody response to HIV infection is slow to appear and antibody responses to B cell mediated vaccines in HIV infected individuals are less robust and less durable than in uninfected individuals. Although B cell abnormalities have been characterized in humans, efforts to link these abnormalities to a specific defect within the B cell compartment have not been entirely successful. The SIV/macaque model of HIV infection of humans provides a means for addressing questions about the naïve and memory B cell populations, whose activity may be differentially compromised by HIV infection, but lacking is the ability to resolve these functionally relevant B cell populations in the rhesus macaque. In this study, we established CD27 as a definitive memory B cell marker in the rhesus macaque. Further, we demonstrated that the naïve and memory B cell populations are depleted from the periphery within 14 days of SIV infection and that the memory B cell population recovered more quickly. We also showed that chronic SIV infection resulted in a loss of CD40 mediated naïve B cell survival, indicating a potential mechanism through which SIV infection may lead to the production of non-reactive or

self reactive antibody producing cells. Together, these findings demonstrated that B cell dysfunctions associated with SIV infection are not limited to the memory B cell population as previously thought, but rather that naïve B cell deficits may be more severe than what has been observed in the memory compartment. Increased focus on abrogating alterations that occur within the naïve compartment have the potential to improve viral control in infected individuals. This study of phenotypic and functional B cell changes over the course of infection will aid in the development of strategies that have the potential to improve prophylactic and therapeutic B cell mediated vaccine efficacy.

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PREFACE

When looking back on my graduate career I am reminded of an episode of the television show Man vs. Wild in which Bear Grylls compares survival to eating an elephant. Mr. Grylls' show is based around him getting stranded in a remote location and figuring out how to make use anything he can find in his immediate surroundings in order to return to civilization. Despite the fact that the average graduate student does not need to wring dung through a T-shirt in order to have "water" to drink, getting lost in an inhospitable wilderness has similarities to writing a dissertation. When looked at as a whole, survival, like writing a dissertation is a massive task. The key is taking one step at a time. Like eating an elephant, it cannot be accomplished in one day, but over the course of many days. The way out of the woods or through graduate school is not often well marked, but with knowledge, creativity and patience, can be found.

My personal journey was made much easier with the help and support of my spectacular friends. Over the past few years it has been my privilege to enjoy numerous outings, dinners and game nights. The friendships built from these interactions will last for a very long time. Additionally, I am very touched that many of these great friends made the trip to Shelter Island to witness my wedding to Kristen.

My family, which has grown considerably during my graduate studies with the birth of my nephew and my marriage, is a wonderful source of encouragement and joy. Thank you Lillian, Mike, Kathy, Matt, Kate, Heiki, John, Linda, Jen, Lauren, Derek and Val. I am so fortunate to be surrounded by so many caring and thoughtful individuals. The ideals they have imparted upon me have made it possible for me to reach this point in my life. To them I am forever indebted.

Most of all, I need to acknowledge my wife, Kristen. She is the most thoughtful, intelligent and beautiful person I know. She is patient and kind and has endured many conversations in which I have extolled the virtues of B cells. She has provided a shoulder to lean on in times of need and has been there to share joys of discovery. I realize that I am a very lucky person to have such a wonderful person in my life and am grateful every day.

1.0 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Globally, millions of people are dying every year due to immune dysfunction associated with human immunodeficiency virus (HIV) infection (2). Despite the massive effort undertaken to develop therapeutic and prophylactic vaccines over the course of the last two and a half decades, no effective vaccination strategy has been designed. Indeed, the recently reported results of a large vaccine trial in Thailand showed that vaccinated individuals were only modestly protected against infection (3, 4). In addition, study participants that became infected demonstrated no reduction in the severity of their disease course compared to non-vaccinated individuals (3).

Although many advances have been made in the understanding of both the immune system and the virus, key aspects of the immunology of HIV infection remain a mystery. Of the interactions between the immune system and the virus, the interaction between B cells and HIV *in vivo* has been one of the most difficult to empirically dissect. B cell abnormalities including hypergammaglobulinaemia, diminished responses of purified B cells to mitogenic stimuli *in vitro*, lymphoma and a depletion of the CD27+ B cell population have been described in HIV infected patients (5, 6), but the mechanism driving these dysfunctions is unknown. In infected patients, the initial antibody response to HIV infection is slow to mature (7), and antibody responses to B cell mediated vaccines in HIV infected individuals are less robust and often shorter in duration than in uninfected individuals (8). Although these generalized B cell abnormalities have been described in humans, efforts to link these abnormalities to a specific defect within the B cell compartment have not been successful.

In this study we explicitly characterize the functional capabilities and fate of naïve and memory B cell subsets during HIV infection through the utilization of the SIV/macaque model. Previous studies that evaluated B lymphocytes in infected animals focused on total B cells, effectively precluding a mechanistic understanding of SIV pathogenesis in specific functional B cell compartments. Here, we establish CD27 as a definitive memory B cell marker in the rhesus macaque in order to analyze B cells during the acute and chronic phases of SIV infection. We then build on the phenotypic analysis by analyzing specific functional characteristics of the naïve and memory B cell populations. This study provides the first explicit information about the functional integrity of the naïve and memory B cell populations following SIV infection, data that will facilitate the understanding of B cell populations of HIV infected humans.

1.2 SPECIFIC AIMS

The following aims and hypotheses frame this dissertation:

Aim 1: To demonstrate that SIV infection results in the rapid and significant loss of naive and memory B cell subsets from the periphery in rhesus macaques and that the naive B cell population does not recover in number as early as the memory B cell population.

Hypothesis: *Naïve and memory B cells are differentially susceptible to SIV infection, and repopulate with different kinetics.*

Aim 1a: To establish that CD27 expression alone accurately and sensitively identifies memory B cells in the rhesus macaque model.

Aim 1b: To establish the kinetics and magnitude of B cell losses in a longitudinal study of SIV infection, with specific attention to the pivotal naïve and memory cell subsets.

Aim 1c: To compare proliferation and activation in naïve versus memory B cell subsets during acute SIV infection.

Aim 2: To demonstrate that chronic SIV infection leads to functional alterations that are inherent to the B cell compartment.

Hypothesis: *Functional alterations that arise in B cells during chronic SIV infection result from changes in B cell reactivity and are not solely due to a loss of T cell help.*

Aim 2a: To establish that chronic SIV infection alters the sensitivity of naïve B cells to CD40 mediated survival.

1.3 HIV BACKGROUND AND B CELL IMMUNOLOGY

1.3.1 HIV as a global pandemic

According to the World Health Organization Statistical Information System, the life expectancy of individuals born in Lesotho, Swaziland and Zimbabwe has been reduced by 2 decades since 1990 (2). The high death rate associated with the HIV/AIDS pandemic especially in these three countries has played a key role in this extreme reduction (figure 1). In the WHO report of deaths due to HIV/AIDS in 2005, each of these countries reported a death rate per year due to HIV/AIDS of one percent of the population (2). Despite the efforts of innumerable health workers, the HIV/AIDS pandemic is ravaging people all over the world. The prevention of new infections and the effective treatment of individuals already infected are necessary to reduce the social and economic impact of the virus in the future.

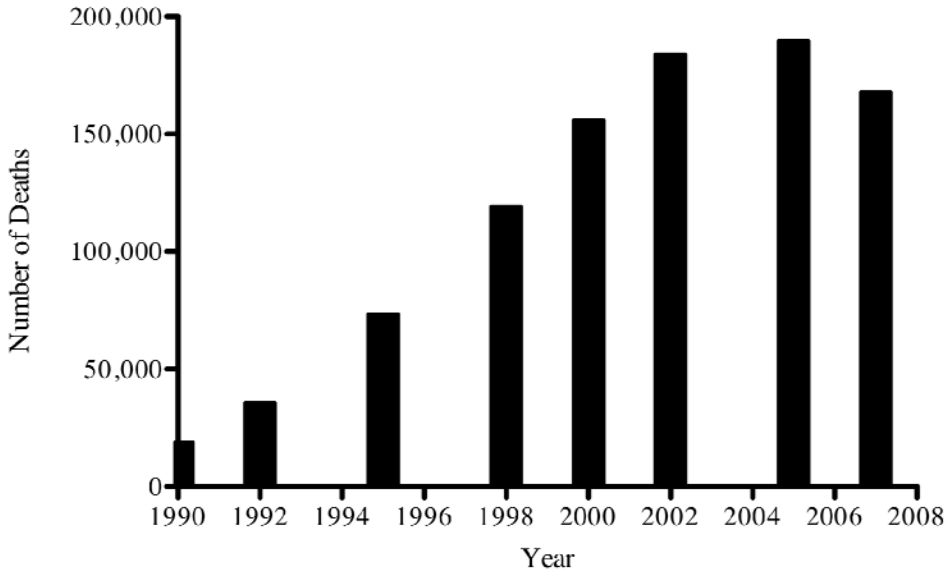

Figure 1. Estimated number of deaths from AIDS in Lesotho, Swaziland and Zimbabwe.

Adapted from UNAIDS, AIDS epidemic update 2009 (1).

The development of an inexpensive, effective vaccine and a massive education effort will be necessary to eradicate HIV. It is of utmost importance for the scientific community to provide an effective, safe, inexpensive and durable vaccine to augment educational efforts focused around the prevention of transmission. Despite almost 3 decades of effort, no vaccine has been developed which meets these criteria. Recent vaccine trials have only demonstrated modest efficacy in disease prevention with no improvements in disease control in individuals that do become infected (3, 9). It is clear that a better understanding of HIV pathogenesis is needed. More information regarding how the virus interacts with immune cell populations, including macrophages, T cells and B cells, will be necessary to design a vaccine that will prevent infection or aid in the clearing HIV from an infected individual.

In the following sections, I detail the nature of HIV pathogenesis and associated immune responses, mechanisms of viral evasion, and the current state of vaccine therapy. Finally, I frame key gaps in the literature that form the basis of this dissertation in which I define B cell defects following SIV infection in a rhesus macaque model. This work represents not only the first longitudinal study to characterize B cell deficiencies in the acute and chronic stages of infection, but also the first to empirically demonstrate the distinct effects of SIV infection on naïve versus memory B cell subsets.

1.3.2 HIV structure and life cycle

Since its discovery in a lymph node biopsy from a patient who had lymphadenopathy and was considered at risk for acquiring AIDS (10), HIV has been genetically and structurally characterized. The virus is classified as a lentivirus, which is a member of the retrovirus family. The lentivirus group derives its name from the fact that all viruses in this group are slow acting with a progressive course of months to years, as compared to other viruses such as influenza, which can progress through infection in about 7 days (11). Retroviruses are enveloped viruses with icosahedral capsid symmetry that contain 2 copies of a positive sense RNA genome. Like all retroviruses, HIV contains the group specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) genes. The *gag* gene codes for the production of capsid and matrix proteins, the *pol* gene codes for the production of reverse transcriptase, protease and integrase enzymes, and the *env* gene codes for the production of gp120 and gp41 envelope proteins. The function of reverse transcriptase and the structural features of gp120 and gp41 are key viral immune evasion features and will be discussed further in section 1.3.5. HIV also contains the accessory genes transactivator (*tat*), regulator of viral expression (*rev*), negative-regulation factor (*nef*), viral

infectivity (*vif*), viral protein R (*vpr*) and viral protein U (*vpu*). The genome of HIV can be read in three reading frames making it possible to produce many proteins from a relatively small amount of genetic material. Mature HIV particles are studded with trimerized gp120 and gp41 proteins and contain a bullet shaped capsid in which the RNA genome, integrase, protease and reverse transcriptase reside (figure 2). Despite difficulties in understanding aspects of HIV pathology, the relative simplicity of the genome has allowed for the virus itself to be well characterized.

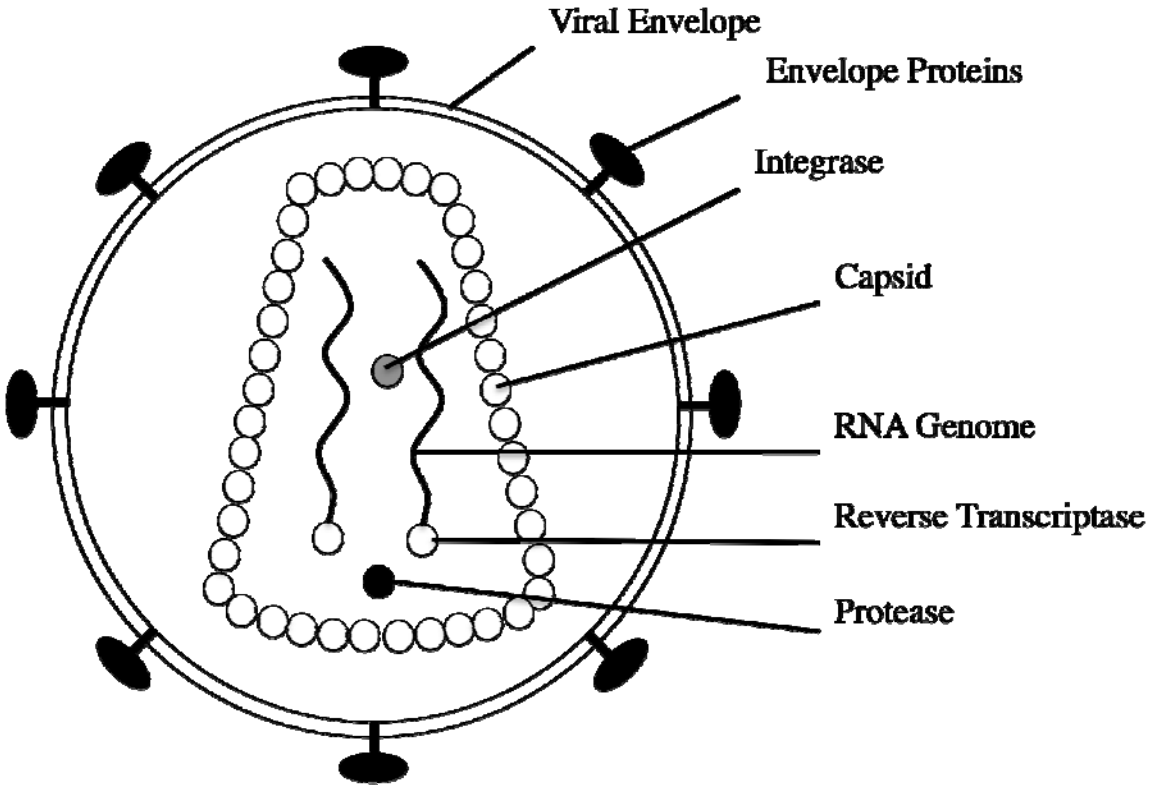

Figure 2. HIV structure and virion components

The viral life cycle of HIV involves multiple stages. Viral particles are able to infect cells through the binding of the gp120 segment of the Env protein to CD4 surface antigen on the host T lymphocytes followed by the fusion of the viral and host cell membranes, which is facilitated

by the binding of the gp41 segment of the Env protein to either chemokine receptor CCR5 or CXCR4. After membrane fusion, the viral capsid is released into the cell and docks on a nuclear pore on the nuclear membrane (12). Prior to being released into the nucleus, reverse transcriptase, which was packaged in the capsid with viral RNA, copies the genome into double-stranded cDNA. After release into the nucleus, the double-stranded viral cDNA can be integrated into the host DNA. Activation of the host T cell or macrophage will result in the induction of proviral transcription followed by splicing in order to form the early genes, *tat* and *rev*. Viral RNA is then transcribed and viral proteins are translated. The viral proteins and RNA genome are assembled into viral particles at the host cell plasma membrane and are then able to bud from the membrane as fully formed particles (figure 3).

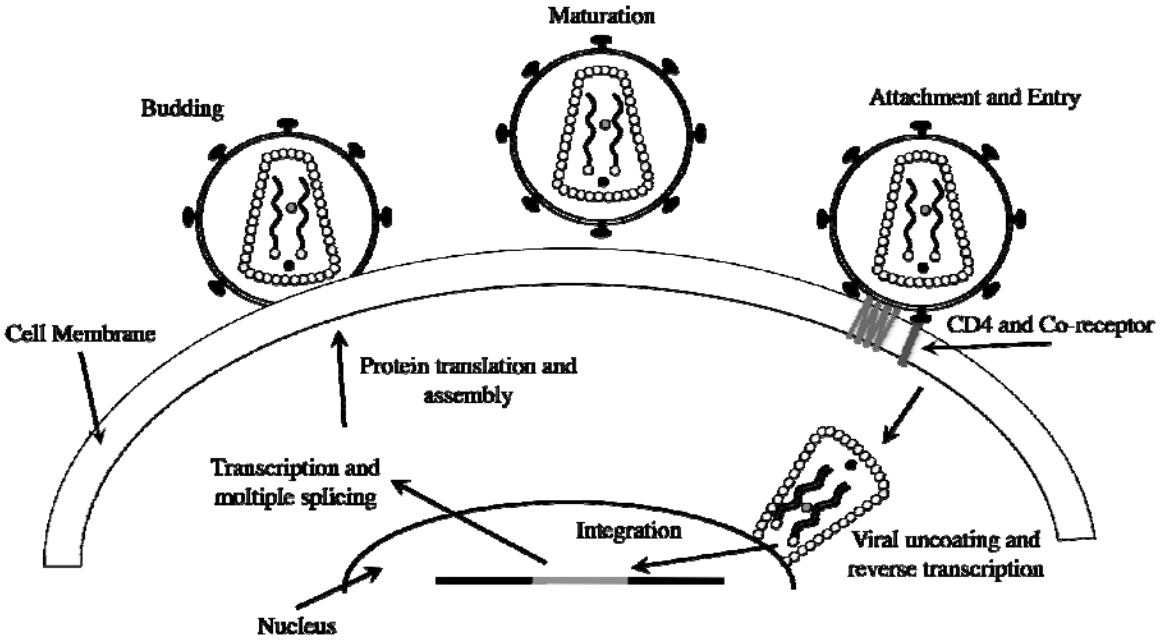

Figure 3. Viral life cycle

HIV is able to persist within the host due to multiple immune evasion strategies. The most confounding means of persistence is the fact that HIV infects CD4 T cells since these cells

are essential for directing strong humoral immune responses. When a humoral immune response is initiated to combat an invading pathogen (either HIV or non-HIV), CD4 T cells proliferate, resulting in more targets available for HIV infection. In addition to infecting T cells, HIV is able to evade immune recognition due to glycosylation shielding conserved regions of the envelope protein and the high variability of immunodominant regions of the envelope protein (13), as well as through the down-regulation of MHC I expression on infected cells through the activity of Nef (14). These tactics prevent immune recognition and make it possible for HIV to continually propagate within the host.

1.3.3 Routes of exposure

HIV infection is most commonly transmitted across a mucosal surface, through sexual contact, or by direct exposure to infected blood. The mode of transmission dictates the cell populations that are initially infected and the immune cell populations responsible for the primary anti-viral response. During sexual transmission, a small amount of free virus or cell associated virus will interact with the epithelial layer of the exposed host and has the potential to be taken up by tissue resident antigen presenting cells including macrophages and dendritic cells. Due to the fact that CCR5 tropic virus primarily infects macrophages, cells that are found in high numbers in mucosal sites, it is the predominant strain associated with mucosal infection (15, 16). This selective pressure dictates that viruses capable of utilizing the CCR5 coreceptor have a greater chance of initiating an infection through the mucosal route (17). After the infection is initiated, the virus population expands. It has been shown in SIV infection of rhesus macaques that mucosal infection leads to propagation of virus in the initially infected cells where founder populations of virus are established (18). These founder populations repeatedly disseminate virus

and are thought to be critical for establishment of productive systemic infections (18). Although mucosal infection can benefit the virus by allowing for the establishment of founder populations, mucosal immunity must be overcome in order for the virus to propagate. As sites that are in contact with the external environment, mucosal surfaces have multiple defense mechanisms including physical barriers and mucin (19).

Non-human primate SIV studies have demonstrated that direct exposure to the blood stream leads to a rapid initiation of infection (20). Although intravenous exposure does occur in humans through i.v. drug use or accidental needle sticks and these exposures lead to infection (21), our knowledge of immune and virus events of the first few days following this mode of infection is limited to non-human primate studies. In i.v. infection, free virus and cell associated virus are introduced directly into the blood stream of the new host, bypassing the epithelial layer defenses that must be overcome in mucosal infection (22). Intravenous infection results in quantifiable viral loads within 3 days of inoculation (23), a rapid progression that is likely due to the fact that no selective pressure is placed on the virus.

Following mucosal infection or intravenous infection, the virus is able to establish long-term reservoirs. Reservoirs are established through the integration of viral cDNA into the genome of non-activated central memory and transitional memory CD4 T cells (24). Regardless of the route of inoculation, chronic HIV or SIV infection is established and no major differences in the long-term clinical disease course have been reported. A majority of SIV infection studies use intravenous infection because it reliably results in a quantifiable infection with a single administration. Mucosal infections can be more difficult to initiate and it can take multiple administrations to establish infection. The SIV/macaque model is explicitly detailed in section 1.3.10. Together, these studies demonstrate that the mucosal and intravenous routes of infection

induce initial immune responses with differing duration between viral inoculation and systemic infection but do not appear to affect the clinical course of chronic infection.

1.3.4 Phases of infection

HIV infection results in three distinct, clinically defined phases of disease progression: acute, chronic and fulminant. The primary stage of infection is often associated with an acute retroviral syndrome that is characterized by anorexia, fatigue, fever, malaise, headache, swollen lymph nodes and ulcers of the mouth and esophagus. Longitudinal modeling of human HIV infection in SIV infected non-human primates has demonstrated that during this primary infection, viral load peaks and then declines (25). There is also a transient reduction of CD4 T cells from the periphery concurrent with the peak in viremia (26) (figure 4). Interestingly, although CD4 T cell numbers do recover following the initial infection, in most cases they do not return to pre-infection levels (20, 27). Following the peak in viral load, there is a decline to lower amount of virus that is maintained at a fairly constant level over the next phase of infection (26).

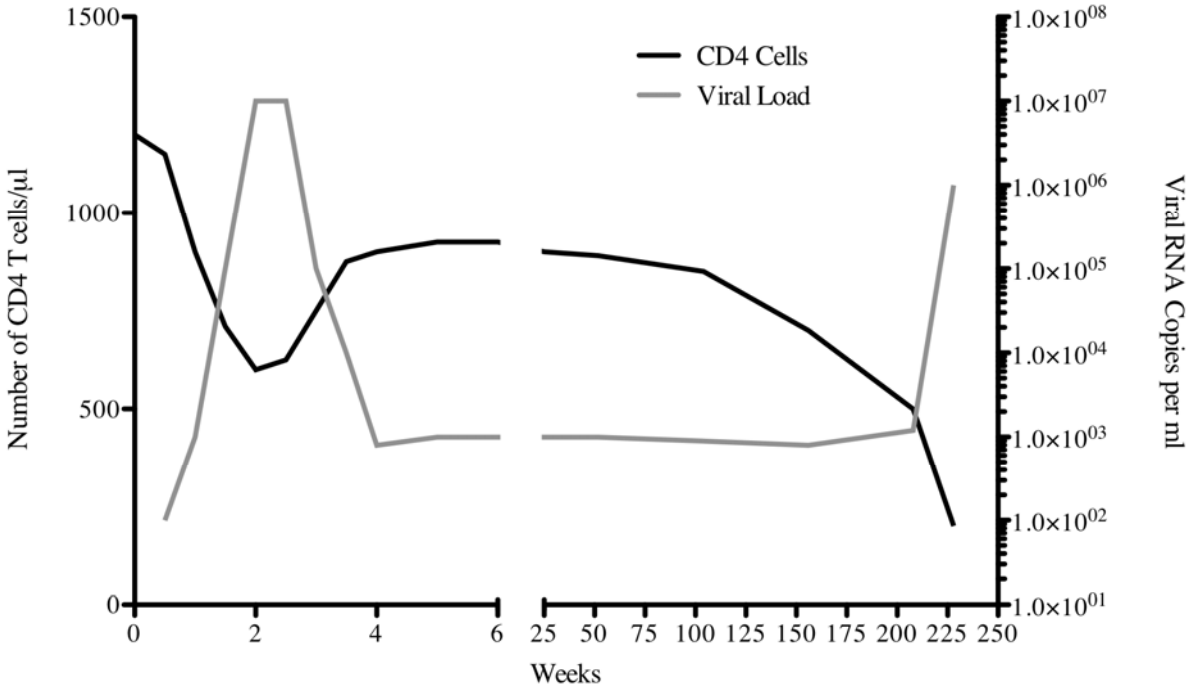

Figure 4. Clinical course of HIV and SIV infection.

Schematic representing the general clinical course of HIV or SIV infection in untreated individuals. There is often much variation between patients. Untreated patients have set point viral loads that range from 200 to 1,000 copies per ml while treated patients can have viral loads that are below the limit of detection. As for the CD4 T cells, the general trend in most patients is similar to what is illustrated in the figure, the range of CD4 T cells prior to infection is usually between 800 and 1200 cells per µl. The chronic phase of infection in untreated patients can range from 2 to 10 years. The clinical course of SIV is similar except that disease progression occurs more rapidly, usually within 6 months to 3 years following infection.

The severity of disease during the primary phase of infection can impact disease progression at later stages of infection. The viral burden following initial decline correlates with time to progression to AIDS. Diminished set point viral load correlates with longer time to disease progression and higher viral set point correlates with more rapid progression to AIDS (28, 29). Immunological control of the initial wave of virus is obtained through activity cytotoxic T lymphocytes (30, 31) and is likely augmented by the activity of B and CD4 T cells as well as innate factors.

Importantly from an immunological perspective, primary infection results in lymphopenia. Data from SIV infection of non-human primates have demonstrated that the number of CD4 T cells and B cells are reduced by about a third in the periphery within two weeks of infection (20). In addition to cell losses in the periphery CD4 T cells are also depleted from the gut, a pivotal site of mucosal immunity (32). The loss of these cells and immune structure within the gut mucosa leaves the host susceptible to opportunistic infection (33) and leads to excess immune activation and potentially low-level sepsis which has been hypothesized as being responsible for the slowly progressing pathology associated with HIV infection (34). In a study by Li *et al*. it was shown that SIV induces apoptosis of intestinal CD4 T cells during early infection through the binding of the GPR15/Bob receptor (35). The depletion of these cells represents a mechanism through which HIV and SIV are able to blunt the initial adaptive immune response.

The beginning of the chronic phase of infection coincides with the leveling off of the viral load after the initial peak. The CD4 T cell population also reaches a steady state at this time that is reduced from pre-infection levels (24-27). The chronic phase of infection is often referred to as clinically latent, but it is clear that the virus does not exhibit viral latency. In this phase of infection, constant viral turnover and prolonged immune activation eventually lead to a loss of viral control and disease progression. In naïve mucosal CD4 T cells, low levels of viral replication have been found (36). In the periphery, resting naïve CD4 T cells act as a viral reservoir (37); they do not exhibit low level viral turnover, but demonstrate viral production when the cells are activated and mature into effector memory cells (36). The infection of resting memory cells results in subsequent immune activation being equivalent to a double-edged sword. The reactivation of an HIV infected memory T cell that is specific for an antigen such as

adenovirus for example, will result in an anti-adenovirus response at the cost of increased HIV production. The viral turnover associated with chronic infection results in continual evolution of the viral *env* and *gag* genes (38), preventing recognition and clearance. These persistence strategies allow HIV to be maintained in a chronic infection.

End stage disease (fulminant AIDS) follows the chronic phase of infection. Fulminant AIDS is clinically defined as CD4 T cell levels less than 200 cells per μl (1). At this stage, viral load levels generally increase rapidly, and the patient becomes highly susceptible to numerous opportunistic infections including *Pneumocystis* pneumonia (39), hepatitis B or C virus (40, 41) and cancer (42). These types of infections that result in significant pathologies are often a primary cause of death. The advent of antiretroviral therapy has been successful in prolonging the time it takes for the disease to progress, but the virus eventually causes a severe enough reduction in immune cells, especially CD4 T cells, that cannot be overcome. Cancer, bacterial infections and liver failure are all common causes of death in HIV infected individuals that are treated with antiretroviral drugs (43, 44).

1.3.5 Viral evolution and immunologic control of HIV during infection

The plasticity of the HIV genome allows the virus to evade immune recognition. The error prone nature of reverse transcriptase and highly variable regions of immunodominant Env proteins allow for the production of progeny viruses that are not identical to the parental strain. HIV, like other retroviruses including Moloney murine leukemia virus and avian leukosis virus all have nucleotide substitution rates of 10^{-4} to 10^{-5} per round of replication which is much higher than the error rate of 10^{-9} to 10^{-10} found in genomic mammalian DNA (45). In addition to random mutations introduced through reverse transcription, a guanine usage bias increases the mutability

of the *env* gene specifically (46). With 10^6 cultured CD4 T cells being able to produce 10^9 viral particles and of those between 10^5 and 10^6 are infectious (47), the odds of producing a mutant virus *in vivo* that is infectious and can evade immune recognition is high. This genomic flexibility provides a means for the virus to evade immune responses from CTL and neutralizing antibodies throughout all phases of established infection (48, 49).

The predominant viral strain within a host often evolves over the course of infection and results in altered susceptibility to infection of primary viral targets, macrophages and CD4 T cells. The initial infection most often occurs with virus that targets CD4 and the chemokine receptor CCR5 in order to bind to and infect T cells and macrophages (50, 51). CCR5 utilizing viruses are classified as macrophage tropic viruses. As the infection progresses and the availability of cells expressing CD4 and CCR5 diminishes (52), the virus can adapt to be able to bind the chemokine receptor CXCR4 in addition to CCR5 (53). This dual tropism often gives way to single CXCR4 tropism during the latter stages of infection. CXCR4 tropic viruses are classified as T cell tropic viruses. T cells, which express both CCR5 and CXCR4 (54), represent viral targets in all stages of infection. These reports demonstrate that HIV infection results in a loss of CCR5 expressing cells within the host which induces changes within the virus, illustrating that the interaction between the virus and the host goes in both directions and is dynamic.

Although HIV is able to adapt very well to its host environment in order to maintain infection, two cohorts of people prove that long-term survival after exposure to HIV is possible without antiretroviral therapy treatment. Long-term survivors and multiple exposed seronegative individuals are seemingly able to control or prevent infection and are the subjects of numerous studies. Long-term survivors, characterized by the maintenance of a relatively high level of CD4

T cells for seven or more years after seroconversion in the absence of antiretroviral treatment, are rare. Indeed, in a large cohort study, only 3.3% of participants were categorized as long-term survivors. In many cases, a specific viral defect or immunological feature has been found to be responsible for long-term control. In terms of viral defects, multiple studies have shown a subset of long-term survivors to be infected with HIV containing deletions in the *nef* gene (55-57). For immunological features that are protective, conserved cytotoxic T lymphocyte epitopes (58), deletions in CCR5 (59), and the presence of antibodies that are able to induce antibody dependent cellular cytotoxicity (60) have all been reported in long-term survivors. Interestingly, stress has also been shown to play a role in survivorship, patients in a study by Ironson *et al*. demonstrated that reduced stress and lowered cortisol levels significantly improved survival time and general health (61). From these studies it is clear that under specific circumstances, HIV infection can be controlled

Sex workers who are known to be exposed to HIV, are seronegative for HIV and show no overt signs of infection make up a cohort that has also been the subject of many studies. Multiple immune mechanisms including strong mucosal IgA neutralizing antibodies (62, 63), robust IFNγ production by NK cells (64), non-cytolytic CD8 responses (22) and gene cluster polymorphisms in the IRF-1 gene (65) have all been shown to play a role in protection from infection in these individuals. These studies provide clear evidence that under the correct conditions, infection can be prevented. The high mutation rate of HIV is an effective persistence trait but that with just the right combination of factors, a strong highly specific antibody or cytotoxic T lymphocyte response, or viral attenuation, infection can be controlled.

For the majority of HIV infected individuals, a combination of cellular and humoral immune responses keeps the virus under control for years but is eventually overcome. Cytotoxic

T lymphocytes are essential for blunting the initial viral load (66) and for maintaining viral control during chronic infection (67). B cells have been found to play a more profound role in the chronic phase of infection, through the production of neutralizing antibodies (68) and are also necessary for maintaining low viral loads (69). The combined effect of B and T cells is greater than either arm of the immune system working alone, for this reason it is necessary to augment the larger body of work focused around T cell responses during HIV infection with more in-depth B cell studies.

1.3.6 B cell maturation and differentiation

In humans, the majority of B cells found in the periphery can be categorized as either naïve or memory cells. Following differentiation in the bone marrow, naïve B cells are released into the periphery. Upon antigen binding through the B cell receptor (BCR), and receiving survival signals such as BCR cross linking or stimulation with CD154 and either IL-2, IL-4 or IL-10, naïve B cells are matured into memory B cells (70-73) (figure 5). Further selection and maturation of the memory B cell population with IL-2 and IL-10 has been shown to lead to the production of plasma cells and long-lived quiescent memory B cells (74) (figure 6). Memory B cells have mutated immunoglobulin genes and in humans have been shown to express CD27 on the cell surface (71, 75, 76). Interestingly, not all laboratories that study B cell populations utilize CD27 to differentiate naïve and memory cells, some use CD38 alone or in combination with CD44 (77). A study of tonsillar B cells by Chaganti *et al*. clearly showed that CD38 did not stain the same population as CD27 and that CD38 staining appeared on generally activated cells (78). Further comparative studies are necessary to test whether this holds true in peripheral B cell populations as well. Memory B cells can be further differentiated based on IgD surface

expression into Ig class switched (CD27+IgD-) and non-switched (CD27+IgD+) populations (79, 80). No labeling strategy for the quiescent memory B cell population has been devised but this population is known to exist *in vivo* from long-term studies that have showed reactivity to vaccines decades after inoculation (81, 82). In humans, CD138 stains a subset of plasma cells (83), but the ability of human CD138 antibodies to differentiate a similar population in rhesus macaques has yet to be proven. These studies demonstrate that surface staining of B cells is an effective means to differentiate B cell populations in multiple phases of maturation.

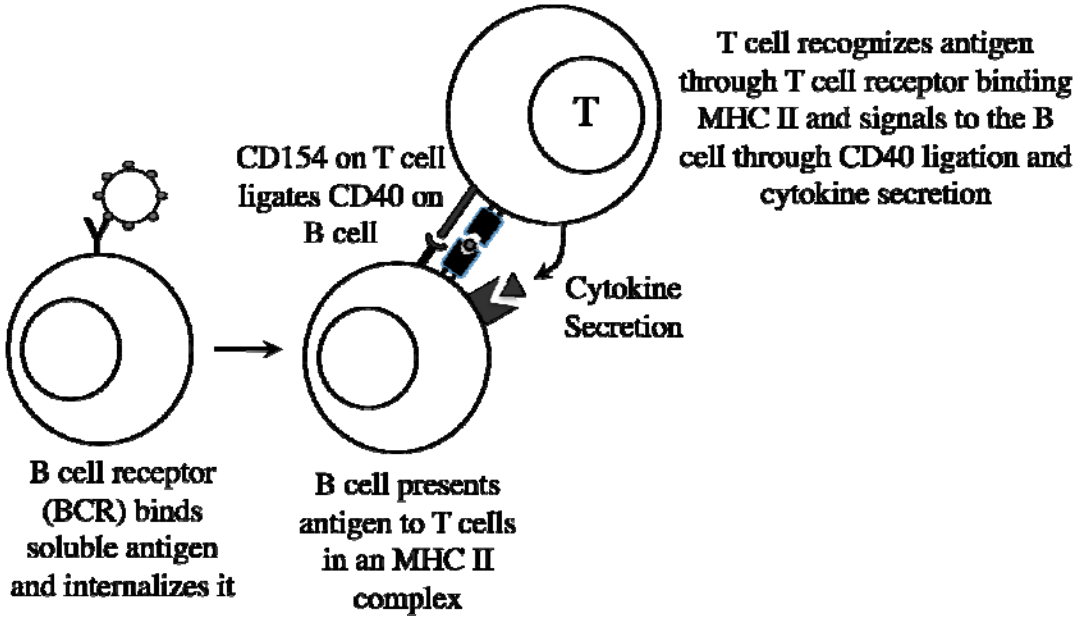

Figure 5. Recognition of antigen by B cell in a T-dependent B cell response.

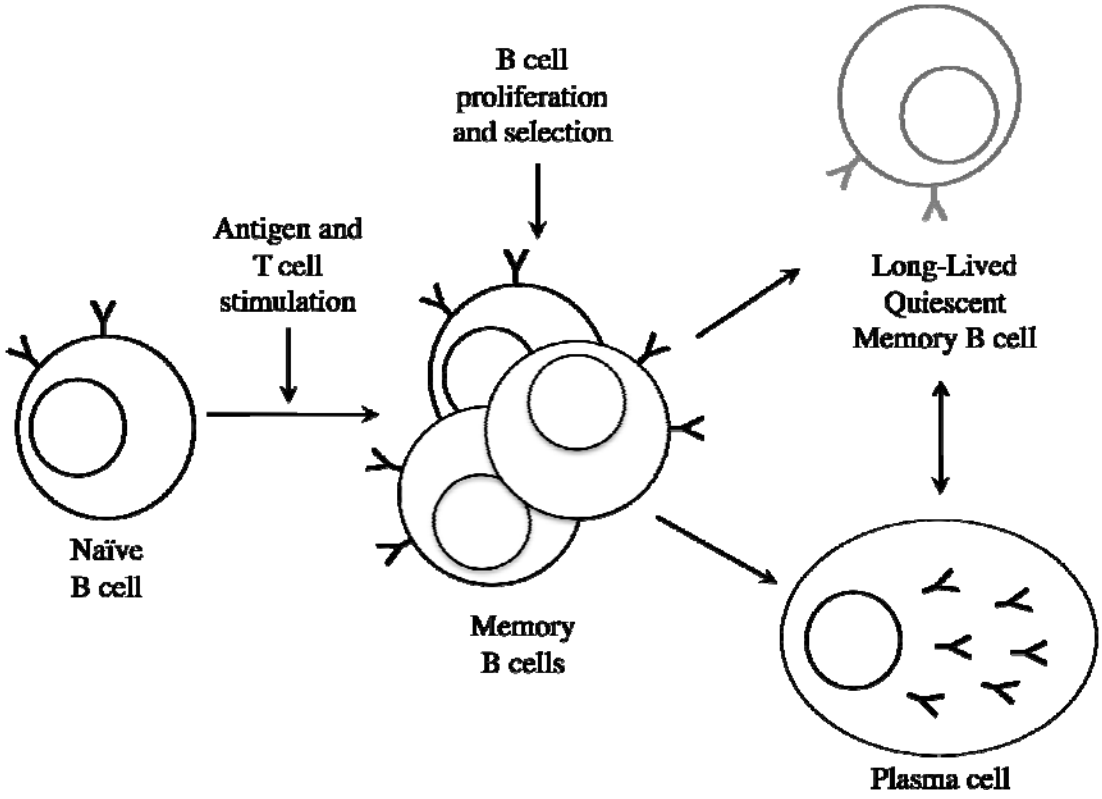

Figure 6. B cell maturation.

Naïve B cells are released into the periphery from the bone marrow and circulate until they encounter antigen. With sufficient T cell stimulation or T cell independent signaling, the cells are able to enter a germinal center reaction in which the cells proliferate and undergo clonal selection. Following selection, plasma cells are produced that produce high quantities of antibodies capable of binding antigen. Once the pathogen is cleared, the pathogen specific B cells are reduced and only a small number of clones are retained as long-lived quiescent memory and plasma cells that are capable of mounting a response to a secondary infection by the original pathogen.

1.3.7 The B cell population during HIV infection

Chronic HIV infection has been shown to result in multiple activity changes in B cells including increased expression of activation markers, increased polyclonal activation, increased cell turnover and hypergammaglobulinaemia (5, 6, 84). Functionally, B cells from HIV infected persons demonstrate an increased proclivity to differentiate into plasmablasts and to produce auto-reactive antibodies through the production of antibodies by cells that express low levels of CD21, a population that is overrepresented in HIV infection (85). In addition, B cell malignancies including non-Hodgkin's lymphoma are not uncommon in the context of HIV infection (84, 86-90). Despite the discovery of these multiple B cell abnormalities, the mechanism or mechanisms through which HIV induces these changes in vivo remain unclear.

At present, there is no evidence to suggest that B cells are directly infected by HIV despite the fact that they express the chemokine receptors CCR5 and CXCR4 (91, 92). Although not actively infected, *in vitro* studies have demonstrated that incubation of B cells with HIV can induce B cell proliferation as measured by thymidine incorporation and differentiation reported in terms of increased antibody production (6). The *in vivo* mechanism for HIV mediated activation is unclear but gp120 has been shown to induce B cell cyclic AMP generation as well as proliferation and antibody secretion (93). Nef has been shown to alter B cell activity *in vitro.* In an elegant study by Xu *et al*. it was demonstrated that Nef is shuttled from infected macrophages to B cells through cellular conduits, and once inside B cells, it attenuates class switching to IgG2 and IgA subclasses (94). These studies demonstrate that despite the fact that B cells are not actively infected by HIV, the virus is still capable of manipulating B cell activity.

In addition to functional differences in B cell activity during HIV infection, the physiological distribution of B cell populations is altered during HIV infection. Initial studies of

B cell populations in humans, with very few longitudinal timepoints, during chronic HIV infection demonstrated that the percentages of memory B cells is decreased (95-99). From these studies it is not possible to ascertain whether the B cells were being slowly depleted or whether all of the cells were lost at one specific time, and were never able to recover. Due to the limited samples available from HIV infected humans and the lack of understanding how HIV induces B cell dysfunction, the mechanism responsible for the loss of memory B cells remains unknown. Even so, the loss of memory B cells has been hypothesized to be responsible for the poor antibody mediated responses induced by vaccination (5).

Human naïve B cells do not appear to be depleted during chronic infection and in fact, have been reported to be greater in frequency (100). However, recent reports demonstrate that this population in HIV infected individuals may not be able to mature into memory B cells as well as in uninfected individuals (101). The presence of naïve B cells and fewer memory cells may be explained in part by the finding that naïve B cells are unable to respond in a normal manner to antigenic stimuli and undergo maturation (102). Immature, phenotypically naïve, CD21 low, B cells in HIV infected individuals have been shown to produce antibodies (101). The antibodies produced likely have decreased efficacy than antibodies from normally matured cells. The inability to mature properly has been hypothesized to be a consequence of poor interaction between B and T cell populations during HIV infection (103). Despite an increased frequency, the naïve B cell population does not appear to be completely functional in HIV infected individuals.

HIV infection is associated with lymphopenia. Despite the fact that the initial loss of CD4 T cells is most often noted in clinical descriptions of viral progression, a longitudinal phenotypic study of rhesus macaques showed that the magnitude of the loss of CD20+ B cells is greater than

the loss of CD4+ T cells (20). The CD4+ cells were depleted from around 900 cells/μl to about 600 cells/μl (averaged value for 6 animals) by day 7 post infection while the B cell population was depleted from around an average of 1300 cells/μl to around 200 cells/μl by day 7 (20). This study is the one of very few published longitudinal assessments of B cells in the SIV model and the best evidence that a similar pattern of depletion would occur in HIV infection of humans. Unfortunately, this study had a small sample size and did not assess B cell subsets. From this study it was not clear how naïve or memory B cells responded to infection and whether or not one of these subsets appears better able to recover following infection. The loss of B cells during acute SIV infection may impact antibody responses in later phases of the infection and needs to be critically analyzed.

Curiously, depletion studies in the non-human primate model have demonstrated that acute phase viral control can be achieved in the absence of B cells. Anti-CD20 antibody mediated B cell depletion during early infection in pathogenic SIV infection does not result in increased peak viral loads, but results in more rapid disease progression during the chronic phase of infection (69). Similarly, Rituximab mediated B cell depletion in non-pathogenic SIV infection does not result in increased viral burden or result in greater loss of CD4 T cells in the acute or chronic phases of infection (104). These studies demonstrate that the B cell population in HIV and SIV infected hosts may be underutilized. If B cell mediated antibody responses are compromised by the virus, reversing viral mediated B cell disruption could result in vastly improved viral control.

1.3.8 Vaccine efficacy in HIV infected individuals

The failure of all anti-HIV vaccine strategies to date to provide more than modest levels of protection illustrates our collective lack of understanding as to how an effective immune response can be mounted against HIV. Recently, a large vaccine trial in Thailand showed that vaccinated individuals had only slightly significantly improved protection against infection compared to non-vaccinated individuals (3, 4) and of the study participants that became infected, no reduction in the severity of their disease course was observed compared to non-vaccinated individuals (3). Early prophylactic vaccine strategies showed promise because it appeared that at least in non-human primates, passive transfer of homologous antibody could confer protection (105-115). Unfortunately, due to the variability of the virus, these studies did not predict future success in humans. T cell epitopes, which are more conserved, also represent a useful target. Recombinant virus based vaccines that induce T and B cell responses have been made with yellow fever virus (116), rabies virus (117) and adenovirus (118), DNA and peptide based vaccines that target T cell responses show promise in non-human primate models (119-121). Unfortunately, only limited effectiveness has been achieved with clinical trials in humans (3, 9, 122, 123). Additional trials are ongoing. A recent study showed that a modified Ankara virus based HIV vaccine was safe and able to elicit a robust anti-HIV response in the form of antibodies to gp120 and T cell production of IFNγ in response to encountering HIV (124-126). In terms of vaccine production, a limitation to T cell mediated vaccines is that the infection must already be underway in order for the anemnestic immune response to be initiated. By contrast, vaccines that elicit productive B cell immunity in addition to T cell immunity, offer the opportunity to slow viral spread prior to infection and target cells that become infected through antibody dependent cellular cytotoxicity, giving the immune system a better opportunity to

prevent the establishment of chronic infection. Since no effective vaccine has been produced after 20 years of effort it has become clear that not enough is known about how the B and T cell populations are affected by HIV or SIV infection and more basic research into how these populations change over the course of infection will pay dividends in the design of future prophylactic and therapeutic vaccine strategies. An in-depth understanding of the immune correlates of protection required to prevent HIV infection is needed to improve the design of anti-HIV vaccines.

In addition to anti-HIV vaccines, vaccines to non-HIV antigens have reduced efficacy in HIV infected individuals. Antibody responses to B cell vaccines in HIV infected persons are less robust and are shorter in duration compared to responses to vaccination in uninfected individuals. Specifically, antibody titers following influenza vaccine are lower in HIV infected people, currently on antiretroviral therapy, compared to uninfected people (127, 128) and the antibody titer is decreased over time more rapidly in HIV infected people (8, 129). A similar lack of protection following primary vaccination with poor to nonexistent anemnestic responses has been reported following hepatitis B vaccination, even in antiretroviral treated individuals (130). Interestingly, a separate study of hepatitis A vaccination demonstrated that HIV infected children on antiretroviral therapy are capable of producing a robust antibody response comparable to uninfected children (131). Even so, another study showed that children who are starting or changing their antiretroviral treatment are unable to mount a recall antibody response to tetanus toxoid (132). Together these studies show that in many cases, B cell mediated vaccines have reduced efficacy in HIV infected individuals. By fully characterizing the phenotypic and functional B cell changes associated with HIV infection, specific dysfunctions can be identified and possibly corrected, leading to the improved efficacy of vaccines in HIV infected individuals.

1.3.9 Antiretroviral therapy

Treatment with highly active antiretroviral therapy (HAART) is an effective means of reducing viral load and boosting the number of CD4 T cells. For example, HAART has been shown to reduce viral loads by 2 $\log_{10}$s and increase the number of CD4 T cells per μl of blood by hundreds of cells (133-135). Amongst B cells, HAART treatment also decreases spontaneous hypergammaglobulinaemia and non-specific B cell activation as determined by induced antibody secretion in the absence of stimulation (103, 136). The improved B cell activity has been hypothesized to be related to improved T cell help available with HAART (5). Despite these improvements, other B cell deficits including a reduction in the percentage of memory B cells (95) and poor antibody responses to vaccines remain even after long-term treatment with HAART (8). The advent of HAART has improved viral control and extended the lives of numerous HIV infected persons but is not a good candidate for a permanent solution due to ability of the virus to develop resistance to components including reverse transcriptase inhibitors (137, 138).

A major drawback to antiretroviral therapy is this high amount of toxicity associated with treatment. HAART has been shown to result in liver damage (139-141). Clinically this demonstrates that the cost of toxicity must be weighed against the potential benefits afforded by treatment. Also, it brings to light the fact that long term HAART treatment, while effective, is not a permanent solution, due to the fact that viral reservoirs remain during treatment (37). Further study of alternative treatment options are necessary in order to design a therapeutic strategy that has the benefits associated with HAART without the collateral damage.

1.3.10 The SIV/macaque model of human HIV infection

Simian immunodeficiency virus (SIV), which infects non-human primates, is a valuable experimental model of HIV-related pathogenesis and disease. The SIV/macaque model provides a means to gain a clearer picture of what happens to the virus and the immune system during acute and chronic infection. Advantages of the system include the ability to have multiple animals infected with the same viral strain at the same time in order to generate statistically relevant data. These animals can also be analyzed longitudinally from preinfection through end stage disease. This type of data collection allows for immunological parameters from each animal to be compared to its own uninfected control level. Despite the genetic similarity between humans and non-human primates, HIV-1 does not generally infect non-human primates. SIV shares approximately 40-50% sequence homology to HIV, is similar in structure with its virion particle demonstrating the same general shape as HIV, and the envelope proteins sharing commonalities in biochemical properties and function between HIV and SIV. SIV infection results in pathogenesis in multiple non-human primate species that resembles HIV infection in humans but with a markedly shorter disease course. The average time of disease progression for pathogenic SIV infection of non-human primates is 1-3 years compared to an average of more than 10 years for HIV infected humans (44, 142, 143).

Despite many years of study, the rhesus macaque model is not completely understood. This was illustrated by the recent failure of the Merck vaccine trial. Vaccine efficacy in non-human primate models of HIV infection did not successfully translate into the human system (9). Not fully understanding the differences between the human and non-human primate model immunologically as well as overly optimistic interpretation of immune protection likely played roles in the poor translation of these studies. As responsible scientists it is of utmost importance

to recognize the limitations of the system in which we work and strive to fill in the knowledge gaps as they become apparent. In the current study, we addressed a specific gap in rhesus macaque immunology, namely the lack of a definitive memory B cell marker, and utilized the knowledge gained from that new understanding to increase the power of the rhesus model in predicting the effects of HIV infection on specific B cell populations.

The SIV/macaque model has led to a greater understanding of B cells during infection through detailed longitudinal characterization of antibody production. The kinetics of maturing neutralizing antibodies has been shown to take months (68), despite the presence of high titer anti-SIV antibodies first being detected within weeks following viral inoculation (144). Also, passive protection studies have demonstrated that antibody mediated protection from infection is possible (105).

In the current study I utilized the SIV/macaque model to gain a clearer picture of how B cells are affected by infection. First, I established that naïve and memory B cell populations in the rhesus macaque could be functionally differentiated based on surface expression of CD27. Then I longitudinally analyzed naïve and memory B cell populations in a cohort of SIV infected rhesus macaques and demonstrated that acute infection resulted in a rapid and dramatic loss of all B cell populations from the periphery followed by slow recoveries in B cell subsets. Finally, I demonstrated that chronic SIV infection results in a loss of naïve B cell sensitivity to CD40 mediated survival. The main implication of these findings is that SIV infection resulted in multiple perturbations of the naïve B cell compartment, a population that is not generally associated with dysfunction during SIV or HIV infection.

2.0 ESTABLISHMENT OF CD27 AS A B CELL MEMORY MARKER

Naïve and memory B cells in the rhesus macaque can be differentiated by surface expression of CD27 and have differential responses to CD40 ligation

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2.1 ABSTRACT

The rhesus macaque model is an invaluable non-human primate model for understanding the fate of B cell populations during pathogenic infections. To date, there has been no definitive study of naïve and memory B cell populations in the rhesus macaque, thereby precluding a rigorous analysis of the generation, persistence and resolution of antigen- or pathogen-specific memory responses. Precise resolution of these populations at the phenotypic and molecular levels will facilitate translation of findings from the macaque model to humans. The present study utilized flow cytometry, DNA sequence analysis and functional assays to demonstrate that CD27 distinguishes between naive and memory B cells. In contrast to CD20+CD27- B cells, CD20+CD27+ B cells had increased expression of the activation markers CD86 and CD95, were larger in size, and preferentially accumulated at effector sites. Critically, direct sequence analysis

revealed that CD20+CD27+ B cells had increased frequency of point mutations in immunoglobulin variable region genes that were consistent with somatic hypermutation, a hallmark of memory B cells. At a functional level, CD40 ligation of naïve but not memory B cells resulted in protection from spontaneous apoptosis *in vitro*. Further analysis revealed that there is a more rapid increase in the memory compared to the naïve B cell population following an initial loss of cells in this population during acute SIV infection. These data provide definitive evidence that the naïve and memory B cell populations of the rhesus macaque can be differentiated using surface expression of CD27.

2.2 INTRODUCTION

The rhesus macaque model is an essential tool utilized in the study of human immunodeficiency virus (HIV) infection and vaccine production. Unfortunately, recent vaccine trials have demonstrated that our ability to predict clinical outcomes in humans based on findings from non-human primate studies is lacking (9, 145). Immunological differences between humans and rhesus macaques may result in different responses to related pathogens and although there are many similarities between rhesus macaques and humans, they are not immunologically identical and should not be analyzed as such. Analysis of B cell subsets has improved our understanding of antibody producing cells in humans (76, 95, 146), but very few studies have focused on B cell subsets in the rhesus macaque. Effective utilization of rhesus macaques for modeling human disease requires the precise resolution and sensitive detection of relevant immune populations. Indeed, ineffective validation of the veracity of cell markers has led to confusion in non-human primate models that have negatively impacted scientific progress. For example, CD56 was utilized as an NK cell marker in rhesus macaques based on its staining profile in humans (147)

until it was demonstrated that CD56 marks monocytes and not NK cells in rhesus macaques (148, 149).

The ability to clearly differentiate between naïve and memory B cell subsets is critical for the detailed study of antibody production and B cell activity during pathogenic infections. Although B cells have been characterized in the human system, to our knowledge, no detailed characterization of naïve and memory B cells has been performed in the rhesus macaque model. The rhesus model is frequently utilized in the study of human pathogens including HIV. Notably, the early depletion of resting memory T cells from the gut in simian immunodeficiency virus (SIV) infected macaques (150, 151) led to improved understanding of the kinetics of the loss of T cells from the gut in human HIV infection (152, 153). A mechanistic understanding of the immunological characteristics of specific B cell populations in the macaque model is needed to facilitate translation of findings from studies in rhesus macaques to application in humans.

Memory and naïve B cells have distinct phenotypic and functional characteristics. In humans, memory B cells are physically larger than naïve B cells (76) and have increased surface expression of activation markers including CD95, CD80 and CD86 (80, 154). Memory B cells in humans have also been shown to express CD27, a TNF-related type II transmembrane protein that is expressed on the surface of multiple lymphocyte populations (75, 155). Previous human studies have demonstrated that CD20+CD27+ B cells have higher rates of mutation within the variable region of the immunoglobulin gene compared to CD20+CD27- B cells, and these mutations are consistent with somatic hypermutation (76, 79). In addition to phenotypic and molecular genetic differences, functional differences have also been described between CD20+CD27+ and CD20+CD27- B cells including different functional response to co-stimulation with CD154 and IL-4. CD20+CD27+ B cells were shown to differentiate into

antibody secreting cells within 4 days of CD40 ligation while CD20+CD27- B cell populations proliferate within 4 days of CD40 ligation and produce antibodies with different kinetics from the CD20+CD27+ B cell population (72). Work from our lab and others demonstrates differential disruption in CD20+CD27+ versus CD20+CD27- subsets in the rhesus macaque model of SIV infection (156), and obtaining a mechanistic picture of how these cells are altered during infection requires definitive resolution of memory B cells.

Although it is generally assumed that B cell subsets in non-human primates are similar to those in humans, fundamental differences are known to exist. For example, anti-human antibodies to both CD19 and CD20 can be used to stain B cells in humans, but only anti-human antibodies to CD20 and not CD19 consistently identify a distinct cell population in the rhesus macaque (157). Additionally, anti-human antibodies that recognize the plasma cell marker CD138 in humans do not reliably stain rhesus macaque B cells (unpublished observation DK and KC). The reason for the failure of these antibodies to stain rhesus B cells has not been identified, but may be related to differences in structural homology between molecules presumed to have similar phenotypic and functional roles or differences in levels of expression. Additional differences between human and rhesus macaque B cells can be found in the structures of the antibodies produced. Although humans and rhesus macaques both have 4 IgG isotypes, the intron lengths are variable between species (158). Lastly, differences in immunoglobulin A (IgA) subtypes also exist. Two subtypes have been identified in humans (IgA1 and IgA2) while rhesus macaques have only 1 IgA subtype which exhibits high levels of heterogeneity (159). These physical differences in antibodies between species may be indicative of physical differences in B cells between humans and rhesus macaques. The considerable value of the rhesus macaque as a surrogate model for human disease and the potential for discrepancies between models

demonstrates the essential need to functionally validate the major markers used to discriminate hematopoietic populations. In addition, understanding the effects of HIV on B cell subsets during the critical acute phase of infection is needed to determine how the initial interactions between the virus and the host immune system set the stage for long-term disease progression in the infected host. The SIV/macaque model provides a system in which to ask these questions.

The goal of the present study was to establish CD27 as a bona fide memory B cell marker in the rhesus macaque, and to define the role of surface CD27 expression in an animal model increasingly exploited for studies of human pathogens. Specifically, we wanted to determine whether CD27 could be used to accurately differentiate naïve and memory B cells, as a step toward elucidating the roles of naïve and memory B cells during chronic viral infection. Our results demonstrated that CD20+CD27+ B cells displayed characteristic size and activation markers associated with memory populations, accumulated at effector sites, bore somatic hypermutations at immunoglobulin variable (IgV) regions, and failed to be protected from apoptosis following CD40 binding. In contrast, CD20+CD27- B cells were smaller in size and had lower activation marker expression, demonstrated a lack of somatic hypermutation and were protected from spontaneous apoptosis through binding of the CD40 receptor. In addition, during acute SIV infection, the increase observed in the number of CD20+CD27+ cells present from days 14 to 41 post infection was significantly higher than the increase observed in the naïve B cell population. Together, these studies empirically validate CD27 as a phenotypic marker of memory B cells in rhesus macaque, provide insight into the role of CD40 ligation in survival and activation of B cell subsets, and allow for the separate analysis of naïve and memory B cell populations during acute SIV infection.

2.3 METHODS

Animals

Peripheral blood, tissue and bronchoalveolar lavage samples were obtained from SIV negative and experimentally SIV infected (SIVmac239, 10,000 infectious units per animal i.v.) colony-bred rhesus macaques (*Macaca mulatta*) of Indian origin at the Oregon National Primate Research Center. Additional blood samples were obtained from normal rhesus macaques awaiting other studies at the University of Pittsburgh. Cord blood from clinical cesarean sections was obtained from the California National Primate Research Center. All animals were maintained and used in accordance with the guidelines of the Animal Care and Use Committees at their respective institutions.

Flow cytometry analysis of whole blood

Flow cytometric analysis was performed on fresh whole blood and umbilical cord blood samples. Two hundred microliters of citrate treated whole blood was obtained in a blood collection tube (BD Diagnostics, Franklin Lakes, NJ) and incubated for 15 minutes with ammonium chloride solution to promote red blood cell lysis. Remaining cells were centrifuged and washed 2 times with Dulbecco's phosphate buffered saline (dPBS). Titrated biotinylated or directly fluorochrome conjugated antibodies to extracellular targets were incubated with cells at room temperature for 15 minutes. Following incubation, cells were washed once with 4 ml cold (4°C) dPBS with 0.1% of fetal bovine serum (FBS) and 5 micro-molar sodium azide (wash buffer). Cells were then incubated with LIVE/DEAD fixable near IR cell stain (Invitrogen, Carlsbad, CA) at room temperature for 10 minutes. Cells were then washed twice with wash buffer and fixed with 1% paraformaldehyde in PBS. Lymphocytes were differentiated using forward and

side scatter characteristics on an LSRII (BD Biosciences, San Jose, CA). B cell populations were analyzed using anti-human CD20 (2H7, eBioscience, San Diego, CA), CD27 (M-T271, BD Bioscience, San Jose, CA), CD40 (5C3, BD Bioscience), CD86 (IT2.2, eBioscience) and CD95 (clone DX2, BD Bioscience). List-mode multiparameter data files were analyzed using the FlowJo software program (Version 8.8.6; Tree Star Inc., Ashland, OR).

Flow cytometry analysis of lymphoid and effector organ sites

Mononuclear cells from peripheral blood, lymph nodes, bronchoalveolar lavage were isolated through centrifugation over Ficoll gradients and either stained fresh or cryopreserved prior to staining. All samples were stained for CD20, CD27 and viability and analyzed on an LSRII.

Blood processing and cell separation based on expression of CD20 and CD27

Purified PBMC were isolated prior to fluorescence activated cell sorting (FACS) based cell separations. Thirty milliliters of citrated whole blood were utilized for purification. PBMC were isolated using Lymphocyte Separation Media (Mediatech, Manassas, VA). Purified PBMC were stained with CD20, CD27 and CD4 in order to identify B cell and T cell populations. The sort was performed in single cell mode (yield mask = 0, purity mask = 32 and phase mask =16) verified at greater than 95% purity in post-sort analysis. Cells were sorted on a BD FACSAria into tubes containing FBS. Following isolation, cells were immediately pelletted and processed for RNA isolation.

Sequence analysis of immunoglobulin gene for somatic hypermutation

Total RNA was extracted from sorted cells using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA) following the manufacturers protocol and the AMV Reverse Transcription System (Promega, Madison, WI) was used to prepare cDNA. Amplification of cDNA was carried out using nested PCR catalyzed by high fidelity Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA). Each round of PCR was performed in a volume of 25µl using oligonucleotide primers with specificity for IgM IGHV1/7 chain family genes for 35 cycles (outer nested reaction) and 30 cycles (inner nested reaction) as previously described (160). PCR products were size selected from electrophoresis in 1.25% agarose and purified using the PureLink Quick Gel Extraction Kit (Invitrogen). IgM H chain libraries were constructed using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) per manufacturer's instructions. Purified plasmids were obtained from *E. coli* (One Shot, Top Ten Chemically Competent cells (Invitrogen) using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The presence of gene insert was verified by gel electrophoresis following a 1 hour digestion with EcoR1 enzyme. Insert containing clones were analyzed for DNA sequence using the M13 forward primer and an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) at the University of Pittsburgh Genomics and Proteomics Core laboratory (Pittsburgh, PA).

Sequence alignment

IGHV1/7 nucleic acid sequences for rhesus macaques (GenBank/EMBL AY161053-71 and AY161078-79 (161)) were used to create a genetic database. Distance based analysis was performed with Geneious software version 4.7.4 (Aukland, New Zealand) using Tamura-Nei distance estimates and a neighbor joining algorithm to create a phylogenetic tree for the

IGHV1/7 genes. Experimental sequences obtained from CD20+CD27+ and CD20+CD27- B cells from rhesus macaques were instilled into the database individually to determine germline sequence identity. Functional IGHV1/7 sequences were next aligned using Geneious Alignment (Geneious software version 4.7.4) to database sequences within an identity cluster. Data are reported as percent homology to germline sequence.

Isolation of PBMC and activation through ligation of CD40

Cells were isolated over Lymphocyte Separation Media (Mediatech), plated at $1x10^6$ cells/ml in a 24 well tissue culture plate (2ml/well) and incubated in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, penicillin and streptomycin. Cells were either treated with anti-CD40 antibody (MAB89, Abcam, Cambridge, MA) at 200ng/ml or were left untreated for 24 hours. After 24 hours, cells were stained for CD20, CD27, CD95 and Annexin V (Caltag, Carlsbad, CA) and treated with a viability dye. Cells were fixed in 1% cold paraformaldehyde and analyzed on an LSRII.

Statistical analysis

Paired Student's T test, T test with unequal variance and 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test were used to compare differences between groups. All Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc. San Diego, CA).

2.4 RESULTS

Phenotypic characterization of CD27 in peripheral blood in rhesus macaques.

Surface molecule expression and size discrimination were utilized for initial phenotypic parsing of B cell subsets. For these studies, peripheral blood cells were stained with antibodies specific for CD20, CD27, CD40, CD86 and CD95, and analyzed by flow cytometry. The CD20+ B cell population was subsequently resolved based on CD27 surface expression, which has been used to define memory cells in humans (76, 79, 162, 163). For the 6 animals analyzed in this study, an average of 6.9% of peripheral blood lymphocytes were CD20+; of those CD20+ cells 44.9% expressed CD27 (figure 7). More CD20+CD27+ cells expressed CD95 (54.9% +/- 10%) ($P < 0.05$) and CD86 (79% +/- 13%) ($P < 0.05$) compared to CD20+CD27- cells, of which 8.3% +/- 3.1% and 71.6% +/- 17.9% expressed CD95 and CD86 respectively. Additionally, more CD20+CD27- B cells expressed surface CD40 (55.4% +/- 11.1%) compared to CD20+CD27+ cells (21.9% +/- 7.3%) ($P < 0.05$). In addition to their activation profiles, these cell populations could also be distinguished based on relative size (figure 8). CD20+CD27+ cells were significantly larger (average geometric mean of FSC = 734.6 +/- 199.7) than CD20+CD27- cells (average geometric mean of FSC = 633.6 +/- 185.6) ($p < 0.05$). These data demonstrated that the pattern of activation marker expression on CD20+CD27+ B cells in rhesus macaques is consistent with a memory phenotype.

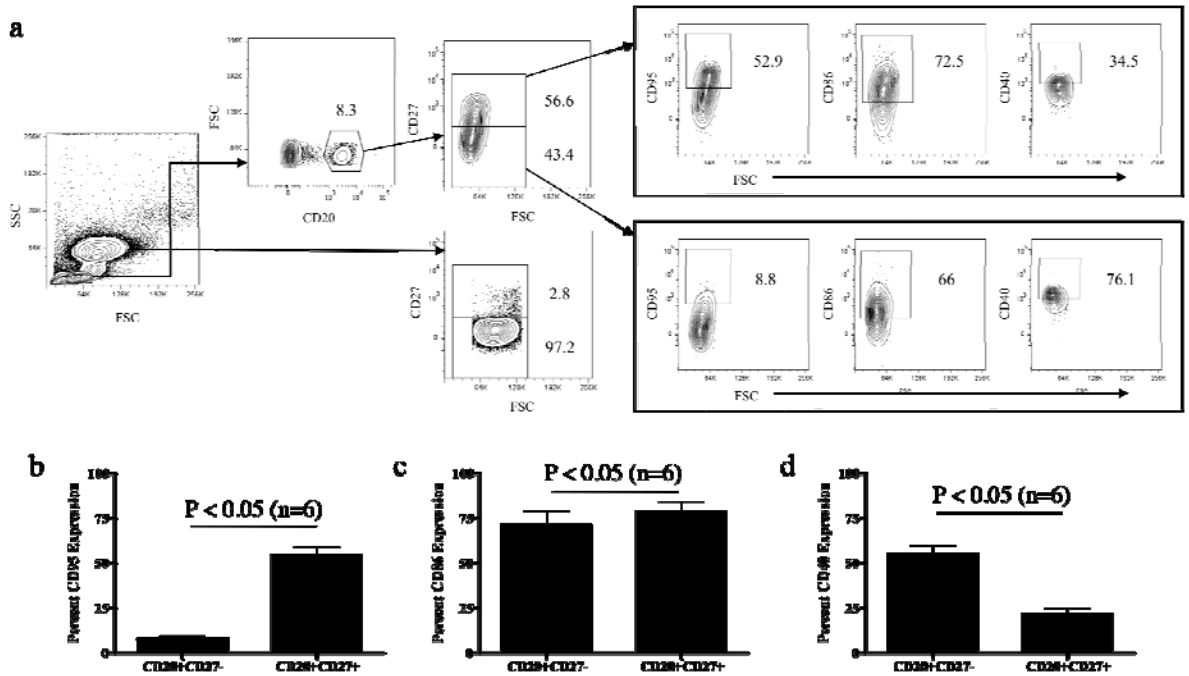


Figure 7. Basic B cell phenotype

Surface expression of CD27 and activation markers on rhesus macaque B cells. (a.) Representative flow cytometry analysis of CD20+ peripheral blood B cells based on CD27 expression. The gate for positive CD27 levels was set against background staining of myeloid cells which are not known to express this marker. The CD27+ and CD27- subsets were subsequently examined for expression of CD95, CD86 and CD40. Averaged results for percent of cells expressing CD95 (b.), CD86 (c.) and CD40 (d.). n= 6 independent animals. Means were compared using paired Student's T test.

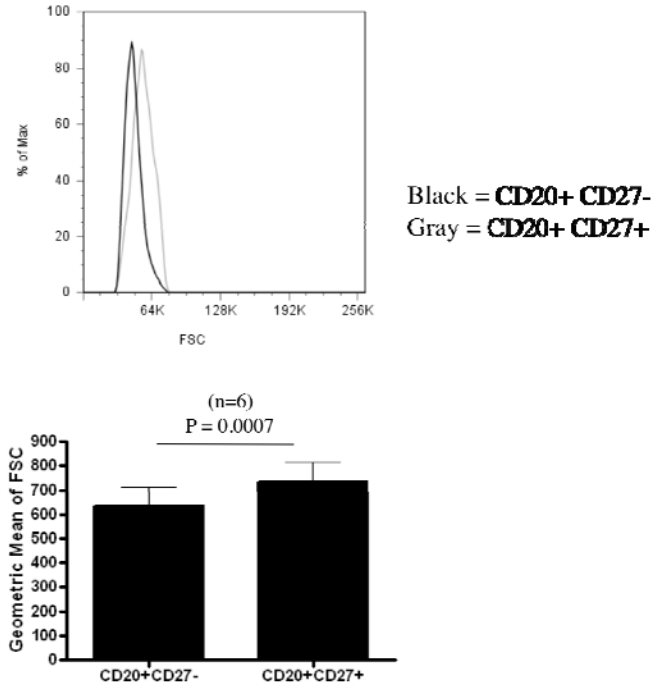


Figure 8. Size comparison of B cell subsets.

CD20+CD27+ Cells are larger than CD20+CD27- peripheral blood cells. (a.) Representative flow cytometry analysis of relative size of CD20+ CD27- (black line) and CD20+ CD27+ subsets (gray line). (b.) Averaged geometric mean values of FSC for 6 independent animals. Means were compared using a paired Student's T test.

Increased surface expression of CD27 on B cells in effector sites

To further validate CD27 as a memory B cell marker in rhesus macaques, the frequency of CD20+CD27+ B cell populations in effector sites were compared to B cells from peripheral blood and lymph nodes. Previous studies in T cells have demonstrated that extralymphoid effector sites have an increased abundance of effector memory T cells (164). Given that interactions between immune cells and pathogens occur at these effector sites, it was anticipated that these locations would include higher frequencies of effector B cells (165). To test this hypothesis, the distribution of B cells expressing CD27 was analyzed in lymphoid and effector sites. Following detailed analysis of tissue from three animals, lymphoid sites (lymph nodes) had fewer CD27 expressing CD20+ B cells (25%) compared with 45% found in peripheral blood.

This was in contrast to effector sites (lungs, through the analysis of bronchoalveolar lavage [BAL] samples, and gut tissue samples), which had 70% and 75% of CD20+ B cells expressing CD27, respectively (figure 9). These data demonstrated that higher percentages of CD27+ B cells were present in effector sites.

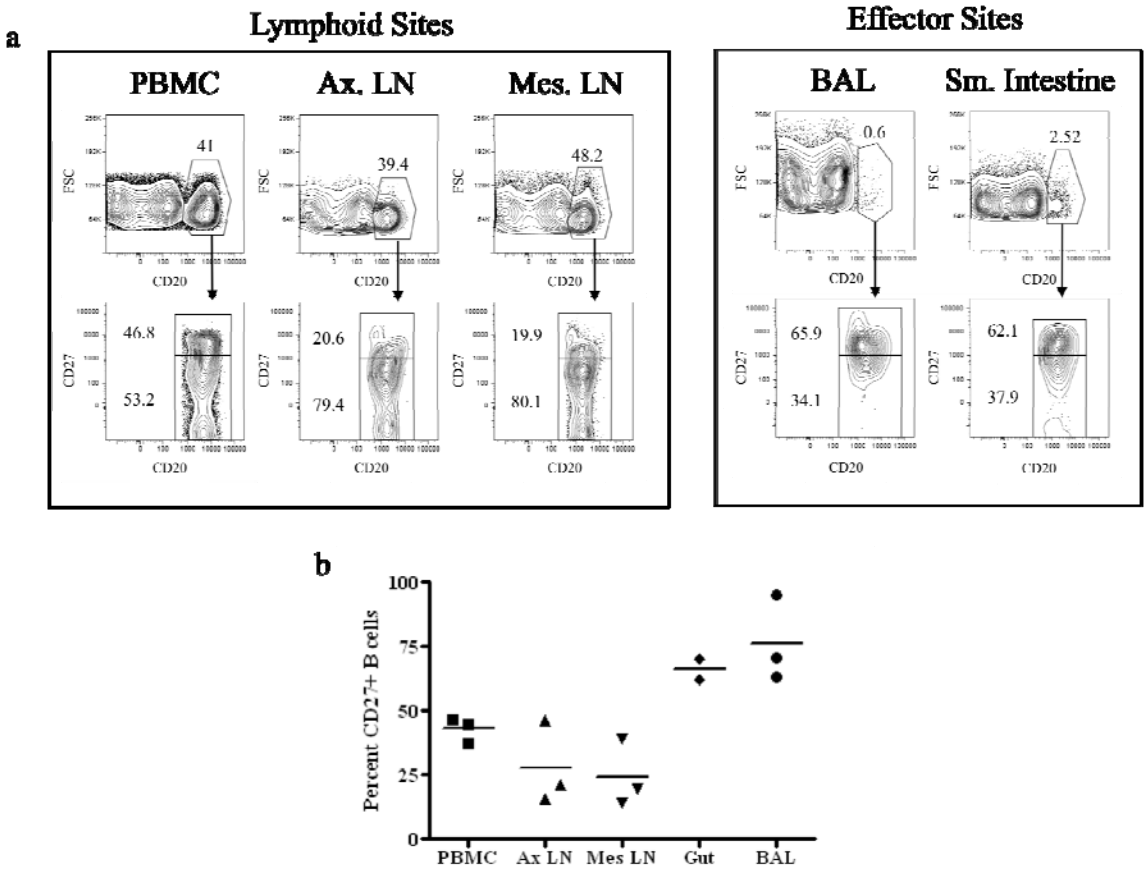


Figure 9. CD27 expression on B cells across immunologic compartments.

Surface expression of CD27 and activation markers on B cells from tissue compartments. (a.) Representative flow cytometry analysis of CD27 expression on CD20+ cells in peripheral blood, Axillary lymph node, mediastinal lymph node, small intestine and bronchial alveolar lavage samples. (b.) Plotted values for each replicate, the line represents the average value for all samples (n=3 independent animals).

B cells from neonatal rhesus macaques do not express CD27

To compare the distribution of CD27 on antigen-naïve versus antigen-experienced B cells, expression of surface CD27 was evaluated in embryonic cord blood samples. By extension from human literature (76), we predicted that very few CD20+CD27+ B cells would be present in cord

blood. Indeed, minimal levels of CD27 were observed in CD20+ B cells from rhesus macaque umbilical cord blood (2.4% +/- 2.5%, average of 3 animals, figure 10) compared to expression observed in peripheral blood B cells in adult rhesus macaques (44.9% +/- 17.1%)($p < 0.05$) (figure 10). These data confirmed that B cells that have not encountered antigen do not express CD27.

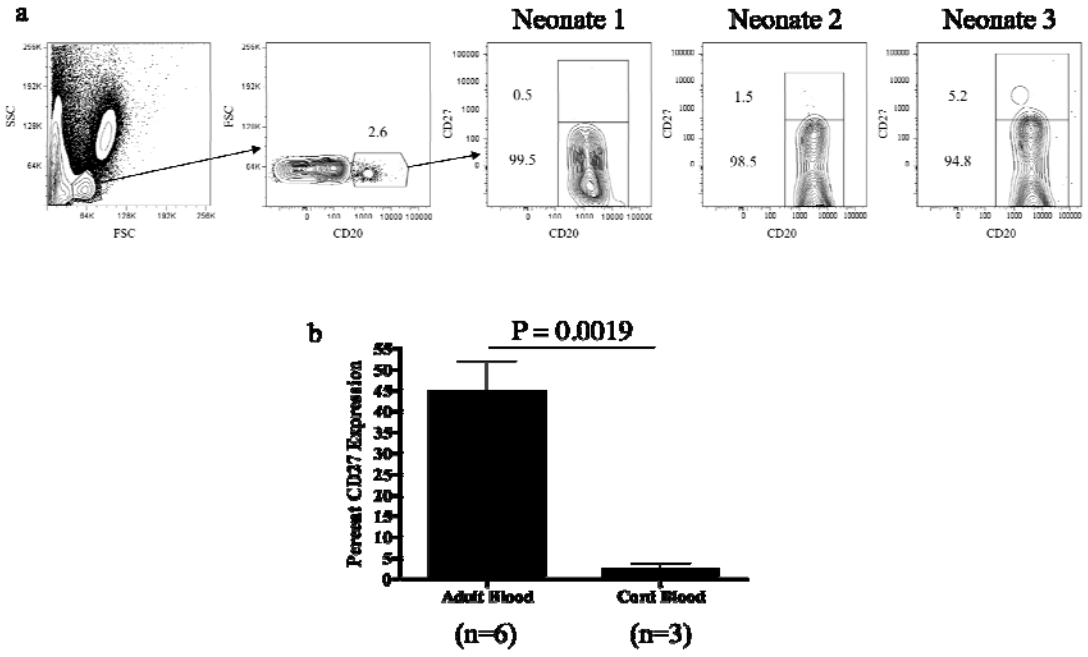


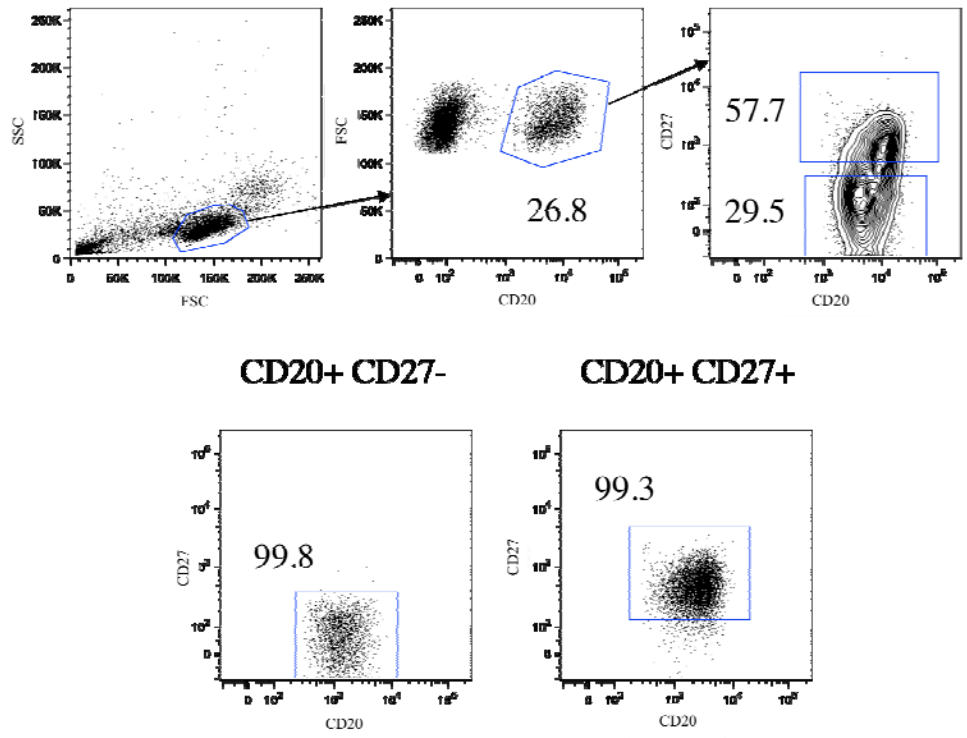
Figure 10. CD27 Stains of umbilical cord blood B cells.

CD20+ B cells from umbilical cord blood do not express CD27. (a.) Analysis of CD27 expression on CD20+ B cells from 3 umbilical cord blood samples. (b.) Averaged CD27+ B cell expression from adult peripheral blood (n=6 independent animals) and cord blood samples (n=3 independent animals). Means were compared using a T test with unequal variance.

Surface expression of CD27 on B cells in the rhesus macaque corresponds with somatic hypermutation of the immunoglobulin gene.

A defining molecular feature of memory B cells is the presence of somatic hypermutation of the immunoglobulin gene variable region (73). In humans, it has been demonstrated that upregulation of surface CD27 occurs on antigen experienced B cells which can be matured into antibody secreting cells (146). Therefore, we examined the somatic hypermutation status of the CD20+CD27+ B cells from rhesus macaques. For these analyses, rhesus macaque peripheral blood cells were sorted by FACS into CD20+CD27+ and CD20+CD27- populations (figure 11a). Following mRNA extraction from these cells, cDNA amplification of the immunoglobulin M heavy chain variable region 1 (IGHV1) was performed using nested RT-PCR (160). Sequences from one representative subgroup (IGHV1p) are shown in figure 11b, while table 1 summarizes all 40 IGHV1/7 family sequences obtained. Sequences from IGHV1c, IGHV1k, IGHV1L, IGHV1p and IGHV7a subgroups were identified and analyzed. Sequence analyses of these amplified regions from 5 independent animals revealed that CD20+CD27+ cells in the rhesus macaque demonstrated significantly less sequence homology to germline sequences (95.5%) compared to the CD20+CD27- cells (99.4%) ($p < 0.05$) (figure 11c and tables 1 and 2). Somatic hypermutation was confirmed by analysis of mutation sites; the majority of observed mutations occurred in and around the complementarity determining regions. These data provided a clear indication at the molecular genetic level that CD27 expression denotes memory B cells.

a


SSC
FSC
FSC
CD20
57.7
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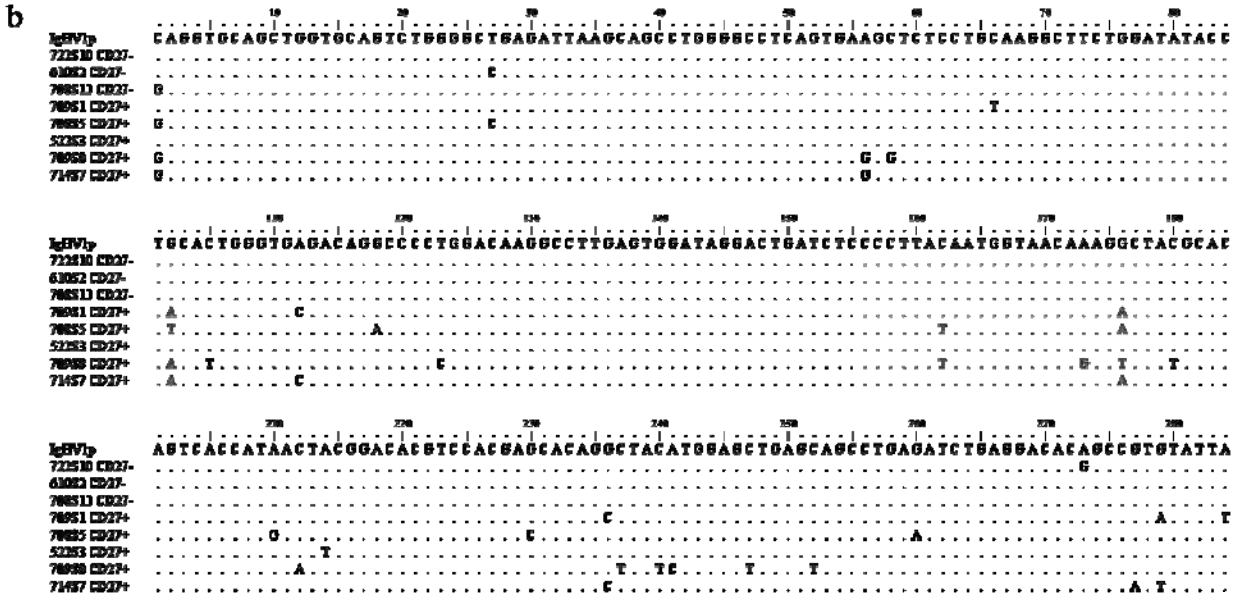
CD20

b


IgHV1p
722510 CD27-
61052 CD27-
70851 CD27+
70855 CD27+
52253 CD27+
70958 CD27+
71457 CD27+

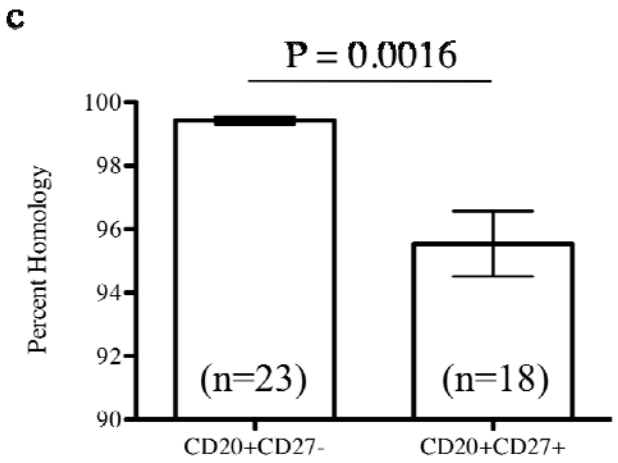


Figure 11. Somatic hypermutation in Ig variable regions of B cells.

CD27+ cells but not CD27- cells demonstrate somatic hypermutations at Ig variable regions. (a) Representative plots for cell sorting parameters and post-sort purity analysis of sorted populations. (b) Representative sequence samples from IgHV1p family genes, shaded areas denote complementarity determining regions. (c) Percent homology to germline sequences for IgHV1 family genes isolated from CD20+CD27- and CD20+CD27+ B cells. Results are representative of 40 unique sequences obtained from 5 individual animals. Means were compared using a T test with unequal variance.

Table 1. Sequence homology to germline IGHV1/7 in CD20+CD27- cells.

Parental Sequence[a]	Animal ID[b]	Framework Mutations[c]	CDR Mutations[d]	Total Mutations[e]	Percent Identity[f]
IGHV7a	RO245	0	0	0	100
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	1	2	99.3
IGHV7a	RO245	1	0	1	99.7
IGHV7a	RO245	1	0	1	99.7
IGHV7a	RO310	4	1	5	98.3
IGHV7a	RO316	3	0	3	99.0
IGHV7a	RO316	1	0	1	99.7
IGHV7a	RO316	1	1	2	99.3
IGHV7a	RO316	1	1	2	99.3
IGHV7a	RO442	4	1	5	98.3
IGHV7a	RO442	1	1	2	99.3
IGHV7a	RO442	1	1	2	99.3
IGHV1c	RO310	1	2	3	99.0
IGHV1L	RO310	0	0	0	100
IGHV1L	RO316	2	2	4	98.6
IGHV1L	RO316	0	0	0	100
IGHV1L	RO316	0	0	0	100
IGHV1L	RO316	0	0	0	100
IGHV1p	RO245	1	0	0	99.7
IGHV1p	RO310	0	0	0	100
IGHV1p	RO316	1	0	0	99.7
Average		1.1	0.6	1.6	99.4
Standard Deviation		1.1	0.7	1.6	0.5

[a] IGHV1/7 germline parental sequence as determined by using Tamura-Nei distance estimates and a neighbor joining algorithm.
[b] Identification for rhesus macaques.
[c] The number of mutations in the Framework regions of the Ig V region gene.
[d] The number of mutations in the complementarity determining regions of the Ig V region gene.
[e] Total number of nucleotide changes from the parental sequence in the immunoglobulin variable region of the sequenced cells.
[f] Relative identity of the experimentally obtained sequence to the germline sequence.

Table 2. Sequence homology to germline IGHV1/7 in CD20+CD27+ cells.

Parental Sequence[a]	Animal ID[b]	Framework Mutations[c]	CDR Mutations[d]	Total Mutations[e]	Percent Identity[f]
IGHV7a	RO245	0	0	0	100
IGHV7a	RO245	0	0	0	100
IGHV7a	RO316	5	4	9	96.9
IGHV7a	RO316	4	4	8	97.2
IGHV7a	RO632	15	8	23	92.0
IGHV7a	RO632	14	8	22	92.4
IGHV7b	RO316	4	4	8	97.2
IGHV7b	RO442	19	5	24	91.7
IGHV1k	RO310	10	1	11	96.2
IGHV1l	RO310	37	16	53	81.6
IGHV1l	RO316	0	0	0	100
IGHV1l	RO442	16	1	17	94.1
IGHV1p	RO245	9	2	11	96.2
IGHV1p	RO245	7	2	9	96.9
IGHV1p	RO310	7	1	8	97.2
IGHV1p	RO310	6	2	8	97.2
IGHV1p	RO316	2	1	3	99.0
IGHV1p	RO442	14	4	18	93.8
Average		9.4	3.5	12.9	95.5
Standard Deviation		9.1	4.0	12.6	4.4

[a] IGHV1/7 germline parental sequence as determined by using Tamura-Nei distance estimates and a neighbor joining algorithm.
[b] Identification for rhesus macaques in study
[c] The number of mutations in the Framework regions of the Ig V region gene.
[d] The number of mutations in the complementarity determining regions of the Ig V region gene.
[e] Total number of nucleotide changes from the parental sequence in the immunoglobulin variable region of the sequenced cells.
[f] Relative identity of the experimentally obtained sequence to the germline sequence.

CD40 ligation protects the naïve but not the memory B cell population

In addition to phenotypic and molecular genetic differences between CD27- and CD27+ B cells, we sought to establish whether the two cell populations had functional differences. CD40 ligation is essential for upregulation of activation markers, class switching and B cell maturation (70, 74, 166). As distinct cell populations, naïve and memory B cells were predicted to respond

to activating stimuli such as CD40 ligation with unequal kinetics. Activation of CD40 with CD40L (CD154) and the addition of interleukin-2 (IL-2), IL-4 and IL-10 for 4 days *in vitro* has previously been shown to result in the expansion of human naïve B cells and the maturation of memory B cells (72, 167). We first tested whether B cell activation through CD40 ligation resulted in expansion of CD20+CD27- or CD20+CD27+ B cell populations. In this study, treatment of B cells with anti-CD40 antibody for 24 hours altered the relative ratios of CD20+CD27- and CD20+CD27+ B cells, but did not result in expansion of either population. Following a 24 hour incubation, there was a significant difference in the percentage of CD20+CD27- B cells between the group treated with anti-CD40 antibody (64.2% +/- 16.7%) and the untreated group (49.3% +/- 18.8%) ($P < 0.05$) (figure 12a). In the CD20+CD27+ B cell population, the percentage of cells decreased following treatment with anti-CD40 antibody compared to untreated cells. An average of 35.9% +/- 16.8% were CD20+CD27+ after 24 hours treatment compared to 50.7% +/- 18.8% after 24 hours with no treatment ($p < 0.05$) (figure 12b).

A significant difference in the absolute number of CD20+CD27- cells was also observed between anti-CD40 antibody treated (42,874 +/- 41,820 cells) and untreated (26,604 +/- 16,707 cells) groups ($p < 0.05$) (figure 12c). In the CD20+CD27+ population, in contrast to the higher number of cells observed in the anti-CD40 antibody treated CD20+CD27- population, fewer cells were present in the treated group (19,430 +/- 18,173) than were in the untreated group (27,956 +/- 20,756 cells) (figure 12d). These data demonstrated differential persistence of CD20+CD27- versus CD20+CD27+ B cell populations occurred following CD40 ligation.

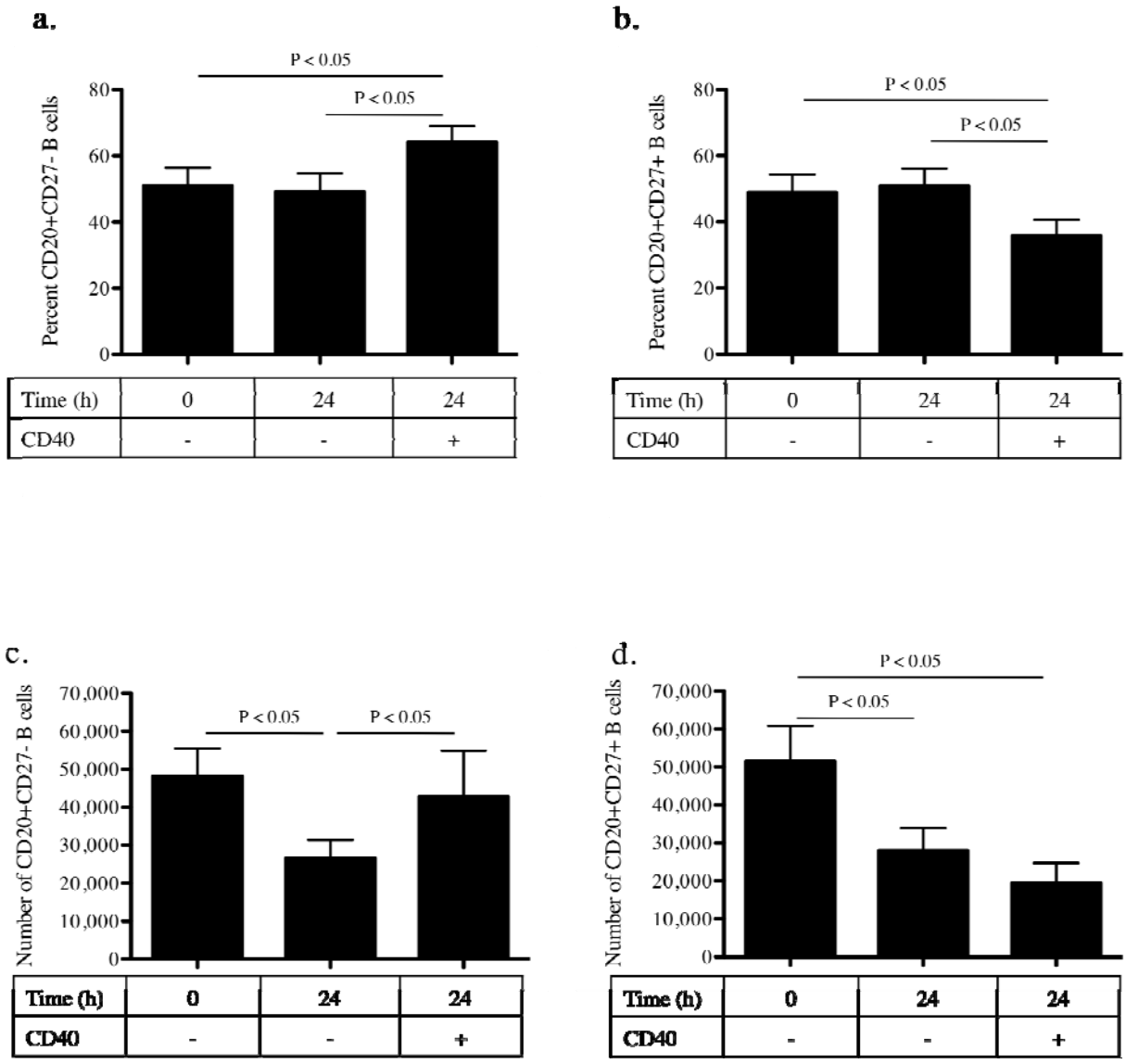


Figure 12. Naive and memory B cell populations after CD40 ligation.

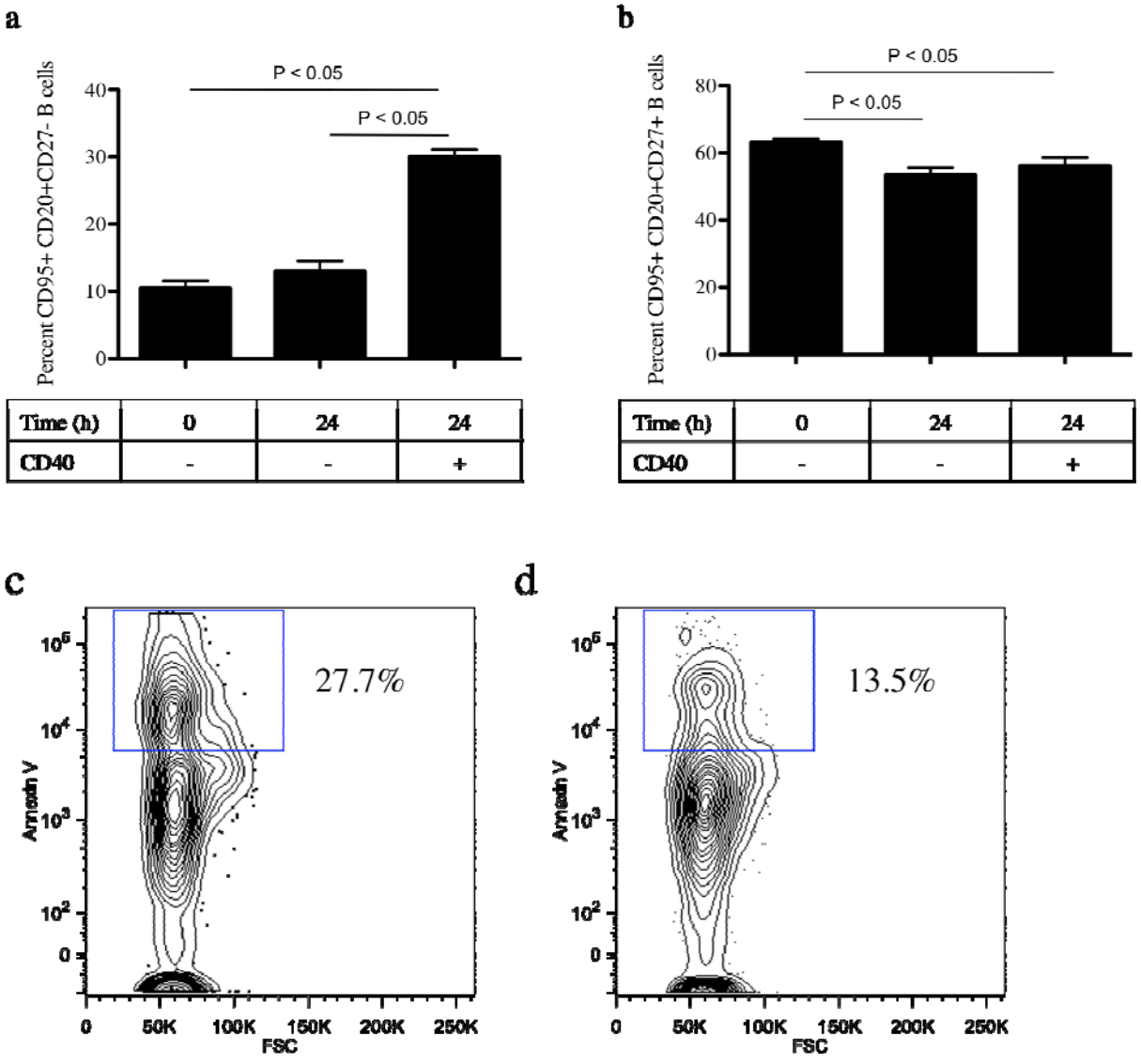
Ligation of CD40 on CD20+CD27- but not CD20+CD27+ B cells results in protection from spontaneous cell losses in a 24 hour culture system. One million PBMC were cultured in the presence or absence of 200ng/ml of anti-CD40 for 24 hours. Cultures were harvested and then analyzed for CD20 and CD27 expression in order to differentiate the percentages and numbers of B cell populations. (a) Percentage of CD20+CD27- B cells and (b) CD20+CD27+ B cells. (c) Absolute number of CD20+CD27- B cells and (d) CD20+CD27+ B cells. Results are representative of 6 independent experiments run in duplicate. Error bars represent the standard error of the mean. Mean values for both culture conditions and freshly isolated cells were compared using 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test.

CD40 Ligation results in a reduction of the percentage of apoptotic CD20+CD27- B cells

To further understand the effects of CD40 mediated activation in the rhesus model, we measured the effects of CD40 ligation on activation and apoptosis. First, we assessed whether anti-CD40 antibody treatment of naïve and memory B cells increased the expression of the activation marker CD95. Treatment with anti-CD40 antibody resulted in a significant ($P < 0.05$) three-fold increase in percentage of CD95+ CD20+CD27- cells from 13% +/- 5.5% in the untreated group to 30% +/- 3.8% in the anti-CD40 antibody treatment ($p < 0.05$) (figure 13a). In contrast to the CD20+CD27- B cell population, no differences were observed between untreated and CD40 antibody treated groups in the CD20+CD27+ B cell population, although these cells had higher basal levels of CD95 expression, 63.1% +/- 4.1% in freshly isolated cells (figure 13b).

A prior *in vitro* study by Hu *et al*. demonstrated that CD40 ligation improves the survival of human tonsillar memory B cells (168). In order to test whether CD40 ligation had differential protective effects on CD20+CD27- and CD20+CD27+ B cell populations in the rhesus macaque, we measured annexin V staining on freshly isolated anti-CD40 antibody treated and untreated cells. Fewer CD20+CD27- B cells appeared apoptotic through analysis of annexin V staining following 24 hour treatment with anti-CD40 antibody compared to untreated CD20+CD27- cells, while no effect of treatment was observed in CD20+CD27+ B cells. In the untreated group, 28.8% +/- 9.4% of CD20+CD27- cells stained with annexin V and in the anti-CD40 antibody, this was reduced to an average of 22.4% +/- 9.3% (figure 13c). In the CD20+CD27+ population, 15.7% +/- 8.8% of untreated CD20+CD27+ cells stained with annexin V compared to 12.7% +/- 2.8% in the anti-CD40 antibody treated cells (figure 13d). Together, these results indicated that CD40 ligation of the CD20+CD27- B cell population resulted in decreased percentage of apoptotic cells and an increase in the percentage of cells expressing CD95 and CD40 ligation of

CD20+CD27+ B cells did not alter the percentage of apoptotic cells or the percentage of cells expressing CD95.



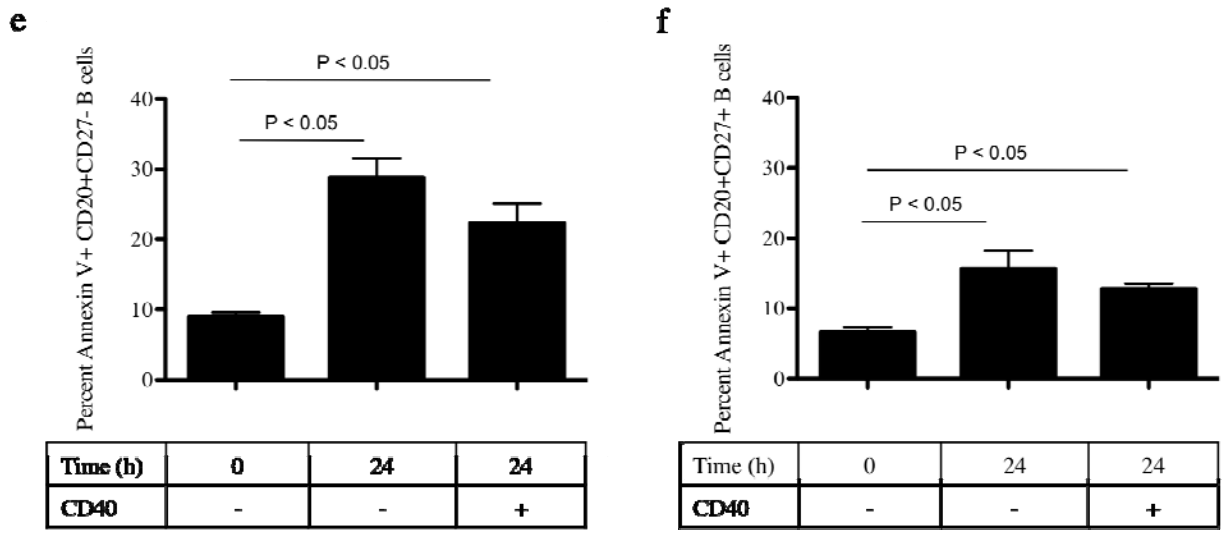


Figure 13. Activation and apoptosis following CD40 ligation.

The percentage of CD20+CD27- B cells expressing CD95 increases and the percentage of CD20+CD27- B cells that bind annexin V decreases after anti-CD40 antibody treatment. PBMC were cultured in the presence or absence of 200ng/ml of anti-CD40 in triplicate for 24 hours. Cultures were harvested and then analyzed for CD95 expression within (a) CD20+CD27- or (b) CD20+CD27+ B cells. Representative flow cytometry plots of the CD20+CD27- B cell population following 24 hour culture (c) without anti-CD40 antibody treatment and (d) with anti-CD40 antibody treatment. The average percentage of annexin V staining for all 6 animals within the (e) CD20+CD27- or (f) CD20+CD27+ B cell populations. Mean values for both culture conditions and freshly isolated cells were compared using 1 way ANOVA with repeated measures and Bonferroni's multiple comparison test.

Different responses to SIV infection between CD20+CD27- and CD20+CD27+ B cell populations.

Establishing CD27 as a bona fide memory marker allows us for the first time to quantify the loss of specific B cell subsets, naïve versus memory, following SIV infection. While previous studies have shown a 6-fold reduction in total CD20+ B cells during acute infection (20), it has not been possible to evaluate the impact to specific B cell subsets, information that is essential for the design of effective vaccine therapy. Ten rhesus macaques were experimentally infected with SIVmac239 (1,000 infectious units i.v.) and individual B cell subsets were analyzed preinfection

and at days 14 and 42 post infection. Strikingly, the memory B cell population was more severely depleted during the first two weeks of infection, but also showed heightened rebound during the onset of chronic infection. The initial loss of CD20+CD27+ cells, a threefold reduction from 749 +/- 556 cells/μl at day 0 to 246 +/- 173 cells/μl at day 14 was much more drastic than the loss that was observed in the CD20+CD27- B cell population, a not quite twofold loss of from 401 +/- 162 cells/μl at day 0 to 259 +/- 129 cells/μl at day 14. Additionally, there was a much larger increase in the number of CD20+CD27+ cells at day 42 up to 506 +/- 291 cells/μl compared to the CD20+CD27- population that exhibited a negligible increase to 275 +/- 123 cells/μl. A similar trend of larger changes occurring within the CD20+CD27+ B cell population was reflected in the percent change of the two B cell populations. A percent change of -62.9% +/- 12.8% from day 0 to day 14 was observed in the CD20+CD27+ B cell population compared to a percent change of -35.7% +/- 18.6% from day 0 to day 14 in the CD20+CD27- B cell population. There was a significant difference ($P < 0.01$) between percent changes of the CD20+CD27+ (154.9 +/- 141.9) and the CD20+CD27- (25.5% +/- 74%) B cell populations from day 14 to day 42 (figure 14). Together, these data demonstrate that the CD20+CD27+ B cell population had acute deficits followed by a greater compensatory rebound than the CD20+CD27- B cell population during the acute phase of SIV infection.

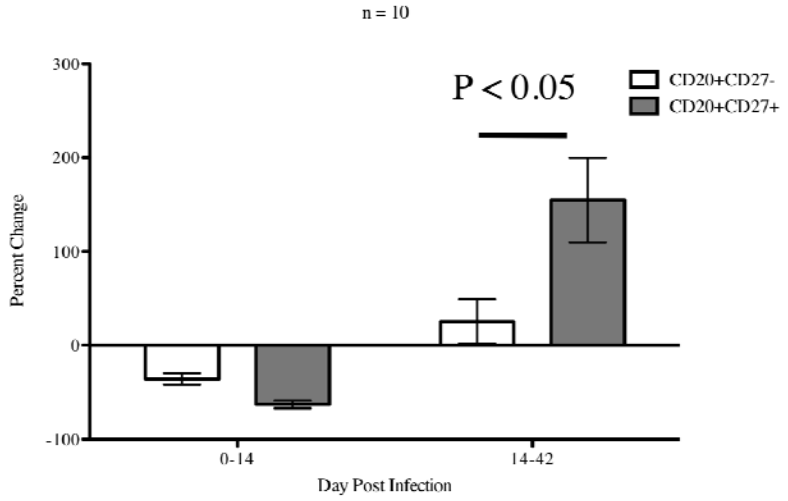


Figure 14. B cell populations in response to SIV infection.

Distinct patterns of B cell responses observed between CD20+CD27- naive and CD20+CD27+ memory B cells in SIV infected rhesus macaques. Ten rhesus macaques were infected with SIVmac239 (1,000 infectious units per animal i.v.) and B cell populations were analyzed by flow cytometry to assess differences in the percent changes of CD20+CD27- and CD20+CD27+ B cells in the periphery in infected animals from days 0 to 14 and from 14 to 42 within the acute phase of infection. Mean values were analyzed using a T test with unequal variance.

2.5 DISCUSSION

The non-human primate model is a valuable tool for the development of improved vaccines and therapeutic treatments for numerous human diseases but fundamentally important immunological parameters have not been definitively characterized. In this study, phenotypic and molecular analysis demonstrated that surface expression of CD27 distinguishes naïve and memory B cell populations, as determined at the phenotypic and genetic levels. Further analysis demonstrated that functional differences including survival, activation and apoptosis following CD40 ligation were present in CD20+CD27- and CD20+CD27+ B cells. Additionally, the CD20+CD27+ population had a greater depletion followed by a more robust rebound during acute SIV infection compared to CD20+CD27- cells. Together, these data will aid in the understanding of the B cell response to pathogenic infections in the rhesus macaque model.

This study established that surface expression of CD27 marks memory B cells in the rhesus macaque model. We showed here that sorted CD20+CD27+ B cells had somatic mutations in the IGHV1/7 region, which occurred in and around complementarity determining regions. Additional data, including the demonstration that CD20+CD27+ B cells have increased expression of activation markers, the increased abundance of CD20+CD27+ B cells in effector sites compared to lymphoid sites, and the increased size of CD20+CD27+ B cells compared to CD20+CD27- B cells further supports this finding. Finally, antigen naïve B cells obtained from rhesus macaque umbilical cord blood had very little CD27 expression. The further characterization of B cells from these findings in the rhesus macaque model provides a basis for additional functional studies of B cell populations.

Functionally, CD20+CD27- B cells were more sensitive to CD40 receptor ligation than the CD20+CD27+ B cell population. CD40 ligation resulted in protection from spontaneous cell reductions in CD20+CD27- B cells and in contrast, exacerbated spontaneous cell reductions in CD20+CD27+ B cells *in vitro*. CD40 ligation also resulted in an increased percentage of CD20+CD27- B cells expressing CD95, but no change the percentage of CD20+CD27+ B cells expressing CD95. Finally, CD40 ligation of the CD20+CD27- B cell population resulted in significant reduction in the percentage of apoptotic cells, as measured by annexin V staining, compared to untreated controls. CD40 ligation of the CD20+CD27+ B cell population did not result in a significant reduction in annexin V staining compared to untreated controls.

CD40 ligation may have multiple mechanistic outcomes depending on the specific B cell population in which it is activated. Previous studies with human B cells have demonstrated that CD40 ligation plays a central role in the maturation and differentiation of naïve and memory B cells. Specifically, CD40 ligation results in increased expression of surface CD95 and is essential

for the development of class switched antibody producing cells (77, 169, 170). CD40 ligation also results in B cell activation through a signaling cascade that includes the activation of the IκB kinase (171). Interestingly, in the current study, CD40 ligation resulted an upregulation of CD95 expression in naïve B cells but not in the memory B cell compartment indicating that analyzing the populations separately provides a clearer picture of the cell populations that become activated. In addition, our results indicated that CD40 mediated changes could be observed within 24 hours which may be more physiologically relevant than prior studies which measured changes after 4 or more days. Further, the present study provides more detailed information over previous studies in the human system about the effect of CD40 ligation on the number of cells present and the percentage of those cells which were apoptotic by analysis of annexin V binding. Interestingly, in this study, it was demonstrated that CD40 mediated effects on the naïve B cell population were not mirrored in the memory B cell population, indicating that the outcome from CD40 ligation is different between the two populations.

The utility of the rhesus macaque is clear in studies of simian immunodeficiency virus (SIV) infection, a system used to model HIV infection in humans. While HIV cannot infect rhesus macaques, HIV and SIV are genetically and structurally similar (172). Thus, understanding the similarities (and differences) between HIV and SIV has led to a greater understanding of human HIV infections, with the rhesus macaque providing an invaluable preclinical animal model for the evaluation of therapeutic and vaccine strategies to prevent infection and/or disease. For example, the rhesus macaque model played a key role in the discovery that acute SIV and HIV infections result in depletion of specific T cell populations from the gut (150, 151, 153). These studies have been extended to other nonhuman primate models and indicate that cells in the gut are highly susceptible to viral infection while not likely

to be major sources of virus (173). Prior SIV studies in rhesus macaques have demonstrated reductions in CD20+ B cell numbers in rhesus macaques during acute infection (20), but none have addressed specific B cell subsets. In the current study we utilized the expression of CD27 to parse B cell populations and demonstrated that B cell subsets do not respond to SIV infection with the same dynamics. These data indicate that the regulation of CD20+CD27- and CD20+CD27+ B cells is different during infection and different SIV induced alterations may be occurring in each population.

The experiments reported here establish CD27 as a marker of memory B cells in rhesus macaques and demonstrate that CD20+CD27+ and CD20+CD27- B cells functionally respond to CD40 ligation differently. CD20+CD27+ B cells demonstrated phenotypic and genetic characteristics of memory cells including increased expression of activation markers and somatic hypermutation. Functionally, CD20+CD27- cells were protected from spontaneous apoptosis through ligation of the CD40 receptor. When analyzed during SIV infection, the CD20+CD27- and CD20+CD27+ B cell populations responded differently to acute SIV infection, providing an experimental platform for development of improved therapeutic vaccines. These critical analyses of lymphoid populations improve our collective understanding of B cells in the rhesus macaque and will enhance our ability to translate findings from the rhesus to the human system.

2.6 ACKNOWLEDGEMENTS

The authors would like to thank Dr. Michael Murphey-Corb for providing normal rhesus macaques, Dr. Brian Healy for his help with the statistical analyses, and Timothy Sturgeon for his assistance with cell sorting. This work was funded by NIH RO1 AI52058 (KSC), R01 AI35522 (DLS), NIH R01 AI079047 (LAB), RR00169 (CNPRC).

3.0 LONGITUDINAL ANALYSIS OF B CELLS DURING ACUTE SIV INFECTION

Evidence of early B cell dysregulation in SIV infection: Rapid depletion of naïve and memory B cell subsets with delayed reconstitution of the naïve B cell population

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3.1 ABSTRACT

Despite eliciting a robust antibody response in humans, several studies in human immunodeficiency virus (HIV) infected patients have demonstrated the presence of B cell deficiencies during the chronic stage of infection. While several explanations for the HIV-induced B cell deficit have been proposed, a clear mechanistic understanding of this loss of B cell functionality is not known. This study utilizes the simian immunodeficiency virus (SIV) infection of rhesus macaques to assess B cell population dynamics beginning at the acute phase and continuing through the chronic phase of infection. Flow cytometric assessment demonstrated a significant early depletion of both naïve and memory B cell subsets in the peripheral blood, with differential kinetics of recovery of these populations. Furthermore, the altered numbers of naïve and memory B cell subsets in these animals corresponded with increased B cell activation

and altered proliferation profiles during the acute phase of infection. Finally, all animals produced high titer antibody, demonstrating that the measurement of virus-specific antibody responses was not an accurate reflection of alterations in the B cell compartment. These data indicate that dynamic B cell population changes in SIV-infected macaques arise very early after infection at the precise time when an effective adaptive immune response is needed.

3.2 INTRODUCTION

Effective B cell responses result in the generation of memory B cell populations which proliferate and produce antibodies that can control primary and secondary insults by microbial pathogens (81). Impaired maturation and timing of B cell mediated immune responses result in the production of ineffective antibodies, which are unable to control infection and may result in the persistence of the pathogen (174). Although human immunodeficiency virus (HIV) infection generally elicits high titer antibody, virus-specific titers do not correlate with delayed clinical progression suggesting that antibodies produced during HIV infection are not sufficient to provide long-term viral control (7). Ineffective antibody production in the context of HIV infection could be a result of numerous T cell and B cell abnormalities induced either directly or indirectly through infection. B cell perturbations, characterized during chronic infection, include hypergammaglobulinemia (136, 175), a diminished in vitro response to mitogenic stimulation (95, 176), diminished antibody responses to vaccination (8, 98), and loss of memory B cell subsets (95, 96, 176). It is highly likely that these B cell abnormalities are linked with the inability of HIV infected individuals to form effective antibody responses to HIV and opportunistic pathogens.

B cell perturbations during acute HIV infection may lead to dysfunctions observed during chronic infection. Despite numerous reports which hypothesize that B cell phenotypic and functional abnormalities arise due to the effects of chronic infection, a limited number of acute infection studies provide evidence that B cell dysfunctions may be initiated much earlier. Studies by De Milito *et al.* and others have reported a decrease in CD27+ B cells associated with chronic HIV infection (95-98, 100, 136, 174-179). The reduction of this population may explain the diminished antibody responses to non-HIV antigens present in HIV infected individuals. However, the mechanism for this loss of memory B cells during chronic infection is unclear. One possibility is that B cell losses are related to reduced T cell numbers. In a study by Titanji *et al.*, a strong correlation between the number of CD4 T cells and the percentage of memory B cells was reported in chronic HIV infection (176). Conversely, others have reported that no correlation could be found between CD4 numbers and memory B cell numbers (95, 96). Interestingly, reductions in percentages of B cells, increased expression of Fas (CD95) on B cells, increased total plasma IgG levels, decreased percentage of IgM memory B cells and decreased B cell responses to antigenic stimulation have been shown to occur within 6 months of HIV infection (174, 176). Disruption of germinal centers in the gut during acute HIV infection may also compromise the humoral immune response (180). While these studies provide insight into virus-induced changes in the B cell compartment during infection, it is difficult to ascertain precisely when these changes occur due to limitations in sample size and numbers during this early period of infection. The conflicting reports reflect the high amount of variability present in human HIV infection and illuminate the need for a model to study B cell populations in which experimental parameters can be more rigorously controlled. Understanding the effects of HIV on the B cell population during this critical early phase of infection is needed to determine how the initial

interactions between virus and host immune system set the stage for long-term disease progression in the infected host. The SIV/macaque model provides a system in which to ask these questions.

Studies in SIV infected macaques have demonstrated that the number of total B (CD20+) cells in the periphery decreases dramatically during the acute phase of infection (20, 181). The loss of these cells coincides with a similar depletion of peripheral CD4 T cells and is associated with primary viremia. Interestingly, the loss of total B cells is greater in magnitude than the loss of CD4+ T cells (20). In order to understand how these cells are being depleted, it is necessary to characterize B cell subsets during SIV infection in the macaque. The present longitudinal study was designed to assess phenotypic changes in B cell numbers during the acute phase of SIV infection, both in the total B cell population as well as in B cell subsets. Our results identified early, rapid changes in B cell subsets that were not apparent in analysis of the total B cell population. Specifically, we identified a significant depletion from the periphery of both the naïve (CD20+CD27-) and memory (CD20+CD27+) B cell populations during acute infection and increased total B cell population activation that may be related to ineffective antibody production commonly associated with SIV infection. Furthermore, these data demonstrated that measurement of envelope-specific antibody responses was not a sensitive reflection of SIV effects on B cell subsets because B cell depletions occurred prior to the formation of the antibody response. These data provide novel information about the timing and dynamics of phenotypic changes in the B cell compartment during SIV infection that may be associated with functional changes observed later in chronic infection. These results can be used to develop more efficacious therapeutic treatments designed to preserve the B cell compartment early in SIV/HIV infection.

3.3 METHODS

Animals

Ten colony-bred rhesus macaques (*Macaca mulatta*) of Indian origin were maintained and used in accordance with the guidelines of the Animal Care and Use Committee at the Oregon National Primate Research Center. Animals were infected at day 0 with SIVmac239 intravenously with 10,000 infectious units. Beginning at day 105 post infection all animals received daily antiretroviral therapy (ART) which included both tenofovir (PMPA) (30mg/Kg until day 134 and then 20mg/Kg subsequently) and emtricitabine (FTC) (50mg/Kg until day 134 and then 20mg/Kg subsequently). Complete blood counts were obtained at each blood draw using a Coulter ACT 5 Diff. 'Open reader' Cell counter (Beckman Coulter, Fullerton, CA).

Viral Quantification.

Assessment of plasma SIV RNA was carried out using a real-time RT-PCR assay (threshold sensitivity <100 SIV gag RNA copy Eq/ml of plasma; interassay CV ≤25%) (66, 182)

Flow cytometric analysis.

Flow cytometric analysis was performed on fresh lysed whole blood samples as described previously (183). Briefly, 100 µl of citrate treated whole blood was obtained in a blood collection tube (BD Diagnostics, Franklin Lakes, NJ). Biotinylated or directly fluorochrome conjugated antibodies to extracellular targets were incubated with whole blood at room temperature for 60 minutes. Following incubation, cells were washed once with 4 ml cold (4°C) dPBS with 0.1% of BSA and 0.02% of sodium azide (wash buffer). Cells were then fixed and

permeablized by 10 minute incubation at room temperature with FACS Lysing solution (BD biosciences, San Jose, CA), washed, and incubated twice with 0.5 ml of FACS Permeabilizing Solution (BD biosciences, San Jose, CA) for 10 minutes at room temperature. Cells were washed twice with cold wash buffer and then stained with directly-conjugated intracellular antibodies at room temperature for 30 minutes, washed once and analyzed by flow cytometry. Freshly stained lymphocytes were differentiated using forward and side scatter characteristics on an LSRII (BD Biosciences, San Jose, CA).

The total B cell population was differentiated using CD20 (eBioscience, San Diego, CA) expression, and was further subdivided according to expression of CD27 (BD Bioscience, San Jose, CA) and IgD (Southern Biotech, Birmingham, AL). B cells were characterized as CD20+CD27+ (memory) or CD20+CD27- (naïve). Further parsing of memory cells using IgD identified IgM secreting (CD20+CD27+IgD+) cells as well as IgG/IgA secreting (CD20+CD27+IgD-) cells. Cell populations were also assessed for surface expression of CD95 (BD Bioscience) and Ki67 expression levels (BD Bioscience, San Jose, CA) as measures of activation and proliferation. CD4 T cell counts were obtained by analyzing expression of CD3 (BD Bioscience, San Jose, CA) and CD4 (BD Bioscience, San Jose, CA) positive cells. List-mode multiparameter data files were analyzed using the FlowJo software program (PC version 7.2.5; Tree Star Inc., Ashland, OR).

SIV envelope-specific antibody endpoint titer.

Antibody reactivity to detergent disrupted SIVsmB7 envelope proteins (184) were determined in a conconavalin A (ConA) ELISA as previously described (68). Briefly, SIVsmB7 viral envelope proteins (gp120 and gp41) were captured onto 96 well microtiter plates (Immulon 2HB; Dynex

Technologies, Chantilly, VA) coated with 5 µg of ConA/well for 1 h at 25°C. After a washing step with PBS, non-specific binding was blocked by the addition of 5% dry milk in PBS (blocking solution) to all wells and incubated for 1 h at 25°C. Heat-inactivated plasma samples were serially diluted in blocking solution and incubated in the SIVsmB7 envelope-coated wells for 1 h at 25°C. After an extensive washing, peroxidase-conjugated anti-monkey IgG (Nordic Immunology Laboratories, Tilburg, Netherlands) was diluted in blocking solution, added to each well, and incubated for 1 h at 25°C and washed. Following the final wash step, all wells were incubated with TM Blue substrate (Seracare, Milford, Mass.) for 20 min at room temperature, color was developed by the addition of 1N sulfuric acid, and colorimetric analysis of antibody binding to SIVsmB7 was performed at an optical density of 450 nm (OD450) using a Spectra Max 340 PC (Molecular Devices, Sunnyvale, CA).

Statistical analysis.

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc. San Diego, CA). Paired T tests were performed, comparing values for individual and longitudinal time points to the time 0 value within a population.

3.4 RESULTS

Acute phase decline in total B cell levels corresponds with peak in viral titer and drop in CD4+ T cells.

Ten Rhesus macaques were infected intravenously with SIVmac239 at the Oregon National Primate Research Center. Peripheral blood samples were analyzed longitudinally during the first 150 days post infection. Viral titers peaked at day 10 post infection (mean: $3.5x10^7$) and declined

to an average set point at day 84 (mean: $7.3x10^6$) (figure 15). Two animals did not control viral replication, maintaining viral loads of 10^7-10^8 copies/ml for 100 days post infection. Five animals demonstrated intermediate levels of control, with setpoint viral titers of 10^6-10^7copies/ml. The remaining three animals had relatively effective viral control, with viral titers that continued to gradually decline for about 60-85 days post infection, reaching setpoint titers of 10^3-10^5copies/ml.

CD4+ T cells decreased during the first 10 days post infection as previously reported (20), reaching the lowest point of approximately 800 cells/µl coincident with the peak in viral load (figure 16). Consistent with a prior study by Roederer *et al*., the peripheral CD20+ B cell population also exhibited a substantial drop in cell numbers, from an average of 1157 cells/µl preinfection to nadir average of 378 cells/µl at day 10 post infection (20). Following this initial drop, the number of peripheral CD20+ (total B) cells rebounded by 30 days post infection to an intermediate plateau, then recovered to preinfection levels between 80 and 90 days post infection. Interestingly, a spike in total B cell numbers was observed following the initiation of ART.

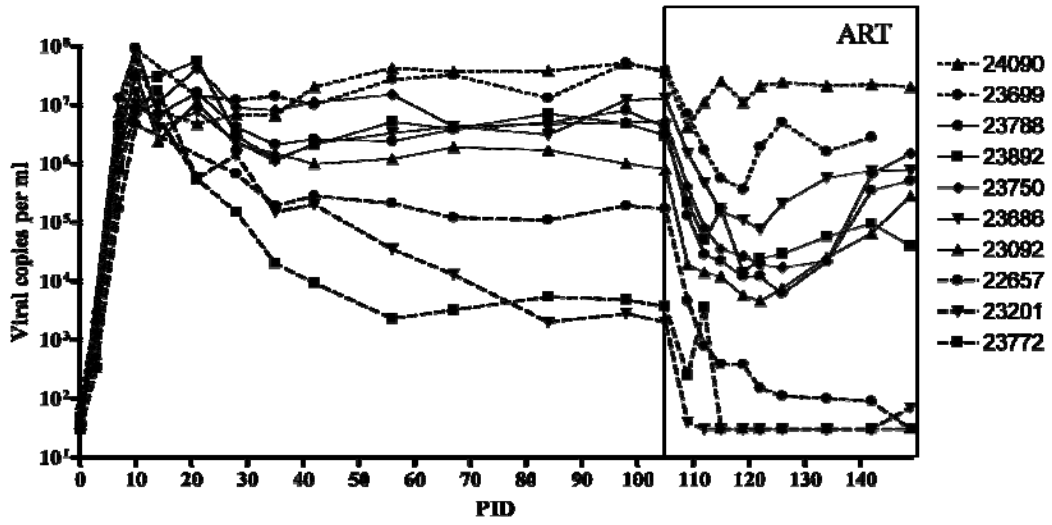


Figure 15. Viral Load

Measurement of viral loads following SIV infection. Ten rhesus macaques were infected i.v. with SIVmac239 and analyzed for the number of viral copies per milliliter of blood from post infection day (PID) 0-149. Viral loads for each animal are plotted individually. All animals were treated with PMPA (30mg/Kg day 105 until day 134 and then 20mg/Kg subsequently) and FTC (50mg/Kg day 105 until day 134 and then 20mg/Kg subsequently), shaded area. High viral load animals are delineated by dotted lines, intermediate viral loads by solid lines and low viral loads by dashed lines.

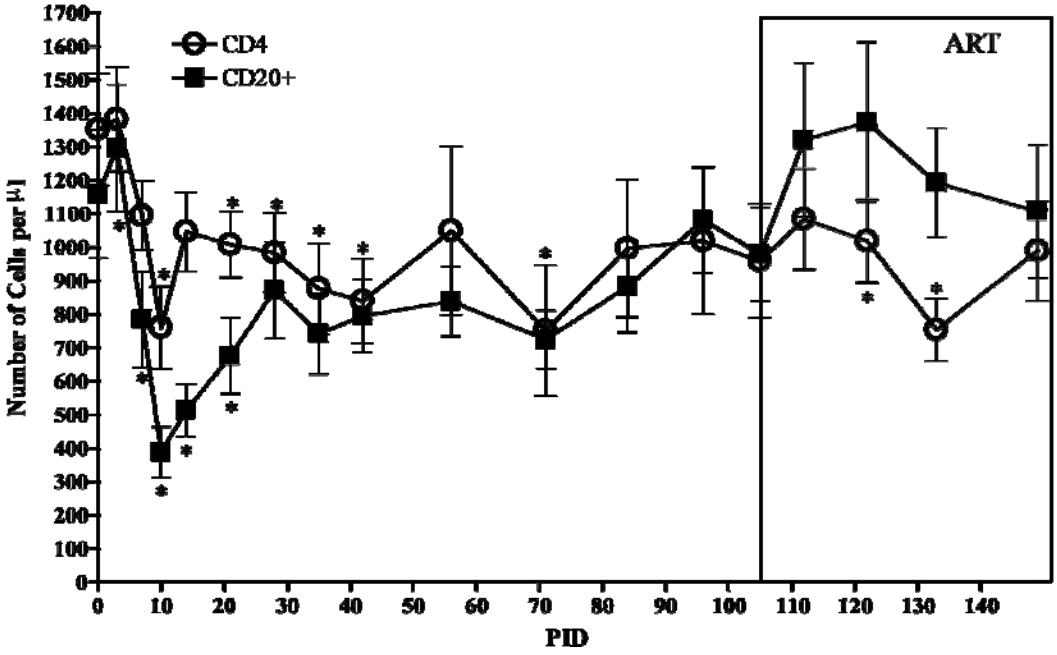


Figure 16. CD4 T cells and total CD20 B cells during SIV infection.

Naïve (CD20+CD27-) B cell numbers remained significantly lower than baseline levels longer than memory (CD20+CD27+) B cell numbers.

Measurement of CD4+ T cell and total CD20+ B cell numbers following SIV infection. The average number of CD4+ T cells and total CD20+B cells for all animals are shown. Data represents the average of 10 animals, with error bars representing the standard error value between the animals for each timepoint. Asterisks denote a significant difference from a particular timepoint back to the day 0 value using a student's T test (* = P<0.05).

To further delineate naïve and memory B cell subsets, total B cells were subdivided based on surface expression of CD27 (figure 17). For these studies, we took advantage of human antibodies that cross-reacted with the rhesus system, utilizing markers previously defined in humans. Thus, putative naïve cells were defined as cells that expressed CD20+CD27-, while putative memory cells were defined as CD20+CD27+, and will be referred to going forward as naïve and memory cells, respectively.

Memory (CD20+CD27+) cell numbers (figure 18b) declined after day 3 post infection to an average of 255.5 cells per µl by day 10 post infection (figure 18c). The number of memory cells recovered to a level that was slightly reduced from preinfection levels (748.8 cells/µl) by

day 30 post infection. The number of naïve (CD20+CD27-) cells (figure 18a) increased significantly ($p<0.005$) from an average of 401.4 cells/µl at day 0 to 519.2 cells/µl at day 3 (figure 18c and table 3). This initial increase was then followed by a significant decrease in cell numbers, reaching the lowest point by day 10 post infection. The naive cell population remained significantly diminished compared to preinfection values out to day 40 post infection, only returning to preinfection levels between day 80 and 90 post infection

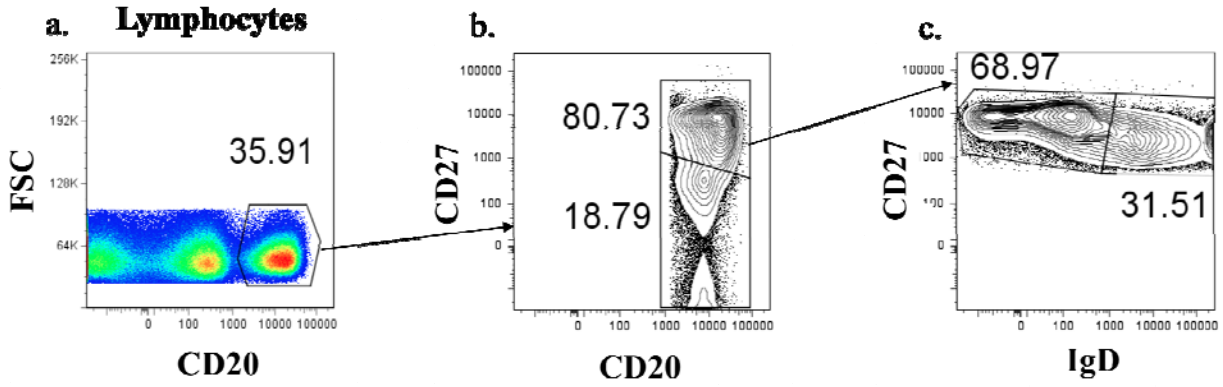
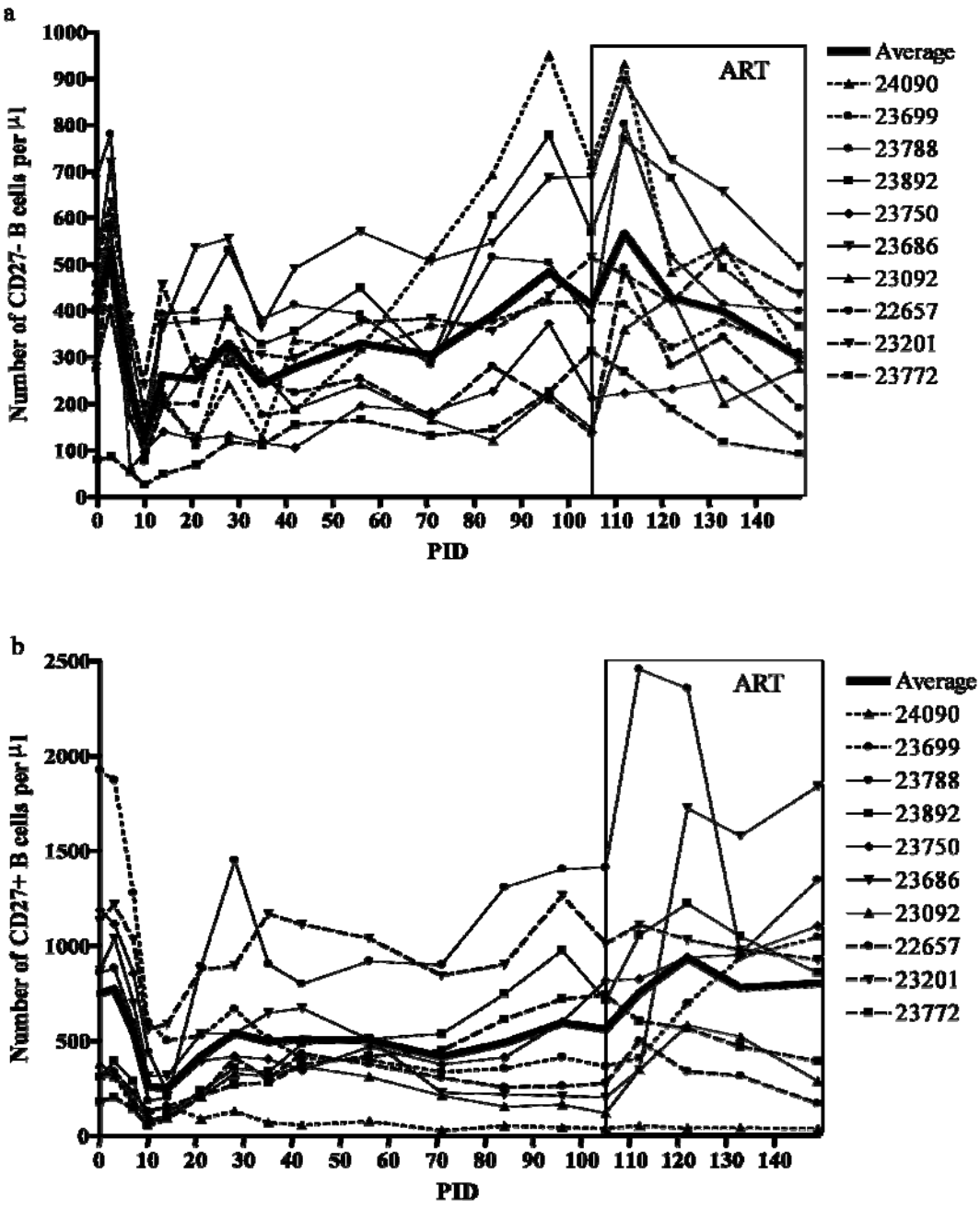


Figure 17. Representative flow cytometry for differentiating B cell populations.

Differentiation of B cell subsets using flow cytometric analysis. Representative plots of whole blood FACS staining and B cell differentiation using antibodies to CD20, CD27 and IgD are shown. (a) CD20+ B cells were differentiated from the total lymphocyte population delineated using side and forward scatter characteristics. (b) Expression of CD27 on B cells was used to separate CD27- (naïve) from CD27+ (memory) B cells. (c) Memory B cells were further subdivided based on expression of IgD into CD20+CD27+IgD+ (IgM secreting) and CD20+CD27+IgD- (IgG/IgA secreting) populations.


a
Number of CD27- B cells per µl
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Average
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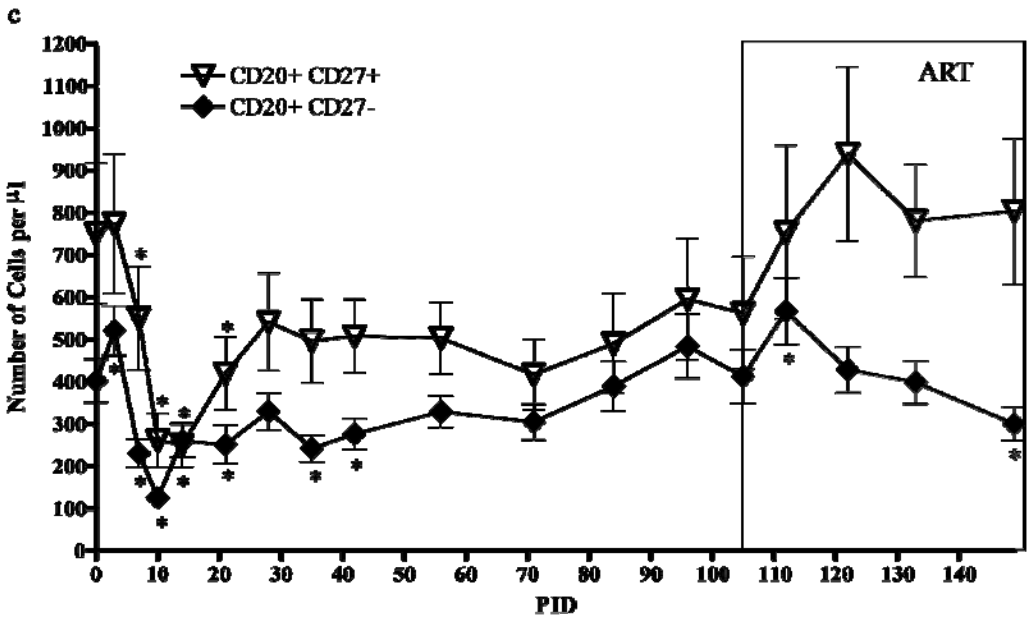


Figure 18. Longitudinal analysis of naive and memory B cells.

Longitudinal analysis of naïve (CD20+CD27-) and memory (CD20+CD27+) B cells. Peripheral blood from rhesus macaques infected with SIVmac239 was obtained at the indicated timepoints following acute and early chronic infection. Cells were stained for surface expression of CD20 and CD27 and analyzed by flow cytometry to differentiate naïve and memory populations. Data representing the number of CD20+CD27- naïve B cells (a) or CD20+CD27+ memory B cells (b) in longitudinal timepoints is shown. The mean value of all ten animals in each group is indicated by the solid line. (c) The average number of naïve (CD20+CD27-) and memory (CD20+CD27+) B cells with error bars representing standard error value for all ten animals. ART treatment is indicated by the shaded area. Asterisks denote a significant difference from a particular timepoint back to the day 0 value using a student's T test (* = $P<0.05$).

Table 3. Change in CD20+CD27- (naive) and CD20+CD27+ (memory) B cell subsets compared to baseline following SIV infection.

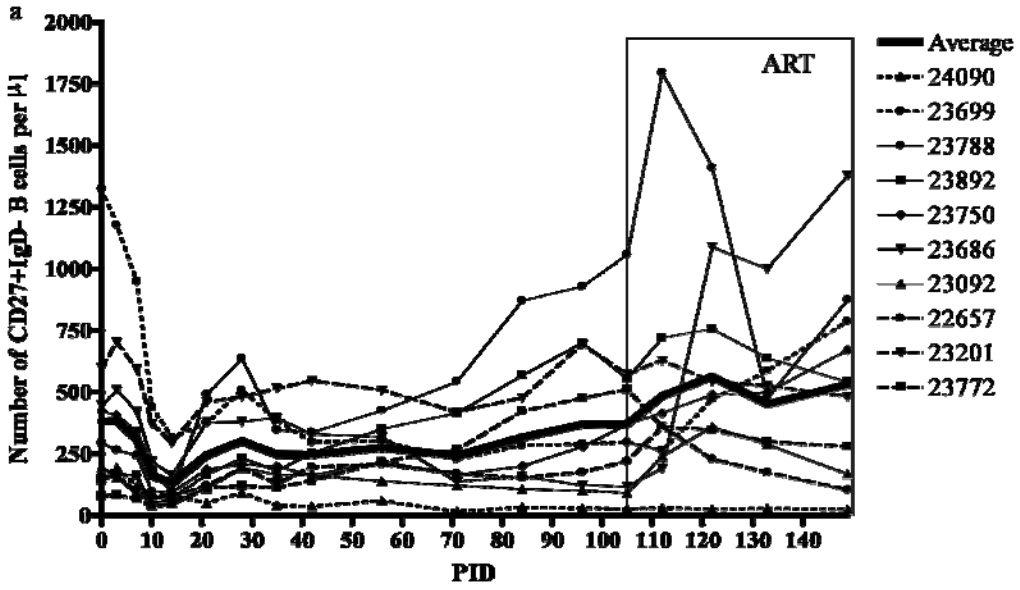
Time Point[a]	CD20+CD27- (±SD)[b]	Percent Change from Time 0[c]	CD20+CD27+ (±SD)[d]	Percent Change from Time 0[e]
0	401.97 (162.22)	0	750.59 (527.57)	0
3	521.3 (186.71)	29.69	773.85 (518.36)	3.10
7	230.65 (103.28)	-42.62	550.82 (389.29)	-26.62
10	125.83 (62.8)	-68.70	261.31 (200.73)	-65.19
14	260.42 (122.81)	-35.21	250.54 (164.8)	-66.62
21	251.4 (144.48)	-37.46	419.47 (270.85)	-44.11
28	329.61 (139.04)	-18.00	541.23 (364.12)	-27.89
35	242.05 (98.21)	-39.78	496.62 (312.34)	-33.84
42	276.65 (116.89)	-31.18	508.11 (276.16)	-32.31
56	329.37 (117.1)	-18.06	503.26 (267.61)	-32.95
71	304.84 (133.94)	-24.16	417.21 (261.9)	-44.42
84	389.86 (187.21)	-3.01	490.67 (373.33)	-34.63
96	484.08 (240.77)	20.43	595.27 (453.83)	-20.69
105[f]	412.8 (200.37)	2.69	562.34 (420.98)	-25.08
112	566.82 (249.95)	41.01	753.31 (647.36)	0.36
122	428.09 (171.65)	6.50	938.74 (650.33)	25.07
133	398.33 (160.9)	-0.91	780.75 (419.74)	4.02
149	300.1 (124.35)	-25.34	803.4 (544.29)	7.04

[a] Days post infection with SIVmac239 (10,000 infectious units, i.v.)
[b] Numbers of CD20+CD27- (naïve) B cells present in peripheral blood at indicated timepoints; values represent the mean of 10 animals (±SD).
[c] Percent change in CD20+CD27- (naïve) B cell number at each timepoint compared to day 0 preinfection timepoint.
[d] Numbers of CD20+CD27+ (memory) B cells present in peripheral blood at indicated timepoints; values represent the mean of 10 animals (±SD).
[e] Percent change in CD20+CD27+ (memory) B cell number at each timepoint compared to day 0 preinfection timepoint.
[f] Initiation of ART (PMPA and FTC)

Incomplete recovery of IgM secreting (CD20+CD27+IgD+) cells.

Memory B cells were further differentiated based upon surface IgD expression (26, 27). In humans, CD20+CD27+IgD+ have been found to predominantly secrete IgM antibodies (80) while CD20+CD27+IgD- cells have been found to predominantly secrete IgG or IgA antibodies (76). Using these markers in the rhesus system, both IgM (figure 19a) and IgG/IgA (figure 19b)

secreting cell populations decreased in total number of cells within 3-10 days post infection. Following the initial decrease in cell number, IgG/IgA secreting cells rapidly increased by day 28 to a mean of 303.8 cells/μl, where they remained steady until day 70 (figure 19c and Table 4). After day 70 this population once again increased, returning to preinfection values by day 96 post infection. In contrast, IgM secreting cells demonstrated a gradual increase in cell number between days 10-28 post infection followed by a gradual decline after day 40 (figure 19c). Interestingly, IgM secreting cells failed to rebound to preinfection values until the initiation of ART.



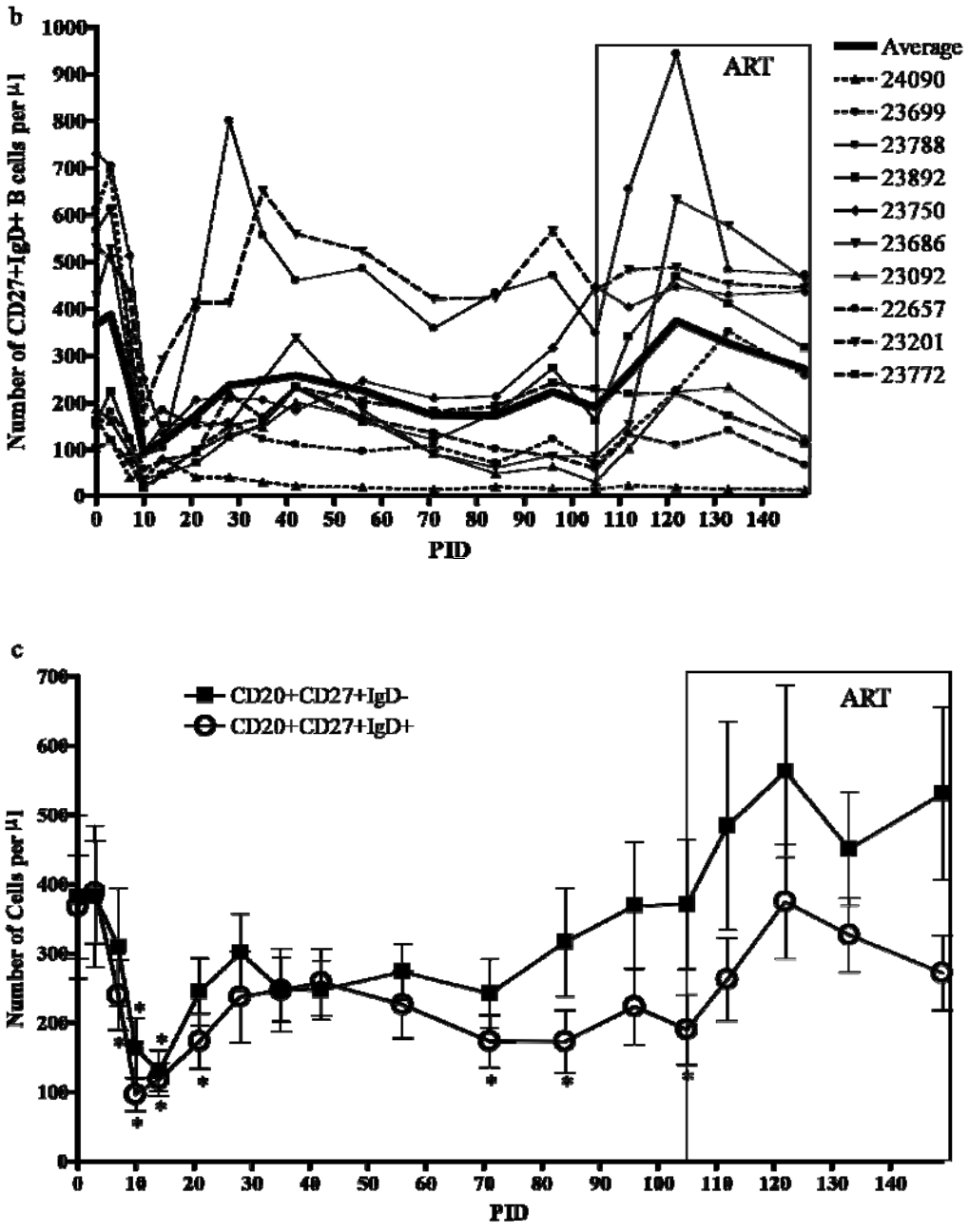


Figure 19. Longitudinal analysis of IgG/IgA secreting and IgM secreting cells.

Longitudinal analysis of IgG/IgA secreting (CD20+ CD27+ IgD-) and IgM secreting (CD20+ CD27+ IgD+) cells. Peripheral blood from rhesus macaques infected with SIVmac239 was obtained at the indicated timepoints following acute and early chronic infection. Cells were stained with the surface markers CD20, CD27 and IgD and analyzed by flow cytometry to differentiate IgG/IgA secreting and IgM secreting cells. (a) Data representing the number of CD20+CD27+IgD- IgG/IgA secreting B cells or (b) CD20+CD27+IgD+ IgM secreting B cells in longitudinal timepoints. The mean value of all ten animals in each group is indicated by the solid line. (c) The average number of IgG/IgA secreting (CD20+CD27+/IgD-) andIgM secreting (CD20+CD27+IgD+) B cells with error bars representing standard error value for all ten animals. ART treatment is indicated by the shaded area. Asterisks denote a significant difference from the day 0 value using a student's T test (* = $P<0.05$).

Table 4. Change in CD20+CD27+IgD+ (IgM secreting) and CD20+CD27+IgD- (IgG/IgA secreting) B cell subsets compared to baseline following SIV infection.

Time Point[a]	CD20+CD27+IgD+ (±SD)[b]	Percent Change from Day 0[c]	CD20+CD27+IgD- (±SD)[d]	Percent Change from Day 0[e]
0	367.05 (222.68)	0	381.34 (370.76)	0
3	387.86 (233.12)	5.67	382.31 (322.4)	0.25
7	239.84 (158.4)	-34.66	308.76 (267.68)	-19.03
10	96.61 (75.95)	-73.68	162.9 (137.29)	-57.28
14	118.51 (74.93)	-67.71	130.77 (93.63)	-65.71
21	173.45 (125.59)	-52.74	244.38 (154.81)	-35.92
28	237.28 (208.05)	-35.35	301.11 (175.27)	-21.04
35	247.08 (188.36)	-32.68	248.03 (145.52)	-34.96
42	258.25 (150.82)	-29.64	246.97 (133.81)	-35.24
56	225.98 (151.65)	-38.43	273.68 (127.14)	-28.23
71	173.37 (120.39)	-52.77	242.19 (156.1)	-36.49
84	173.03 (141.83)	-52.86	316.11 (247.01)	-17.11
96	223.57 (175.13)	-39.09	368.66 (291.33)	-3.33
105[f]	190.14 (159.56)	-48.20	370.48 (294.29)	-2.85
112	262.29 (189.73)	-28.54	484.12 (474.99)	26.95
122	374.26 (261.66)	1.96	562.67 (392.73)	47.55
133	326.22 (169.03)	-11.12	450.53 (258.54)	18.14
149	271.89 (171.05)	-25.93	530.46 (394.17)	39.10

[a] Days post infection with SIVmac239 (10,000 infectious units, i.v.)
[b] Numbers of CD20+CD27+IgD+ (IgM secreting) B cells present in peripheral blood at indicated timepoints; values represent the mean of 10 animals (±SD).
[c] Percent change in CD20+CD27+IgD+ (IgM secreting) B cell number at each timepoint compared to day 0 preinfection timepoint.
[d] Numbers of CD20+CD27+IgD- (IgG/IgA secreting) B cells present in peripheral blood at indicated timepoints; values represent the mean of 10 animals (±SD).
[e] Percent change in CD20+CD27+IgD- (IgG/IgA secreting) B cell number at each timepoint compared to day 0 preinfection timepoint.
[f] Initiation of ART (PMPA and FTC)

Dramatic and Early Increase in CD95 expression in all B cell subsets.

To assess whether differences in B cell recovery between memory subsets was associated with increased cell activation, surface expression of CD95 was analyzed. All examined B cell populations demonstrated increased CD95 surface expression following infection, despite differences in the basal level of CD95 expression. The magnitudes of the increases were also

different between B cell subsets (figure 20). For example, the lowest level of basal surface expression of CD95 (1.64%) was observed in the naïve B cell population and a significant increase in the percentage of these cells expressing CD95 (3.62%) ($p<0.05$) was observed by day 7 post infection (figure 20, closed circles). The percentage of CD95+ naïve B cells remained significantly elevated above baseline from day 7 through the entire study period. IgM secreting cells expressed moderate basal levels of CD95 (17.9%) with a significant increase in the percentage of CD95 expressing cells to 27.0% by day 7 post infection ($p<0.05$). The percentage of IgM secreting cells expressing CD95 remained significantly higher than preinfection levels for the duration of the study period, with the maximum percentage observed being 44.8% (figure 20, open triangles). Finally, 66.1% of IgG/IgA secreting cells expressed CD95 preinfection (figure 20, closed squares), with a significant increase in expression ($p<0.05$) by day 3 post infection. As seen in the other populations studied, the increased expression remained significantly elevated for the duration of the study with a maximum of 86.5% of IgG/IgA cells expressing surface CD95. These data demonstrated that B cells from SIV infected animals had significantly higher percentages of cells with an activated phenotype.

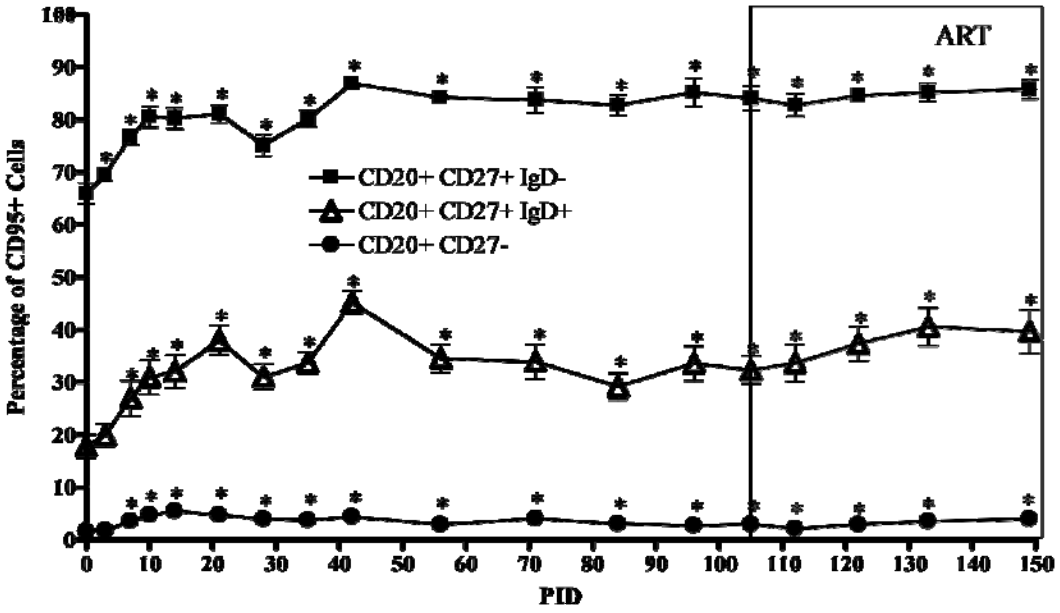


Figure 20. Activation of B cells during SIV infection.

Expression of CD95 in B cell subsets following SIVmac239 infection. The average percentage of IgG/IgA secreting (closed squares), IgM secreting (open triangles) and naïve (closed circles) B cells expressing CD95 was followed in longitudinal peripheral blood samples from 10 SIVmac239 infected rhesus macaques. Lines represent the average percent of cells expressing CD95 for each cell population with error bars representing standard error value across all 10 animals. Asterisks denote a significant difference from the day 0 value using a student's T test (* = $P<0.05$).

Contrasting proliferative responses to SIV infection between B cell subsets.

To assess whether decreased proliferation played a role in acute B cell depletion, B cell subsets were further analyzed for expression of the Ki67 proliferation antigen. The proliferative responses observed were remarkably different when comparing the naïve, IgM secreting and IgG/IgA secreting populations (figure 21). The percentage of Ki67+ naive cells slowly decreased for the entire duration of the study from 15.1 percent preinfection to 4.8 percent at day 149. ART did not alter the decline in the percentage of Ki67+ cells in this naïve B cell population. In contrast, memory B cells exhibited a spike in the percentage of Ki67+ cells at day 21. The IgG/IgA secreting population demonstrated a significant increase from 16.6 percent preinfection

to 40.2 percent at day 21 ($p<0.005$), followed by a moderate decline with the average steady state level of Ki67+ cells being maintained at a higher level than preinfection until the initiation of ART. In contrast, a significant increase in the percentage of Ki67+ IgM secreting cells was observed between preinfection and day 21 (19.5 percent to 32.2 percent, respectively, $p<0.05$). This increase was followed by a significant decline, with the percentage of cells being maintained at a lower level than preinfection levels until initiation of ART. These data demonstrated that memory B cell populations had higher levels of proliferation but that these increased levels were only maintained into the chronic phase of infection in the IgG/IgA secreting memory B cell subset.

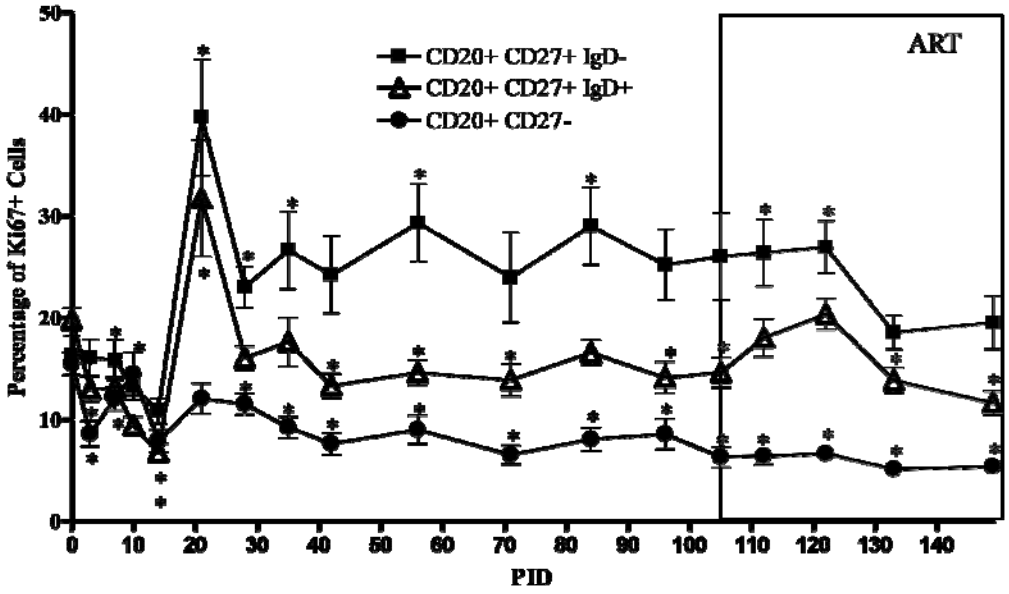


Figure 21. Proliferation in B cell subsets during SIV infection.

Intracellular expression of Ki67 following infection with SIVmac239. Peripheral blood from rhesus macaques infected with SIVmac239 was obtained at the indicated timepoints during acute and early chronic infection. Lymphocytes from whole blood analysis were analyzed for intracellular Ki67 expression. Lines represent the average percent of Ki67+ cells within each cell population present across all 10 animals. Asterisks denote a significant difference from the day 0 time point using a student's T test (* = $P<0.05$).

Differential effect of ART treatment on B cell subsets.

In order to test whether decreased viral load and improved CD4 T cell counts would alter the B cell populations during early chronic infection, the animals were treated with antiretroviral therapy. Treatment with PMPA (50mg/Kg) and FTC (30mg/Kg) was initiated at day 105 post infection in all animals. The dosage of PMPA was reduced at day 134 to 20mg/Kg at the same time the dosage for FTC was reduced to 20mg/Kg. Following initiation of ART, all animals demonstrated an initial drop in viral setpoint with the non-controllers and intermediate controllers rebounding within an average of 15 to 25 days after treatment, respectively. In contrast, the three animals demonstrating the lowest viral setpoint had the most dramatic drop following initiation of ART, with 2 of the 3 animals reaching undetectable levels of virus within 10 days after treatment and the third animal becoming undetectable within 45 days of treatment initiation (figure 15). Antiretroviral therapy resulted in a slight increase in the number of total B cells (figure 16). However, changes within B cell subsets were more dramatic. The naïve B cell population demonstrated an initial, moderate increase in cell numbers following the initiation of ART (figure 19), from 408 cells/µl at day 105 to 562.6 cells/µl at day 112. The increase in naïve B cell numbers at the initiation of ART was then followed by a steady decline to 298 cells/µl by day 149. Memory B cells also demonstrated pronounced increases in cell numbers (figure 18) from 571 cells/µl at day 105 to 954.3 cells/µl at day 122. In contrast to the naïve B cell population, the number of memory cells remained higher than pretreatment numbers out to day 149. Interestingly, ART did not elicit changes in the surface expression of CD95 on any of the B cell populations studied (figure 20). While ART had no effect on the Ki67 expression in the naïve B cell population, it did result in a transient increase in the percentage of IgM secreting B cells expressing Ki67 and a sustained decrease in the percentage of IgG/IgA secreting cells

expressing Ki67 (figure 21). ART resulted in modest improvements in the memory B cell subsets but only induced transient improvements in the naïve B cell population.

Antibody production during early SIV infection.

To determine how analysis of B cell subsets correlated with the production of SIV specific antibody production, SIV envelope-specific antibody endpoint titers were measured in longitudinal serum samples in a concanavalin A ELISA. All animals with the exception of one rapid progressor demonstrated high titer SIV specific antibody by 4 weeks post infection, and these titers were sustained for the duration of the study (figure 22). Early timepoints (days 0-56) were not available for endpoint titer analysis. However, results were consistent with data previously published from our laboratory demonstrating that antibody titers from historical controls infected with SIV rapidly rise and peak within 4-12 weeks post infection (7, 68). These data demonstrated that there was little variation in antibody production between animals.

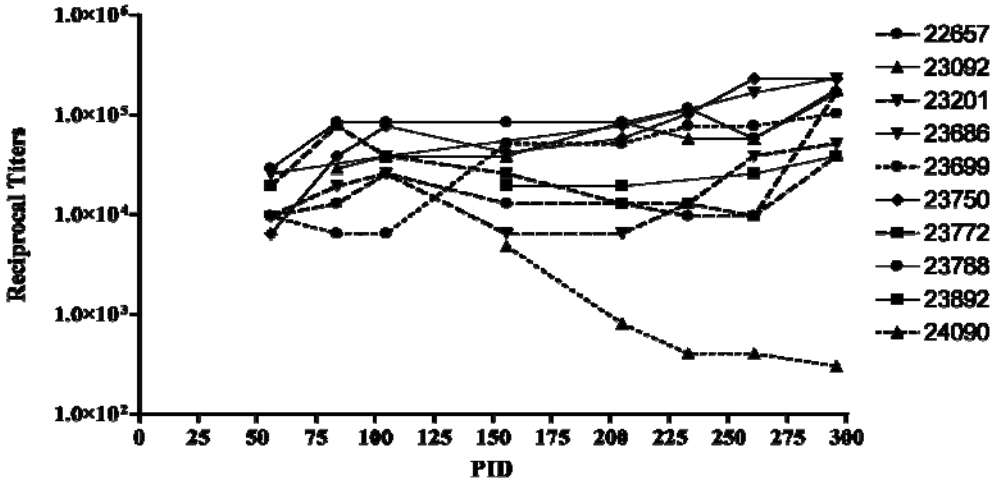


Figure 22. Total SIV antibody production following infection.

Measurement of SIV-specific antibody endpoint titer by concanavalin A ELISA. Envelope-specific antibody titers were measured to concanavalin a-captured SIVsmB7 envelope proteins in longitudinal samples from rhesus macaques infected with SIVmac239. Endpoint titers are reported as the last twofold dilution above the cutoff of the assay. Lines represent the $\log_{10}$ reciprocal endpoint titers from individual animals. Dashed lines are representative of animals with low viral setpoints (10^3-10^6), solid lines are representative of animals with intermediate setpoints (10^6-10^7) and dotted lines are representative of animals with high viral setpoints (10^7-10^8).

3.5 DISCUSSION

This study is, to our knowledge, the first detailed, longitudinal analysis of B cell subsets during acute and early chronic SIV infection. Novel changes were identified very rapidly following SIV infection within specific B cell subsets that have not been observed in previous studies when analyzing total B cells. While all B cell subsets in this study demonstrated a similar and profound depletion in cell number concordant with peak viremia, differences in the timing of recovery to preinfection values were observed among specific populations. The depletion of multiple B cell subsets from the periphery during acute infection may indicate a compromise in the early B cell response to SIV. Further, these depletions may play a role in abnormal B cell maintenance and functionality observed during later stages of infection. For example, from these studies it is not clear whether the B cells are being depleted due to direct or indirect viral effects, or whether cells

are trafficking from the periphery to tissues. Additional studies to determine the mechanisms for this depletion are needed.

It is likely that a combination of B cell depletion and redistribution to lymphoid organs occurs during acute SIV infection. The current study only addressed B cell populations in the periphery and could not directly demonstrate whether depletions from the periphery were reflected in the spleen or lymph nodes. Prior studies in cynomolgus and rhesus macaques indicate that redistribution of a portion of B cells to the spleen and lymph nodes occurs during acute infection (156, 185). Germinal centers have been shown to be reservoirs for virus and are areas where virus and viral proteins are likely to interact with B cells (185). As such, interactions in lymphoid organs may also lead to B cell dysfunction since there is evidence that the presence of proliferating B cells within the germinal centers of lymph nodes decreases as early as day 20 post infection (185). Thus, the depletion of peripheral B cells from the periphery, either via redistribution to lymphoid organs or cell death, would have a detrimental effect on the B cell population.

Several B cell functional abnormalities have been associated with HIV infection, including hypergammaglobulinemia (95, 96, 136, 175, 176, 186, 187), increased basal activation accompanied by diminished reactivity to mitogenic stimulation (85, 103, 136, 175, 188, 189), and depletion of the memory B cell subset (95, 96, 98, 136, 175, 176, 178). However, due to the difficulty in obtaining longitudinal samples from HIV-infected patients, especially during the early stages of infection, studies in HIV-infected patients have predominantly represented a snapshot of the B cell repertoire at one or very few timepoints during the chronic phase of infection. Host-virus interactions that occur during the acute phase of HIV infection are known

to heavily influence disease progression (174). Thus, the SIV/macaque model provides an effective means for a longitudinal B cell analysis during acute infection.

The present study identified dynamic changes in the memory B cell population during acute infection. The number of memory B cells within the periphery significantly dropped within 7 days of infection. The IgG/IgA secreting B cell population recovered to a level just below preinfection numbers within 30 days, while the IgM secreting B cell population recovered more slowly. The human B cell population which is analogous to the IgM secreting B cell population in rhesus macaques has been shown to be critical for the production of antibodies to newly emerging viral mutants and opportunistic infections, and is also very important in the production of T independent responses to pathogens including pneumococcus (190-193)**.** Thus, a slow recovery in this B cell subset has the potential to slow the initial response to SIV/HIV during acute infection and to render the host more susceptible to opportunistic infections later on. Defining the mechanism underlying the loss of this B cell subset could aid in the design of targeted immune therapy to protect the host from opportunistic infections.

Measurement of total antibody production has been a gold standard by which investigators measure the functionality of the B cell compartment. However, data from our lab and others have demonstrated that the production of quantitative levels of virus-specific antibody may not necessarily indicate that the antibody produced is qualitatively effective at limiting virus replication (7, 68, 144, 194)**.** Thus, the functional relevance of the antibody response in controlling infection may still be compromised despite measurement of a robust antibody titer. In a recent study by Scheid *et al*, IgG clones derived from memory B cells in HIV-infected patients demonstrating broad neutralizing activity were comprised of clonal responses to diverse envelope epitopes (195). In general, high affinity antibody (binding) did not correlate with

neutralization sensitivity (functionality). These data support our current study where we demonstrated that fewer B cells were present during the initiation of the antibody response. Thus, it is likely that a small number of B cell clones were responsible for the antibody response and that the breadth and potency of the response was limited. Additionally, the paucity of memory cells during acute infection when the initial antibody response is formed could have lead to diminished efficacy of the antibodies produced. With a reduced memory population, antibody responses have to be formed de novo, effectively resulting in each successive SIV clone to appear as a new antigen to the antibody mediated immune response. These data demonstrate that measurement of antibody titer alone is an insufficient means for assessment of B cell activity and needs to be combined with in depth analysis of the B cell compartment.

The depletion of naïve B cells from the periphery during acute SIV infection is also likely to play a significant role in the slow development of the B cell response. Naïve cells dictate the breadth and efficacy of the antibody response, requiring a multitude of signals, including BCR activation, CD40 stimulation and cytokine signals to initiate activation, maturation and proliferation (73). Thus, virally induced disruption of any of these signals could lead to decreased potency of the antibody response. The early depletion of naïve B cells renders the infected host more vulnerable to SIV during the initial, critical host-pathogen interaction. Further, the failure of the naïve population to rebound with ART suggests a limited ability for the host to recognize viral variants or new pathogens, resulting in a diminished B cell response to both SIV and other opportunistic pathogens. Early antiviral treatment or therapeutic vaccination focused on preventing the loss of naïve B cells during acute infection would render this critical cell population more effective during the chronic stage of infection.

Finally, in addition to rapid alterations in population dynamics, increased activation was observed in all examined B cell subsets. Although prior studies have demonstrated increased CD95 expression on B cell populations during chronic infections (85, 179), the precise timing of this increase relative to infection was unknown. Our data demonstrated that the increase in B cells expressing CD95 occurred almost immediately following SIV infection, was maintained through the acute phase of infection and was unaffected by ART treatment. This increased B cell activation is of importance as activation has also been implicated as a potential mechanism for altered B cell activity i.e. poor responses to B cell mediated vaccines (5). Therapeutic strategies to inhibit chronic activation, either via CD95 or other pathways, can be further explored using the SIV non-human primate model. In contrast to activation measured by CD95 expression, proliferative responses assessed using Ki67 were variable among B cell subsets, indicating the potential for differential regulation between the memory and naïve B cell subsets. Alterations in proliferative capacity may be related to how these specific subsets are able to respond to antigenic stimulation, and warrant further functional studies.

Data from the present acute study and others have demonstrated that significant changes in total B cell numbers occur in the periphery following acute SIV infection. The current study provides novel information about alterations in specific B cell subsets during acute SIV infection that were not revealed when analyzing the total B cell population. Furthermore, the data presented in this study clearly demonstrate that measurement of virus-specific antibody titer is not reflective of alterations in subset composition in the B cell compartment. It is important to monitor antibody specificity and functionality during SIV infection to fully understand the extent of the damage to the immune system. Further studies are warranted to identify potential

mechanisms for these phenotypic alterations and to test whether concurrent functional changes in B cell subsets occur during the same time frame.

3.6 ACKNOWLEDGEMENTS

The authors wish to thank Dr. Edmundo Kraiselburd for kindly providing the SIVsmB7 cell line. This work was funded by NIH/NIAID grants R01 AI52058 (KSC) and R01 AI35522 (DLS).

4.0 NAÏVE B CELL DYSFUNCTION FOLLOWING CD40 LIGATION

Naïve B cells from SIV infected rhesus macaques are insensitive to CD40 mediated protection from apoptosis

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4.1 ABSTRACT

B cell dysfunction has been known to be associated with HIV infection, but the mechanisms of specific functional perturbations have yet to be elucidated. The SIV/macaque model provides a system that is pathogenically similar to HIV infection but has greater availability of clinical samples and more control over virological parameters. Recent studies in the macaque model have demonstrated that B cell abnormalities are not confined to the memory B cell subset and that potentially more devastating functional changes may occur within the naïve B cell population. In the current study we sought to assess naïve and memory B cell functionality in response to CD40 ligation. Our findings demonstrated that the naïve B cell population from SIV infected animals did not respond in the same way as the naïve B population from uninfected animals. Specifically, naïve B cells from SIV infected animals did not demonstrate CD40

ligation mediated protection from apoptosis but were able to upregulate CD95 in response to CD40 ligation. These data demonstrated that CD40 signaling could occur within naïve B cells from SIV infected animals but that a portion of the signal was not being transmitted effectively.

4.2 INTRODUCTION

B cell functional abnormalities including hyperactivation, hypergammaglobulinemia and a paradoxical reduction in the response to activating stimuli are hallmarks of human immunodeficiency virus (HIV) infection (84). Despite the fact that these abnormalities have been well established, the mechanisms through which HIV induces these perturbations *in vivo* remain unclear. Multiple potential mechanisms have been hypothesized including direct B cell stimulation by HIV proteins (94) and generalized non-specific activation induced through long-term chronic infection (196), but these hypotheses cannot explain all the B cell abnormalities that have been associated with HIV infection. In order to mechanistically understand the functional failures within the B cell compartment, a more complete picture of B cell activity at the molecular level is necessary. Although a major focus of B cell studies during HIV infection has been on the memory population, there is increasing evidence to suggest that the naïve B cell population is also affected. Since memory cells arise from naïve B cells, it is of utmost importance to fully characterize mechanistic alterations within the naïve B cell population during HIV infection.

The simian immunodeficiency virus (SIV)/macaque model provides a means for testing B cells in the context of an infection that is similar to HIV infection in humans. Using the SIV/macaque model, experimental parameters including timing and dose of viral inoculation and assessment of clinical samples can be rigorously controlled and since naïve and memory B cells

in the rhesus macaque have been extensively characterized, findings from these studies will likely translate well in studies of HIV infection in humans. Prior studies in the rhesus macaque have demonstrated that acute SIV infection results in rapid depletion of both naïve and memory B cell populations from the periphery, and that these populations slowly recover in number as the disease progresses to the chronic phase (23). Functionally, like HIV infected humans, SIV infected monkeys are unable to mount a humoral immune response to B cell mediated vaccines that is as robust and durable as the response elicited in uninfected controls (8, 197, 198).

As a critical interactive links between B and T cells, CD40, expressed on B cells, and its ligand, CD154, which is upregulated on activated CD4+ T cells, play major roles in the development of highly specific antibody responses. CD40 mediated activation has been shown to be important for multiple factors in B cell maturation including cell proliferation, germinal center formation and class switching (166). Although it has been demonstrated that T cells from HIV infected people are less able to sustain surface expression of CD154 (199), other HIV studies have demonstrated that B cell proliferation and antibody production in long-term cultures in response to co-stimulation with CD154 and other either cytokines or mitogens is diminished (103, 187). These data suggest that impaired T cell expression of CD154 and B cell recognition of signaling through the CD40 receptor both play a role in the formation of ineffective antibody responses found in the context of HIV infection.

The current study was designed to investigate the responses of naïve and memory B cells in the rhesus macaque to CD40 mediated activation as a potential mechanism for altered B cell function during SIV infection. Results from this study demonstrated that naïve B cells in SIV infected animals are insensitive to CD40 ligation mediated effects on cell survival, and further

indicated that a key B cell selection pathway in SIV infected animals is critically altered during chronic infection.

4.3 MATERIALS AND METHODS

Animals

Peripheral blood was obtained from colony-bred rhesus macaques (*Macaca mulatta*) of Indian origin maintained and used in accordance with the guidelines of the Animal Care and Use Committee at the Oregon National Primate Research Center. Animals were infected with SIVmac239 at 10,000 infectious units intravenously.

FACS analysis of whole blood

Flow cytometric analysis was performed on fresh whole blood samples. Two hundred microliters of citrate treated whole blood was obtained in a blood collection tube (BD Diagnostics, Franklin Lakes, NJ) and incubated for 15 minutes with ammonium chloride solution to promote red blood cell lysis. Remaining cells were spun down and washed 2 times with Dulbecco's Phosphate buffered saline (dPBS). Titrated fluorochrome conjugated antibodies to extracellular targets were incubated directly with cells at room temperature for 15 minutes. Following incubation, cells were washed once with 4 ml cold (4°C) dPBS with 0.1% of FBS and 5 micro molar sodium azide (wash buffer). Cells were then incubated with a viability dye at room temperature for 10 minutes. Cells were washed twice with cold wash buffer and fixed with 1% paraformaldehyde in PBS. Freshly stained lymphocytes were differentiated using forward and side scatter characteristics on an LSRII (BD Biosciences, San Jose, CA). B cell populations were analyzed using anti-human CD20 (eBioscience, San Diego, CA), CD27 (BD Bioscience, San Jose, CA)

and CD40 (BD Bioscience, San Jose, CA), CD86 (eBioscience, San Diego, CA) and CD95 (BD Bioscience, San Jose, CA). List-mode multiparameter data files were analyzed using the FlowJo software program (Version 8.8.6; Tree Star Inc., Ashland, OR).

Isolation of PBMC and activation through ligation of CD40

Cells were isolated over ficoll, plated at $1x10^6$ cells/ml in a 24 well tissue culture plate (2ml/well) and incubated in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, penicillin and streptomycin. Cells were either left untreated or were treated with anti-CD40 antibody (Abcam, Cambridge, MA) for 24 hours. After 24h, cells were stained for CD20, CD27, CD95 and Annexin V (Caltag, Carlsbad, CA) and treated with a viability dye. Cells were fixed in 1% ice cold paraformaldehyde and analyzed on an LSRII.

Statistics

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc. San Diego, CA). One way ANOVA with repeated measures and unpaired T tests with Welch's correction were used to compare differences between groups.

4.4 RESULTS

Loss of CD40 mediated survival in SIV infected animals

We were interested in determining whether CD40 ligation of B cells from SIV+ animals would have the same functional output as CD40 ligation of B cells from uninfected animals. We first confirmed the expression of CD40 on naïve and memory B cells in SIV infected and uninfected rhesus macaques. Peripheral blood cells from 6 uninfected and 2 SIV infected (chronic phase of

infection) animals were stained following red blood cell lysis with antibodies specific for CD20, CD27 and CD40 and analyzed by flow cytometry. CD27 expression within the CD20+ population was used to differentiate between CD27+ memory and CD27- naïve cells. In 6 uninfected animals, 55.4% +/- 11.1% of naïve B cells and 21.9% +/- 7.3% of memory B cells were found to express CD40 (figure 23a). In 2 SIV+ animals 49.4% +/- 0.8% of naïve and 14.2% +/- 6.1% of memory B cells were found to express CD40 (figure 23b). These data in a small number of animals demonstrated no gross differences in the percent expression of CD40+ B cell populations between SIV+ and uninfected (SIV-) animals.

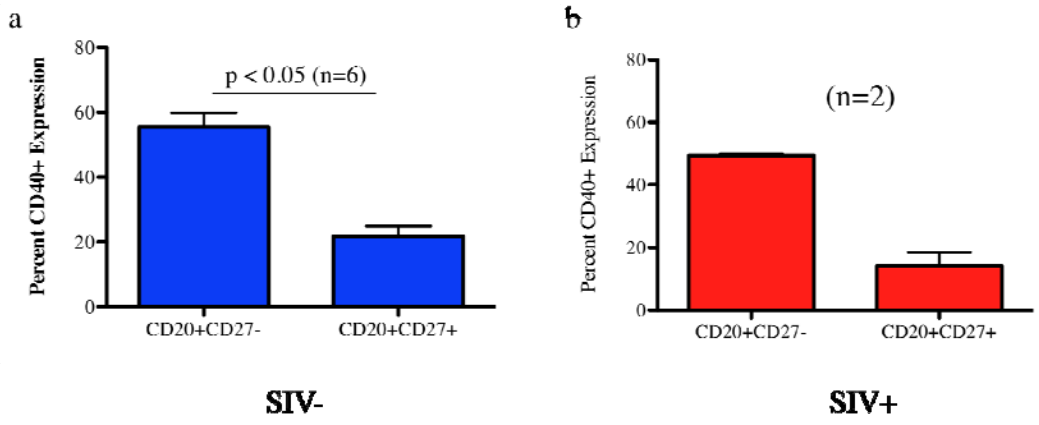

Figure 23. CD40 expression on B cell populations.

No difference in the percentage of naïve or memory B cells with CD40 surface expression between SIV infected and uninfected animals. Naïve (C27-) and Memory (CD27+) B cells were stained for CD40 expression. The percentage of CD40+ cells is represented. Differences between groups were analyzed with a Student's T test.

To analyze the effects of CD40 ligation on naïve and memory B cells, PBMC were isolated from SIV+ and SIV- animals and divided into treated and untreated groups. The untreated groups were cultured in supplemented RPMI alone and the treated groups were cultured in supplemented RPMI and treated with or without anti-CD40 antibody for 24 hours. In freshly isolated cells, an average of 51% +/- 18.7% from uninfected animals (n=6) and an

average of 38.9% +/- 16.3% in SIV infected animals (n=8), had a naïve phenotype (figure 25a). Per $1x10^6$ lymphocytes isolated, an average of 48,184 +/- 25,430 naïve B cells were isolated from uninfected animals and 36,864 +/- 6,676 naïve B cells were isolated from SIV infected animals (figure 24a). SIV infection resulted in reduced ratios and numbers of naïve B cells.

After 24 hour incubation of $1x10^6$ lymphocytes per well in culture with anti-CD40 antibody, an average of 64.2% +/- 16.7% of B cells from uninfected animals (n=6 animals, 2 wells per animal) had a naïve phenotype and 54.7% +/- 18.3% of B cells from SIV infected animals (n=8 animals, 2 wells per animal) had a naïve phenotype (figure 25a). In absolute number following anti-CD40 antibody treatment, an average of 42,874 +/- 41,820 naïve cells were observed in cultures from the uninfected animals and an average of 32,660 +/- 7,256 naïve cells were observed in cultures from the infected animals (figure 24a). After 24 hour incubation without treatment, an average of 49.3% +/- 18.8% of B cells from uninfected animals had a naïve phenotype and an average of 49.7% +/- 17.6% of B cells from SIV infected animals had a naïve phenotype (figure 25a). Without treatment, an average of 26,604 +/- 16,707 naïve cells were observed in cultures from the uninfected animals and an average of 32,729 +/- 7,863 naïve cells were observed in cultures from the SIV infected animals (figure 24a). Significant differences ($p<0.05$) were observed between the number of naïve B cells in the CD40 antibody treated group and the untreated group as well as between the percentage of naïve B cells between treated and untreated groups. These data demonstrated that more naïve B cells were present in anti-CD40 antibody treated cultures compared to untreated cultures from uninfected animals but that the same treatment does not induce a similar response in naïve B cells from SIV infected animals.

In freshly isolated cells, an average of 51,532 +/- 32,408 memory B cells per $1x10^6$ lymphocytes were present in uninfected animals and 88,719 +/- 78,520 memory B cells were

present in SIV infected animals. Following 24 hour treatment with anti-CD40 antibody, an average of 19,430 +/- 18,173 memory B cells was observed in cultures from uninfected animals and an average of 35,080 +/- 28,398 memory B cells was observed in cultures from SIV infected animals. Slight differences were observed between treated and untreated groups in the uninfected animals and no differences were observed between treated and untreated groups in the SIV infected animals (figure 24b and tables 5 and 6). These data demonstrated that there were significant ($p<0.05$) reductions in the numbers of memory B cells after 24 hours in culture in both uninfected and SIV infected animals but that CD40 ligation did not abrogate the loss of memory cells in either group of animals.

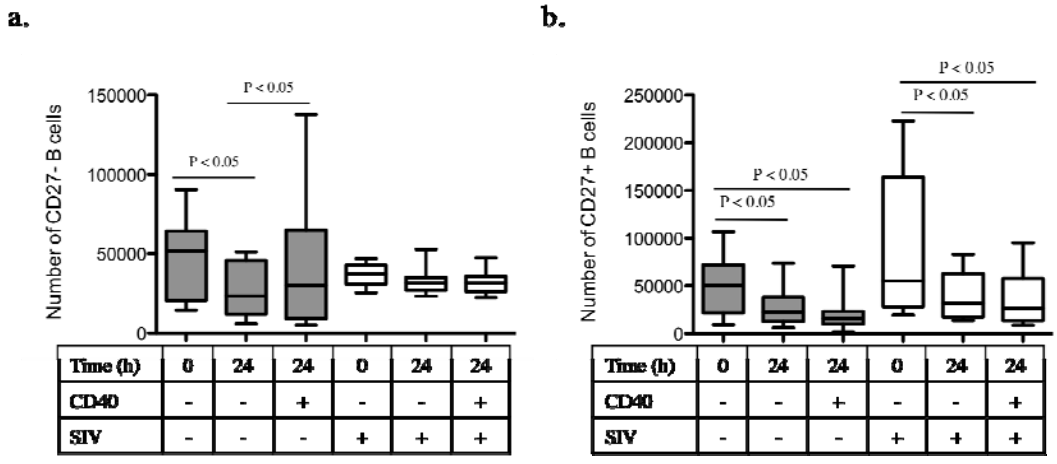

Figure 24. Number of naive and memory cells following CD40 ligation.

Spontaneous loss in naïve B cell numbers is mitigated through ligation of CD40 receptor in uninfected but not SIV+ animals. The number of naïve (a) and memory (b) B cells per well at time 0 and from cells incubated for 24 hours with and without anti-CD40 antibody were analyzed. Data represent average from 2 wells per animal, N=6 uninfected and 8 SIV infected animals. Error bars span from the minimum to the maximum values observed within a group, the box represents the middle 50% of the values and the solid line represents the median value. Differences between treatments were analyzed with a one way ANOVA with repeated measures.

Table 5. CD40 mediated population changes in the memory B cell subset.

Treatment	Number of Memory B cells Uninfected	Number of Memory B cells SIV Infected
0 Hour	51,532 +/- 32,408	88,719 +/- 78,520
24 Hour Untreated	27,956 +/- 20,756	38,195 +/- 23,900
24 Hour Treated	19,430 +/- 18,173	35,080 +/- 28,398

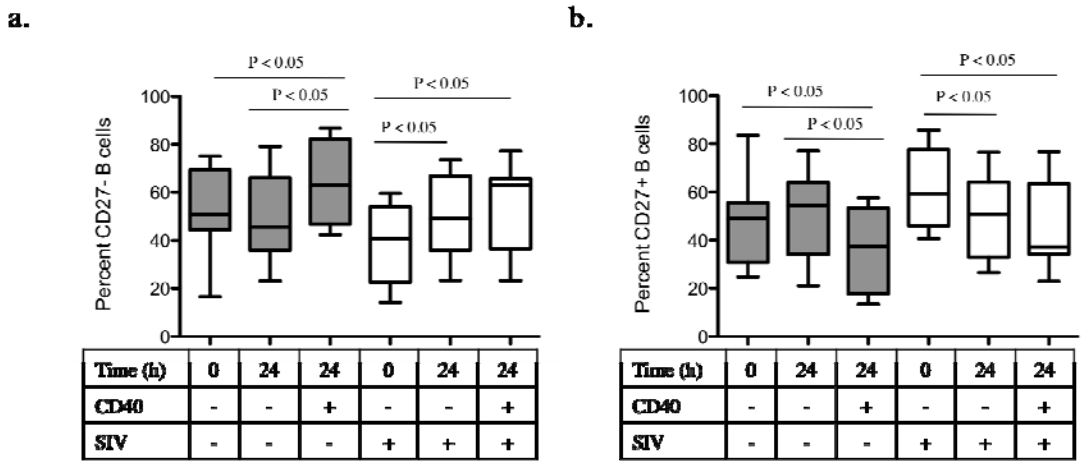

Figure 25. Percentage of naive and memory cells following CD40 ligation.

Increased percentage of naïve B cells following CD40 ligation in uninfected but not SIV infected animals. The percentage of naïve (a) and memory (b) B cells from freshly harvested and cells incubated for 24 hours with and without anti-CD40 antibody. Error bars span from the minimum to the maximum values observed within a group, the box represents the middle 50% of the values and the solid line represents the median value. Differences between treatments were analyzed with a one way ANOVA with repeated measures.

Table 6. CD40 mediated changes in the percentage of memory B cells

Treatment	Percent Memory B cells Uninfected	Percent Memory B cells SIV Infected
0 Hour	49.0% +/- 18.7%	61.1% +/- 16.3%
24 Hour Untreated	50.7% +/- 18.8%	50.3% +/- 17.6%
24 Hour Treated	35.9% +/- 16.8%	45.3% +/- 18.3%

CD40 ligation results in an increase in the ratio of CD95 positive naïve B cells in SIV infected animals.

Since the naïve B cell population in SIV infected animals did not demonstrate a survival response to CD40 ligation as measured in terms of cell number, we sought to test whether another known CD40 ligation mediated effect, namely an increase in the expression of CD95, would occur. The expression of CD95 has been shown to be upregulated on the surface of B cells following CD40 ligation and is associated with an increase in activation (169). Treatment with anti-CD40 antibody in uninfected and SIV infected animals resulted in a significant increase in the percentage of naïve B cells expressing CD95. As shown in figure 26a and table 7, following a 24 hour incubation without treatment, an average of 12.9% +/- 5.5% of naïve B cells expressed CD95 on the cell surface. This was in contrast to the anti-CD40 antibody treated cells in which significantly ($p < 0.05$) more cells, an average of 30% +/- 3.4%, expressed CD95 on the surface. Similar upregulation was observed in cells from SIV infected animals, after 24 hour incubation without treatment an average of 9.4% +/- 5.3% expressed CD95 and after 24 hours with treatment, 32.8% +/- 9.4% expressed CD95.

In the memory population, reductions in expression of CD95 were observed between freshly isolated cells and 24 hour cultures, but no differences were observed between treated and untreated cells (figure 26b and table 8). In freshly harvested cells from uninfected animals, 63.1% +/- 4.1% of memory B cells expressed CD95 and after a 24 hour incubation without treatment, 53.4% +/- 8% expressed surface CD95. A similar pattern was observed in cells from SIV infected animals in which 84.5% +/- 5.7% of freshly isolated cells expressed CD95 and 70.2% +/- 12.4% of cells incubated for 24 hours with no treatment expressed CD95. These data demonstrate that SIV infection does not alter the naïve or memory B cell response to CD40 ligation with regard to alterations in CD95 surface expression.

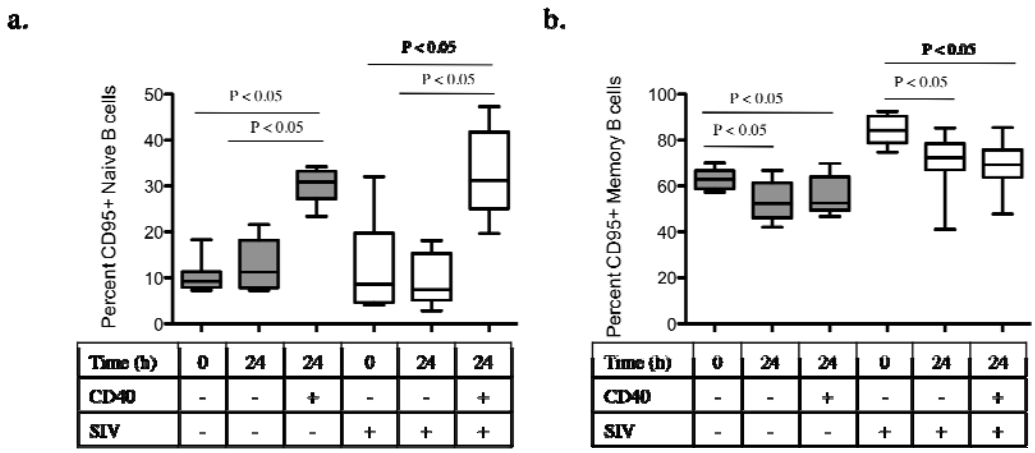

Figure 26. CD95 expression following CD40 ligation.

Ligation of CD40 in uninfected and SIV infected animals results in increased percentages of CD95+ naïve B cells. The percentage of CD95+ naïve (a) and memory (b) B cells at time 0 hours and cells incubated for 24 hours with and without anti-CD40 antibody. Error bars span from the minimum to the maximum values observed within a group, the box represents the middle 50% of the values and the solid line represents the median value. Differences between treatments were analyzed with a one way ANOVA with repeated measures.

Table 7. CD40 mediated upregulation of CD95 in naive B cells.

Treatment	Percent Naive B cells Uninfected	Percent Naive B cells SIV Infected
0 Hour	10.5% +/- 3.8%	12.1% +/- 9.5%
24 Hour Untreated	12.9% +/- 5.5%	9.4% +/- 5.3%
24 Hour Treated	30.0% +/- 3.4%	32.8% +/- 9.4%

Table 8. CD40 mediated upregulation of CD95 in memory B cells.

Treatment	Percent Memory B cells Uninfected	Percent Memory B cells SIV Infected
0 Hour	63.1% +/- 4.1%	84.5% +/- 5.7%
24 Hour Untreated	53.4% +/- 8.0%	70.2% +/- 12.4%
24 Hour Treated	56.1% +/- 8.5%	69.0% +/- 10.7%

CD40 ligation fails to protect naïve B cells from SIV infected animals from spontaneous apoptosis.

We evaluated the effects of treatment with anti-CD40 antibody on naïve and memory cells by measuring surface staining of annexin V gated on live cells (figure 27 and table 9). Naïve B cells from SIV infected animals had significantly ($p < 0.05$) higher basal level of apoptosis (27% +/- 23%) compared to naïve B cells from uninfected animals (8.9% +/- 2.3%). In memory cells, significantly ($p < 0.05$) higher percentage of apoptotic cells from SIV infected animals (12.8% +/- 8.8%) compared to the percentage of apoptotic cells from uninfected animals (6.6% +/- 2.6%). In naïve cells from uninfected animals, fewer 24 hour anti-CD40 antibody treated cells (22.4% +/- 9.3%) were annexin V positive compared to untreated cells (28.8% +/- 9.4%). In contrast, in cells from SIV infected animals, the percentage of annexin V positive naïve B cells was higher in 24 hour anti-CD40 antibody treated cells (37.1% +/- 11.4%) compared to 24 hour untreated cells (30.6% +/- 10.4%).

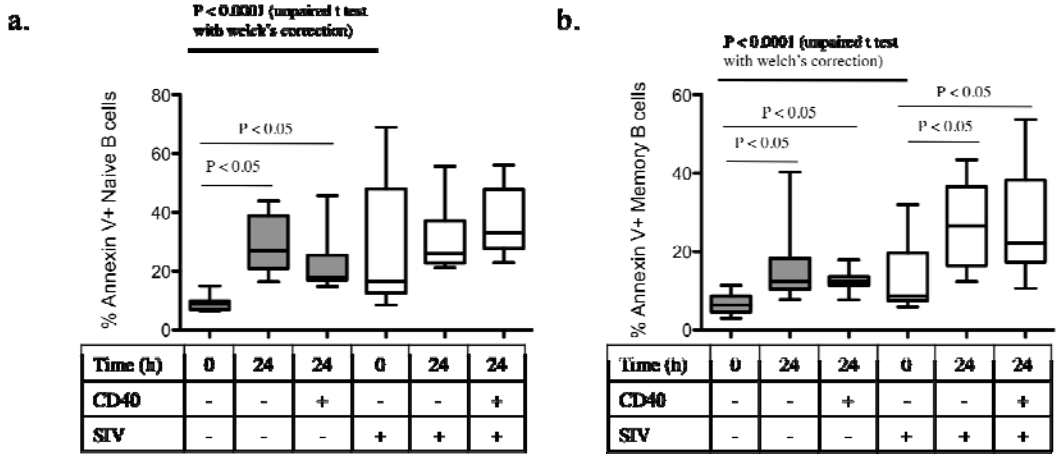

Figure 27. Apoptosis following CD40 ligation.

CD40 ligation of naïve B cells in uninfected but not SIV infected animals results in protection of these cells from apoptotis. The percentage of annexin V+ naïve (a) and memory (b) B cells at time 0 hours and cells incubated for 24 hours with and without anti-CD40 antibody. Error bars span from the minimum to the maximum values observed within a group, the box represents the middle 50% of the values and the solid line represents the median value. Differences between treatments were analyzed with a one way ANOVA with repeated measures and differences between freshly isolated cells of uninfected and SIV infected animals were analyzed with an unpaired T-test using Welch's correction.

Table 9. CD40 mediated protection from apoptosis in naive B cells.

Treatment	Percent Annexin V+ Naive B cells: Uninfected	Percent Annexin V+ Naive B cells: SIV Infected
0 Hour	8.9% +/- 2.3%	27.0% +/- 23.0%
24 Hour Untreated	28.8% +/- 9.4%	30.6% +/- 10.4%
24 Hour Treated	22.4% +/- 9.3%	37.1% +/- 11.4%

Table 10. CD40 mediated protection from apoptosis in memory B cells.

Treatment	Percent Annexin V+ Memory B cells: Uninfected	Percent Annexin V+ Memory B cells: SIV Infected
0 Hour	6.6% +/- 2.6%	12.8% +/- 8.8%
24 Hour Untreated	15.7% +/- 8.8%	26.7% +/- 10.4%
24 Hour Treated	12.7% +/- 2.8%	27.2% +/- 12.9%

4.5 DISCUSSION

The present study demonstrated that naïve B cells from SIV infected animals had an impaired response to CD40 ligation. Most strikingly, naïve B cells from SIV infected animals were insensitive to CD40 mediated survival. CD40 ligation did not reduce the level of apoptosis in naïve B cells from SIV infected animals as was found to occur in naïve B cells from uninfected animals. Despite the lack of CD40 ligation driven survival in SIV infected animals, CD40 ligation mediated increases in activation were similar between naïve B cells from SIV infected and uninfected animals. Altered survival as seen in the naïve B cells between SIV infected and uninfected animals was not readily apparent in the memory B cell compartment.

In this study, CD40 ligation did not affect the survival of naïve B cells from SIV infected animals. In uninfected animals, CD40 ligation resulted in decreased apoptosis of naïve cells. CD40 ligation is one of several signals naïve B cells receive in addition to B cell receptor stimulation and binding IL-2, IL-4 or IL-10 which collectively induce the maturation into a

memory cell (71, 72, 74, 77). Naïve B cells have a high rate of turnover and will be negatively selected in the absence of positive signaling. In uninfected animals, naïve B cells spontaneously underwent apoptosis *in vitro* and cell numbers were reduced if CD40 ligation did not occur. In the presence of CD40 ligation, fewer cells became apoptotic and the cell population numbers were preserved. In SIV infected animals, there was no difference in spontaneous apoptosis between cells that have experienced CD40 ligation for 24 hours and those that have not. Taken together, these data demonstrated that naïve B cells from SIV infected animals lacked the positive selective pressure associated with CD40 ligation of naïve B cells in uninfected animals. Naïve B cells that were matured in the absence of selective pressure would have an increased risk of not being specific for an antigen.

In addition to being insensitive to CD40 mediated survival, the naïve B cells from SIV infected animals had higher basal levels of apoptosis. There was a three-fold difference in the percentage of apoptotic cells from SIV infected animals compared to uninfected animals. The increased apoptosis likely plays an additional role in the inability of B cells to form effective antibody responses during HIV or SIV infection. Since this level of apoptosis is found in the naïve B cell population, it is increasingly likely that the majority of antibody mediated responses generated by this cell population would only be effective in the short term. B cells naturally succumb to apoptosis in order to prevent a single clone from taking up too much of the B cell niche, which could potentially prevent the formation of antibodies against new pathogens. Predisposition to apoptosis makes this cell population susceptible to premature cell death, which would occur prior to the formation of fully efficacious antibody responses that culminate in the production of highly selected quiescent memory and plasma cells.

Despite evidence of an obvious defect in the CD40 mediated B cell survival in SIV infected animals, not all downstream effects of CD40 ligation were altered in naïve B cells from SIV infected animals. In this study, CD40 ligation resulted in increased surface CD95 expression, indicative of activation, in naïve B cells from uninfected and SIV infected animals. In the context of SIV infection, these results are promising in that they demonstrate that at least a portion of the CD40 mediated signaling pathway is intact. Further analysis of the CD40 mediated signaling pathway including the dissection of signaling molecules involved in naïve B cell selection and increased activation could lead to the production of therapeutic agents designed to correct CD40 signaling dysfunction induced by SIV infection.

Functional CD40 signaling is critical, without it the formation of high titer, highly specific and highly efficacious antibodies through the activation of B cells is impaired. Selection of B cell clones that are capable of making specific antibodies occurs through interaction between B and T cells that are both able to recognize the same pathogen. Long term CD40 ligation by T cells has been shown to result in proliferation (72), which will increase the potential amount of antibody that can be produced and increases the odds that B cell selective events including somatic hypermutation will produce a higher quality antibody secreting cell. As a key element in the formation of germinal center reactions, CD40 ligation also aids in the ability of B and T cells to co-localize in lymphoid tissue in a manner that is conducive to propagating high-affinity antigen-specific B cell populations (200). These previous data, combined with the current study, which described a short term protection of cell numbers through CD40 ligation, demonstrate the critical need for positive CD40 mediated signals in the formation of an antibody mediated immune response. This study also highlights the fact that in uninfected animals,

negative selection of naïve B cells occurs readily in the absence of CD40 ligation, illustrating an additional positive selection method utilized by the CD40 receptor.

The current study also adds to a growing body of evidence that altered CD40 mediated signaling is a central component of impaired B cell activity during HIV and SIV infection. Activation through CD40 induces B cell proliferation (201) and germinal center formation (202), two B cell activities that are known to be altered during HIV and SIV infection (84). Although not fully verified with *in vivo* testing, the viral protein Nef has been implicated in altering B cell activity that is normally regulated in part by CD40 including class switching (203). Although the loss of CD4+ T cells and T cell mediated signaling could be responsible for some alterations in B cell populations during HIV and SIV infection, these reports and the current study, which show altered B cell responses independent of T cell populations, demonstrate that it is increasingly likely that the CD40 mediated defects are inherent to the B cell population.

Proper B cell maturation is tantamount in the formation of high quality antibody mediated immune responses to viral pathogens. By altering the maturation process, SIV is able to prevent the formation of antibodies that are able to clear the virus from an infected host. The data presented here delineate one potential mechanism for B cell population defects associated with SIV and HIV infection. This study demonstrates a need for increased understanding of the naïve B cell population during SIV and HIV infection. By fully understanding how SIV infection alters naïve B cell function, novel therapeutic targets can be identified.

4.6 ACKNOWLEDGEMENTS

The authors would like to thank Brian Healy for his critical review of the manuscript and his help with the statistical analyses contained herein. We would also like to thank Lisa Borghesi for

her insight and guidance in designing experiments for this manuscript. This work was supported by the Center for Vaccine Research at the University of Pittsburgh School of Medicine and NIH R01 AI52058 (Cole).

5.0 GLOBAL DISCUSSION

5.1 OVERALL FINDINGS

In this study it was established that surface expression of CD27 on CD20+ cells in the rhesus macaque corresponds with phenotypic and molecular characteristics consistent with a memory B cell population. In addition, we phenotypically and functionally analyzed naïve and memory B cells during the acute and chronic phases of SIV infection. Further, we demonstrated that B cell perturbations in the rhesus macaque induced by SIV infection occur in the naïve as well as the memory B cell population and that SIV induced alterations are apparent within days following infection. Longitudinal phenotypic data demonstrated that all B cell subsets examined are significantly depleted from the periphery during acute infection and that the recovery of the naïve B cell population did not mirror the recovery in the memory B cell population. In addition, it was demonstrated that the survival of naïve B cells in response to CD40 ligation is altered during chronic infection. Taken together, these data provide evidence that the SIV/macaque model is a highly valuable tool for understanding the role of B cells in HIV infection in humans and that B cell alterations following infection, which are most often attributed to dysfunction of memory B cells, are exacerbated by phenotypic and functional abnormalities present within the naïve B cell compartment.

5.2 ESTABLISHMENT OF CD27 AS A MEMORY MARKER IN RHESUS MACAQUES

A clearer understanding of B cell populations in the rhesus macaque was gained from the establishment in this study that the expression of CD27 on the surface of CD20+ B cells in the

rhesus macaque marks a population that is consistent with memory B cells. Parsing of naïve and memory cells provides a succinct picture of specific B cell subsets during pathogenic infection, and how they respond differentially during the acute versus chronic phases. It is important to note that although CD27 expression can be used to differentiate memory cells in both humans and rhesus macaques, the pattern and intensity of staining is not identical between the species. In rhesus macaques, careful parsing of CD27 stained cells using the myeloid cell population to set the gating parameters is necessary to differentiate between CD27+ and CD27- cells, while these populations are readily identifiable in similar staining of human B cells (76, 162). With this understanding, our data indicate that B cell populations are phenotypically and functionally similar between rhesus macaques and humans, and that findings from rhesus studies will likely translate to human experiments. Critical analysis of phenotypic and functional changes in B cell subsets in rhesus macaques during all phases of SIV infection will have improved relevance to the human model due to improved insight into naïve and memory B cells afforded by this study.

Our study also establishes an essential framework within which the activity of naïve and memory B cells can be studied *in vivo* as a key step in improving vaccine therapy. The template we provide will be useful in further parsing of the B cell compartment. In addition to naïve and memory cells, parsing quiescent memory B cells and plasma cells would provide additional information on the B cell population in pathogenic models of infection. Thus far, little is known phenotypically about quiescent memory B cells due to multiple experimental hurdles that must be overcome to study them even in the mouse model. Numerous iterations of adoptive transfer studies into irradiated mice have begun to shed light on this population and has to date, verified its existence and demonstrated interplay between the quiescent memory B cell population and plasma cells (204-206). Long-term vaccination studies have demonstrated that B cell memory

populations are capable of mounting substantial antibody responses decades following primary inoculation (81, 82), and it may be useful to specifically generate these quiescent memory B cell populations in SIV challenged rhesus macaques. To further analyze memory B cell populations in the SIV/macaque model, B cells from animals vaccinated with an antigen such as influenza could be strategically sorted based on expression of CD27 and activation markers, for example, CD20+CD27+ cells with high CD95 expression versus CD20+CD27+ cells with low CD95 expression, and then tested for antigen reactivity using an influenza hemagglutinin and neuraminidase protein targeting ELISPOT system. Analyzing which sorted population is able to respond with a strong anemnestic response, and comparing these responses back to uninfected animals could aid in the understanding of the phenotypic characteristics of these cells. For plasma cells, CD138 has been shown as a marker of cells with a plasmacytic phenotype (207), but a recent study in humans has shown that as much as half of the plasma cell population do not express surface CD138 (83). Characterization of B cells with additional markers including CD10 and CD38 as have been used in the human system to differentiate additional B cell populations including plasma cells and utilization of these surface markers would improve our current abilities to parse B cell populations in the rhesus macaque. With the improved characterization of B cell subsets, careful comparison of B cells in SIV infected and uninfected animals will aid in our understanding of why these cells act differently during SIV infection and will improve the design of therapeutic strategies targeting B cell dysfunction.

5.3 PHENOTYPIC ALTERATIONS OF NAÏVE AND MEMORY B CELLS DURING ACUTE AND EARLY CHRONIC SIV INFECTION

In this study, significant depletions of naïve and memory B cell populations from the periphery were observed within 2 weeks of SIV infection and the depletion within the naïve population was unexpected due to the fact that memory B cell depletion is a primary focus in human HIV studies (5, 8, 84, 95, 101, 189). These B cell depletions occurred during the same time frame as previously reported CD4 T cell depletions (20), indicating that acute infection is a time of profound activity within the T and B cell arms of the adaptive immune system. What is perplexing is that the B cell numbers were affected to a greater extent than CD4 T cells. A key difference between populations, specifically that CD4 T cells express CD4 and are viral targets and that B cells do not express CD4 and are unlikely to be direct viral targets, suggests that the mechanism responsible for the loss of CD4 T cells and the loss of B cells during acute infection was not the same for each population. Additional analysis demonstrated that the repopulation of memory B cells in the periphery was not mirrored in the repopulation of naïve B cells, providing evidence that the mechanism responsible for controlling naïve and memory B cells during SIV infection is also different.

The depletion of B cells from the periphery during acute SIV infection was most likely due to either cell death or redistribution to tissues. Conflicting evidence from the literature makes it difficult to predict whether death or redistribution is favored. Since B cells are not directly infected by HIV or SIV, the mechanism by which peripheral blood B cells are depleted is not apparent. Redistribution of B cells would likely result in trafficking to the lymph nodes. Although lymphadenopathy has long been viewed as a symptom of acute HIV infection (208), events occurring within the lymph node following infection have not been fully characterized.

Lymphadenopathy suggests that there is an increase in cells in the lymph node but studies in the SIV/macaque model have demonstrated depletions of B cells from lymph node germinal centers during acute infection (185). Parallel studies of peripheral blood and lymph nodes during acute SIV infection would provide a clear picture of how B cells traffic between the compartments during infection and absolute numbers of cells in the blood and lymph nodes would be needed in order to make strong comparisons. To clearly demonstrate that trafficking from the periphery to the lymph node is occurring, as opposed to increased proliferation within the lymph node leading to greater numbers of cells, the proliferation marker Ki67 would be utilized to assess whether proliferation increases during acute infection. Augmenting these studies would be analysis of the apoptotic state of the B cell population during acute infection by measuring annexin V binding and molecular assessment of caspase activity. Together these additional studies would better define the roles of apoptosis and redistribution in depleting B cell populations from the periphery during acute SIV infection.

Expansion of B cell studies to include tissue from the gut would also improve our understanding of B cell populations during HIV and SIV infection. As a location of T cell population destruction, especially during early infection (34), there is a high probability that B cell disruption would be found in the gut. Gut biopsies and analyses of IgA producing B cells during SIV infection would show if B cells are depleted from the gut during acute infection and would demonstrate whether B cell functionality in the gut is maintained. B cells that reside within the gut are key components of the mucosal immune system and represent a population that is likely susceptible to depletion during HIV or SIV infection.

The current findings, that B cells are depleted from the periphery of SIV infected animals very early in acute infection, are likely to be reflected in human HIV infection. Although studies

of primary HIV infection are limited, at least one study has shown that patients with known HIV exposure in the previous 3 months and symptoms of acute retroviral syndrome have significant depletions of total B cells, an increased expression of activation markers including CD95 on naïve and memory B cells and hypergammaglobulinaemia (174). An additional study of peripheral B cells utilizing a large cohort of individuals who are at risk of infection would be needed in order to confirm that the naïve B cell population is depleted within 2 weeks of initial infection and recovers more slowly than the memory B cell population as demonstrated in our rhesus macaque study. Despite the potential to be able to translate the current non-human primate model findings to humans, more study of the non-human primate model is also warranted. The current understanding of naïve and memory B cell depletion and recovery from the periphery is based solely on i.v. inoculation of the virus, which required one large single dose, 10,000 infectious units, for infection. Expansion of the current model to include low dose repeated exposure, for at least 13 weeks, 2 inoculations per week at 10^3 50% tissue culture infective doses vaginally, would more closely resemble the type of infection associated with sexual transmission of HIV between humans (209). This type of infection regimen would demonstrate whether the same pattern of B cell depletion occurs when virus is not directly introduced into the blood stream.

5.4 DETRIMENTAL EFFECT OF DISRUPTION OF THE INITIAL ANTIBODY RESPONSE TO SIV INFECTION

The loss of B cells from the periphery during acute SIV infection likely plays a role in the inability of B cells to form neutralizing antibodies needed to clear HIV or SIV. Vaccination studies in the non-human primate model have demonstrated that an antibody mediated immune

response can prevent SIV infection (105-115), however, antibodies are only effective when the vaccine strategy used to generate the antibody response utilizes proteins that are homologous to the viral strain used to infect. This suggests that with the proper survival, activation, proliferation and maturation, B cells have the potential to control virus. The virus infection itself appears to play a key role in the prevention of the formation of a robust, highly efficacious immune response. The early loss of B cells may be a mechanism through which HIV and SIV are able to overcome the humoral arm of the immune system.

Because of the ability of HIV or SIV to mutate, a slow initial response to infection results in an increase in the number of quasispecies present in infected hosts. The variation between viral strains within a host ensure that even if an antibody is formed, it is likely specific for an epitope that is not shared by all viral strains present. The fact that the virus is able to change envelope protein structure during infection dictates that the antibody response must be able to evolve as well. With the initial depletion of B cells from the periphery, the antibody-mediated recognition of extracellular virus would be slow to form. Indeed, the slow formation of the primary antibody response to HIV and SIV has been documented (7). In SIV infection, B cell depletion is already detectable by day 7 and does not reach a nadir until day 14 (23). In acute viral infections such as influenza, the antibody response is established within 7 days of infection (8) indicating that the initial antibody response to SIV would be formed over the time period when viral loads are very high. High viral loads increase the probability that multiple variants will be present, making direction of antibodies against a predominant strain more difficult. In effect, the mutation driven evolution of envelope proteins is established prior to the maturation of the primary antibody response and any additional viral mediated slowing of B cell maturation after the primary response could make recognition of newly emerging epitopes less effective. In

this context, increasing the time it takes for naïve B cells to mature could be enough to prevent the formation of high quality antibodies. As further evidence of the inability of B cells to form efficacious antibodies during early infection, a B cell depletion study of pathogenic SIV infection has demonstrated that there is no difference in viral load and CD4 T cell loss between animals that have had their B cells depleted with anti-CD20 antibody and those that have not during the acute phase of infection (69). Interestingly, a similar study in a non-pathogenic model demonstrated that viral loads were controlled and CD4 T cell levels were maintained in the absence of B cells (104). Two short-term studies are necessary to gain a better understanding of how the acute cell loss affects the B cell population. First, naïve and memory B cells need to be phenotypically analyzed in an acute infection to clarify what happens to these cells during a B cell mediated immune response that results in viral clearance. A short-term infection, such as influenza H1N1 could be used. B cell populations could be analyzed pre-infection and at days 2, 4, 6, 8 and 10, to assess whether acute B cell depletion, seen in SIV infection, is associated with a functional antibody mediated response. The pattern of B cell distribution in the influenza model could then be compared to the SIV model. This experiment should demonstrate aspects of B cell activity, such as precise timing of trafficking and proliferation that are incorporated in an effective B cell response. With the analysis of differences between infections, SIV induced alterations in the initial B cell response can be identified and potentially abrogated in additional acute SIV trials. Second, a functional study assessing the ability of the B cells that are present during the acute phase of SIV infection to proliferate and mature, is necessary to pinpoint longitudinally whether B cell proliferation, activation and maturation occurs with normal kinetics. Since chronic infection is associated with dysfunction in these B cell processes, a longitudinal assessment will pinpoint when changes in function first arise. Following infection

with SIV, peripheral cells would be collected at days 3, 7 and 14 post infection and assessed for their ability to respond to B cell stimulation such as BCR and CD40 signaling. A finding of altered B cell functionality during acute infection will indicate that conditions within the host during acute infection preclude the normal functionality of B cells and that B cell dysfunction arises soon after infection and is not due to prolonged infection. A lack of B cell functional changes would indicate that the population itself is intact during this phase of infection and further studies could be initiated to assess how long the B cell compartment could be functionally maintained with early antiretroviral intervention. Proliferation by BrdU staining and protection from apoptosis could be analyzed longitudinally and compared to pre-infection results.

The early loss of multiple B cell populations during the acute stages of both HIV and SIV infection supports the hypothesis that virus directly induces disruption during the initial stage of infection. A major argument for how HIV is capable of devastating the immune system is through the slow progression of a constitutively active chronic infection that eventually results in immune exhaustion and an inability to recognize and clear pathogens (196). This theory is based on the fact that there is not a complete loss of immune functionality from any single compartment but multiple small effects of viral infection that slowly result in loss of immune control. In analyzing the phenotypic alterations in the B cells there is substantial evidence against this theory. Most strikingly, the alteration within the B cell population is drastic and it occurs within days of infection. And, although the cells do rebound in the periphery and are able to produce antibody, because virus is never cleared, it appears that after infection they are never able to form antibody responses with the same efficacy possible before infection. So, despite the fact that the ultimate demise of the immune system may take years to occur, a profound immune

disruption, either virally directed or induced by another mechanism, occurs at the initial stage of infection. While the loss of CD4 T cells during primary infection has been well established, the greater magnitude and severity of the B cell loss combined with the T cell loss bolster the hypothesis that these depletions are major events that will have lasting effects over the course of infection.

5.5 FUNCTIONAL ALTERATIONS OF NAÏVE B CELLS FROM SIV INFECTED RHESUS MACAQUES IN RESPONSE TO CD40 LIGATION

In the current study it was demonstrated that naïve B cells from SIV infected animals were insensitive to CD40 mediated survival. The interaction between the CD40 receptor expressed on B cells and CD40 ligand (CD154), which is upregulated on activated T cells (210), provides multiple signals capable of inducing specific functional changes in both the B and T cells involved in the interaction (166). The importance of this interaction is clear from analysis of patients that lack either CD40 or CD154, who are unable to produce immunoglobulin of any isotype other than IgM, and are unable to mount robust T cell dependent immune responses to numerous pathogens (202). In the current study, a large proportion of naïve B cells from uninfected animals spontaneously underwent apoptosis over 24 hours unless treated with anti-CD40 antibody. In SIV infected animals, no loss of cells was observed after 24 hours in either treated or untreated groups, indicating that the cells were insensitive to CD40 ligation under the conditions tested. Interestingly, naïve B cells from SIV infected animals, like naïve B cells from uninfected animals were able to upregulate CD95 surface expression following CD40 ligation. In addition, freshly isolated naïve B cells from SIV infected animals were significantly more apoptotic compared to naïve B cells from uninfected animals, which was paradoxical considering

the fact that there was very naïve B little cell loss following 24 hour culturing of these cells under either experimental condition. These data indicated that although CD40 mediated survival was impaired in naïve cells from SIV infected animals, the same cells were still able to respond to CD40 ligation and at least a portion of the CD40 signaling pathway remained intact.

As the building block from which all B cell mediated antibody responses arise, the naïve B cell population is a critical immunological component. The consequences of poorly controlled maturation range from the benign, production of irrelevant antibodies that are unable to bind antigen (5, 136, 174, 175, 189, 211, 212), to extremely harmful, production of self-reactive antibodies whose binding results in damage to the host (180, 213, 214). We hypothesize that HIV and SIV induced disruptions of the naïve B cell population prior to or during antibody maturation and production are responsible for the poor antibody mediated immune response to the virus and to opportunistic pathogens. Memory B cell dysfunctions associated with chronic HIV infection could reflect altered survival and proliferation within the naïve B cell compartment. Poor negative selection of non-reactive naïve B cells, or unregulated positive selection of naïve B cells could lead to the overproduction of non-specific antibodies associated with hypergammaglobulinaemia. Maintenance of the memory B cell population requires repopulation through the maturation of naïve B cells. Blockade of naïve B cell maturation would result in fewer B cells becoming memory cells, and the reduction of memory cells observed during chronic HIV infection.

The CD40 experiments described in the current study provide a basis for numerous lines of inquiry. Clearly demonstrated from the current study was the fact that SIV distorts responses to CD40 ligation within naïve B cells. The next step in analyzing the naïve B cell population would be to compare gene expression between sorted naïve cells from SIV infected and

uninfected animals. Genes of interest include those involved with the apoptosis cascade including caspases 2, 3, 6, 7, 9 and 10 and genes involved in the CD40 signaling cascade such as TRAF 1, TRAF 3, STAT 3 and NF-κB. These analyses will provide gene targets that can be experimentally manipulated in future studies to potentially reverse detrimental SIV induced effects on the naïve B cell population. siRNA knockdown of genes found to be upregulated by SIV infection or drug therapy to block the effects of SIV on B cells could be utilized to mitigate disruption within the B cell population. Such an approach has been used to control the generation of antibodies in B cells in a cancer cell line (215).

In addition to gene array analysis, further study of the CD40 mediated activation described in this study is warranted. Utilizing a more sensitive proliferation measurement such as BrdU incorporation would provide a clearer picture of how CD40 ligation alters the naïve B cell population. Also, CD40 stimulation alone is not generally thought of as physiologically relevant as CD40 stimulation in conjunction with other signals including B cell receptor binding. The current studies in uninfected and SIV infected animals could be augmented by the addition of BCR stimulation in addition to stimulating cytokines including IL-2, IL-4 and IL-10 to assess whether these molecules are capable of increasing the activation profiles of these cells. These stimuli are chosen because they have been proven to induce proliferation and maturation in B cells (71, 72, 77). B cells can be activated through BCR and CD40 stimulation and either IL-2, IL-4 or IL-10 signaling and each combination should be thoroughly investigated. As the downstream result of B cell activation and proliferation, antibody production is a key component that should also be assessed in these experiments. Total antibody production should be assessed in addition to SIV specific antibody. Both ELISA for total antibody produced and ELISPOT for antibody production on a per cell basis would provide excellent data. Multiple envelope proteins

could be utilized in ELISA and ELISPOT assays to assess the specificity and breadth of the antibody response.

The enumeration of a distinct functional abnormality in the naïve B cell population is interesting because HIV induced B cell abnormalities in humans have been most commonly associated with memory B cell maturation, differentiation and proliferation. The discovery that the percentage of memory B cells is reduced in HIV infected persons (95) gave valuable information to the research community, and knowledge about naïve B cell defects is likely to be equally informative. The lack of memory B cells does not necessarily imply that the memory B cell population is the source of B cell dysfunction during HIV infection, but it has been used as justification for further analysis of memory B cell populations in numerous studies. The studies reported here and at least one other (102) are driving the search for the mechanism of B cell dysfunction toward the naïve B cell population. These studies demonstrate that it is increasingly likely that the B cell population dysfunction is established prior to the formation of memory B cells.

In order to definitively test whether B cell dysfunction is predominant in the naïve or canonical memory population, B cell memory to a non-SIV antigen would need to be established prior to SIV infection. To establish a long-lived functional memory population, animals should be vaccinated with a non-SIV antigen such as influenza prior to infection with SIV. A phenotypic analysis of the naïve and memory B cell populations in these animals would be used to set a baseline for how B cell population numbers are altered in the animals in response to the vaccine in the absence of SIV infection. This could be compared to a second set of animals that was vaccinated with influenza after SIV infection. Given the findings presented here, we predict that the vaccine response will be more robust in the uninfected animals but that the protection

mediated by the vaccine will be lost upon SIV infection, due to the loss of memory B cells associated with acute infection. The comparison would show how naïve B cells respond to the vaccine under each condition and would show if the response was altered, slowed or less robust in SIV infected animals. After SIV infection of the first group, the anemnestic response to influenza antigens could be assessed using ELISA and ELISPOT assays, and influenza proteins could be utilized in conjunction with CD40 to activate B cells for assessment for activation and proliferation following stimulation.

5.6 PROPOSED MODEL

Infection of rhesus macaques with SIV results in the formation of antibody mediated B cell responses that are less robust and less durable than analogous responses from uninfected animals. Data from the current study demonstrated that phenotypic alterations occur in the naïve and memory compartment during acute and chronic infection and that functional responses to CD40 ligation are altered during chronic infection. Together, these data indicate that SIV induced alterations within the B cell compartment occur very rapidly following infection, and that B cell alterations are inherent in the population itself and not the result of T cell losses alone. Based on the data obtained in this study, we believe that one mechanism for the lack of strong B cell responses lies in the abnormal selection of B cell clones during SIV infection. Under normal circumstances in a T cell mediated immune response, naïve B cells have their fate dictated by their ability to bind antigen and the presence of signals from T cells. Cells that bind antigen and receive survival signals are able to mature and proliferate in a germinal center. Cells that do not bind cognate antigen are negatively selected for depletion through spontaneous apoptosis. Additional signals from T cells and antigen drive the mature B cells to increase antibody

specificity and become memory and plasma cells. In the case of B cells from SIV infected animals, our data suggest that a possible mechanism for negative selection of naïve B cells is lost. In healthy animals, ligation of CD40 helps protect naïve B cells from depletion, however, in SIV infected animals CD40 ligation does not exhibit a protective effect as assessed *in vitro*. Based on our in vitro studies from the periphery, we hypothesize that that this less stringent selection ability within germinal center reactions will induce the production of less efficacious B cell clones. Combined with a higher level of spontaneous apoptosis in the naïve and memory B cell populations from SIV infected animals, the cells that are able to mature will likely not form long term memory populations. It is this lack of selection in B cells from SIV infected animals that drives the functional issues within the humoral immune compartment (figure 28).

Although it is not possible to isolate and therefore test for dysfunction in long-term memory B cell populations with the current technology, hints as to the extent of dysfunction within these populations can be gleaned from experiments with the currently resolvable B cell populations. Early depletions of measurable B cell populations from the periphery during acute infection indicate that the production of true memory B cell populations would be slowed. Having fewer naïve and memory cells available means that fewer cells would be able to mature and that quiescent memory and plasma cells would be selected from a smaller pool of cells, increasing the likelihood that these cells would not be matured to their full potential. In addition to acute losses, altered communication between B and T cell populations indicated by compromised CD40 signaling would likely lead to the maturation of cells that were unable to respond to all the signals that are available for properly directing the production of highly effective antibodies. This could lead to fewer cells being matured, effectively decreasing the true

memory B cell populations, or could lead to the maturation of true memory cells with questionable efficacy.

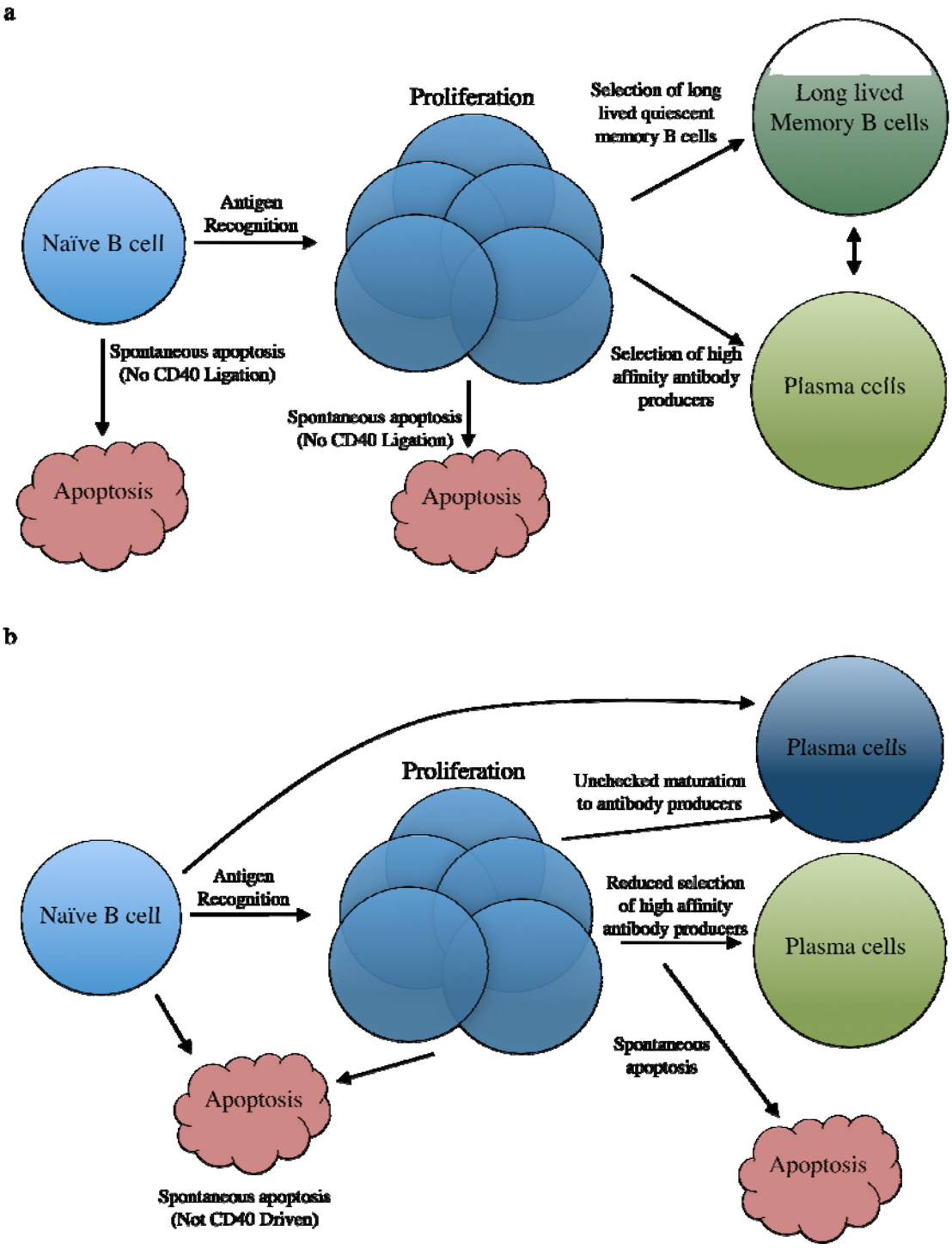

Figure 28. Proposed mechanism for dysfunctional B cell maturation during HIV infection.

Mechanism of disruption of B cell maturation. B cell maturation in (a) uninfected animals results in the production of high affinity antibody producing cells. B cell maturation in (b) SIV infected animals lacks the CD40 selective pressure found in uninfected animals and results in the production of less efficacious antibody producing cells.

5.7 CONCLUSION

This study demonstrated that naïve and memory B cell numbers are depleted from the periphery within days of SIV infection and that naïve B cells from SIV infected animals are unable to respond to CD40 mediated survival signals. These findings extend our current understanding of B cells in the context of SIV infection by demonstrating that phenotypic and functional deficiencies are apparent in the naïve B cell population. It is highly likely that the deficiencies that arise in the naïve B cell population are linked to, or are in fact responsible for dysfunction that has been described within the memory B cell compartment. Further analysis of the naïve B cell population during HIV and SIV infection will likely illuminate clear targets for therapeutic strategies designed to augment the production of effective antibody responses to HIV or opportunistic pathogens.

APPENDIX A

ADDITIONAL METHODS

PBMC isolation

Numerous procedures in this dissertation utilized cell separation by either Percoll or Lymphocyte separation medium. We utilized Percoll separations fairly successfully until we started in on B cell activation experiments. For those it appeared that the Lymphocyte Separation Medium from Mediatech worked better.

The basic procedure using either method is the same. Fifty ml conical tubes that were used for the separation were loaded with 15ml of separation buffer. Approximately 10ml, 1 tube full of blood was obtained from a vacutainer tube (either citrate treated or heparin treated worked well) and diluted to a final volume of 30ml with Hank's Balanced Salt Solution (HBSS). The blood was carefully overlayed upon the separation medium. Tubes were then spun in a centrifuge at 2500rpm for 25 minutes at 25°C. After the spin, the lighter colored top portion of the overlay was aspirated from the tube to within 5ml of the visible buffy coat and discarded. Then a 10ml pipette was used to carefully remove the buffy coat. The buffy coat was transferred to a clean 50 ml conical tube and HBSS was added to the tube to the 40ml line. These cells were spun down at 1700rpm for 7 minutes, the supernatant was poured off, and the cells were resuspended in 40ml of HBSS. After the first wash, multiple tubes (up to 6) from the same animal could be combined. Cells were then spun down once again at 1700rpm for 7 minutes, resuspended and counted. These cells were then ready for staining, sorting or functional assays.

Percoll

The percoll was prepared by mixing 53% percoll, 37% sterilized water and 10% 10X HBSS. The percoll was always prepared the day of the cell isolation, but could be made a few hours before it was needed. Importantly, the percoll needed to be a room temperature when the blood was overlayed.

Lymphocyte separation medium

Mediatech's lymphocyte separation medium worked very well and was extremely easy to use. The medium itself does not need to be refrigerated so was always ready to be used. An added bonus is the fact that lymphocyte separation medium does not need to be diluted and was just added directly to the separation tube.

Flow Protocol

Staining

The staining procedures used in the completion of this dissertation project were almost exclusively used to separate B cell populations in the periphery of rhesus macaques, but will work to stain other cell populations in the human or rhesus macaque models. Prior to staining, cells were purified from red blood cells (RBC) either by cell separation as described above or using RBC lysis. The advantage of RBC lysis is that it is much faster than using cell separation. In cell separation, the cells are isolated first and then stained, while in the lysis protocol, whole blood samples are stained and then the RBC are lysed.

RBC lysis

Lymphocyte populations were stained in whole blood using a RBC lysis protocol. Two hundred µl (can use as little as 100µl and still get reasonable results) of whole blood (ACD treated or heparinized blood both work) need to be used within 12 hours of draw for best results. Flow antibodies were added directly to the blood and incubated for 15 minutes at room temperature in the dark. Following incubation, 3ml of ACK buffer was added to the sample and incubated for 10 minutes. Samples were then centrifuged at 1700 rpm for 7 minutes. Following centrifugation, samples were washed twice by decanting supernatant, adding 3ml of dPBS and centrifuging at 1700rpm for 7 minutes.

Staining Protocol with Live/Dead differentiation

When staining whole blood or separated cells, 3µl of each directly conjugated antibody and 1µl of biotinylated antibody were added per tube. For IgD, the biotinylated antibody worked the best, the signal was difficult to detect with directly conjugated antibodies. The standard B cell tube was CD20 (pacific blue), CD27 (PE), CD95 (FITC), IgD (pacific orange) and Live/Dead stain (near IR) (Table 11). Following a 15 minute incubation at room temperature in the dark, cells were washed with Facs buffer and spun down. Cells were then resuspended in a 1:1000 dilution of Live/Dead stain and incubated for 10 minutes in the dark. Following incubation, cells were washed twice with Facs buffer and resuspended in 1% paraformaldehyde in PBS and run on an LSRII.

Table 11. Antibody Panel.

Antibody	Fluorochrome	Clone	Vendor
CD20	Pacific Blue	2H7	eBioscience
CD27	PE	M-T271	BD Bioscience
CD40	None	5C3	BD Bioscience
CD86	PE-Cy5	IT2.2	eBioscience
CD95	FITC	DX2	BD Bioscience
IgD	Biotin (Pac Orange SA)	Polyclonal	Southern Biotech
Live/Dead	Near IR		Invitrogen

Population differentiation

All data were obtained on an LSRII and were analyzed using FlowJo. As shown in figure 29, side scatter and forward scatter characteristics were used to differentiate lymphocyte and myeloid cell populations. The lymphocyte population is smaller in size and has less granularity than the myeloid population. The lymphocytes are analyzed through a viability gate to ensure that the cells analyzed after this point are all alive. CD20+ B cells are then selected from this live cell population and this population is segregated based on CD27 expression using the myeloid cells to set the gate. Myeloid cells are mostly negative for CD27 so it is easier to see the break between populations using these cells. Following CD27 differentiation, the CD20+CD27+ cells can be differentiated based on IgD expression. In most cases, a definitive split will be seen between IgD+ and IgD- samples. If it is difficult to see the split, the CD20+CD27- B cell population can be used as a guide to set the IgD gate due to the fact that the vast majority of CD20+CD27- B cells are IgD+.

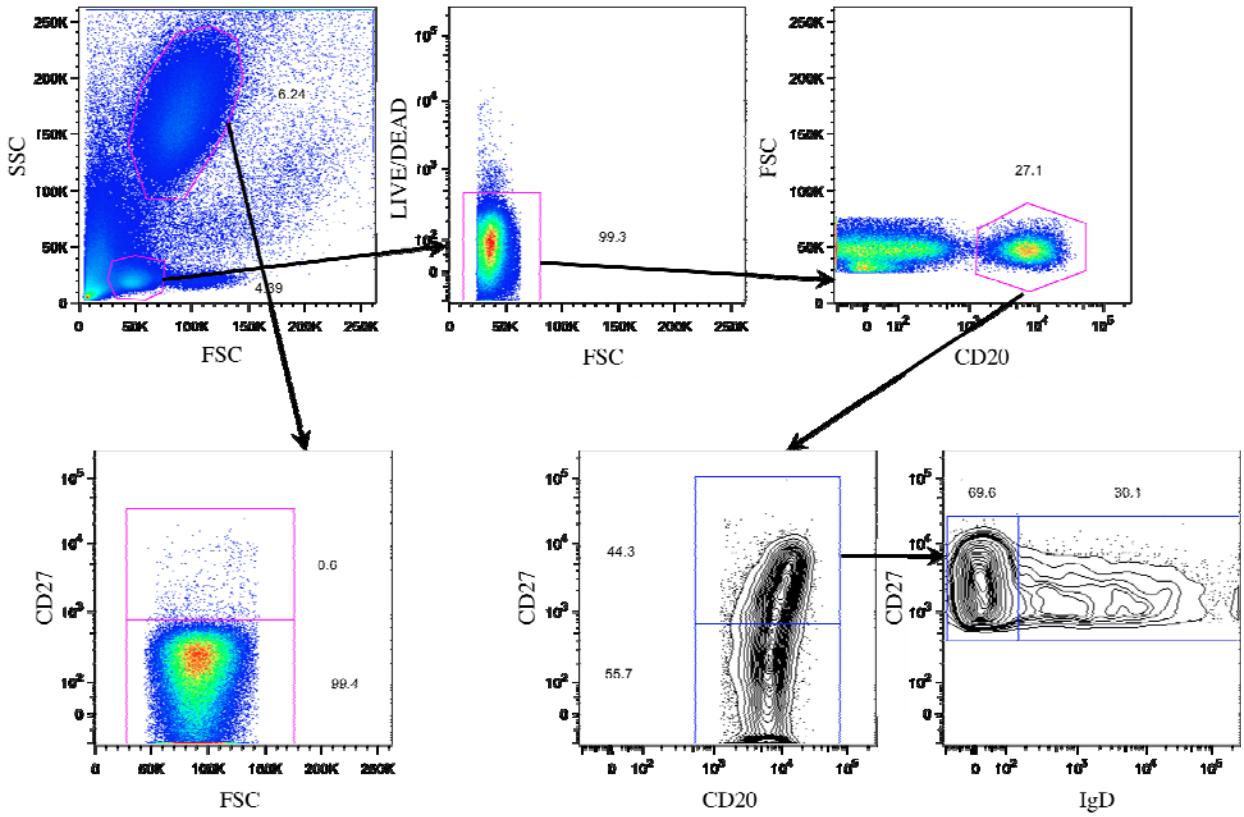

Figure 29. Gating strategy for differentiating B cell populations.

Sorting Protocol

In these studies cells were sorted based on expression of CD4, CD20 and CD27. PBMC were isolated from at least 20ml of whole blood using lymphocyte separation medium prior to staining. After separation, cells were resuspended in 2ml sterile dPBS with 4% FBS (sort buffer). Because of the high number of cells present, 10µl of each antibody were added to each sample. Following 15 minute incubation, the cells were washed twice with sort buffer and then resuspended in at least 2ml of sort buffer. Compensation beads were used to set up the FACSAria prior to the sort. Cells were sorted into collection tubes containing FBS.

CD40 ligation: Induction of naïve B cell activation and protection from apoptosis.

In this dissertation a new protocol was devised in order to assess the activity of B cell populations in normal and SIV infected rhesus macaques. Anti-CD40 antibody treatment was used as an agonist to CD40 on B cells and cell activation and protection from apoptosis were analyzed following treatment. Cells from uninfected and SIV infected animals were both treated the same way. PBMC were separated from RBC using the separation technique described above over lymphocyte separation medium. One million cells were plated into a singe well of a 24 well plate in RPMI. Samples were run in duplicate and half were treated with the anti-CD40 antibody and the other half were left untreated. Following 24 hour incubation, the cells were stained with B cell and activation marker antibodies as well as annexin V and analyzed on an LSRII. Clear annexin V stains were only achievable if the cells were kept ice cold following the addition of annexin V and they were run on the LSRII as soon as possible following fixation.

CD95 Ligation: Induction of apoptosis

Prior to discovering B cell selection alterations following CD40 ligation, we attempted to induce B cell apoptosis through ligation of CD95. We successfully induced apoptosis in Jurkat cells (figure 31) but were unable to induce apoptosis in B cells. PBMC were isolated over percoll gradients and plated at 1x106 cells/ml in a 24 well plate. Anti-CD95 antibody was added to the experimental wells and all cultures were incubated for 24 hours. A time course (6, 12 and 24 hours) was analyzed in the B cell population and no apoptosis was observed.

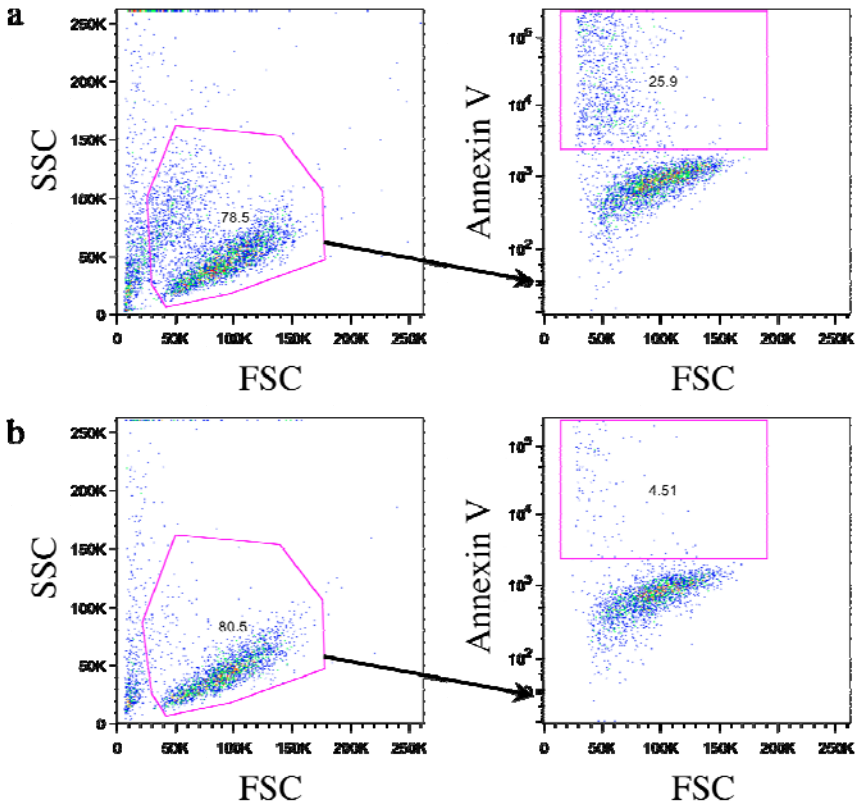

Figure 30. CD95 induced apoptosis in Jurkat cells.

Apoptosis was induced in Jurkat cells through the ligation of CD95 after 24 hours in culture. (a) Jurkat cells treated with anti-CD95 antibody and (b) untreated Jurkat cells.

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