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Rachael D. Aubert Date

Interplay between T and B cell responses during chronic viral infection

By

Rachael Dwyer Aubert
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

Rafi Ahmed
Advisor

Rama Amara
Committee Member

Christian Larsen
Committee Member

Aron Lukacher
Committee Member

Ifor Williams
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

Date

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By

Rachael Dwyer Aubert
B.S., University of Wisconsin-Madison, 2000

Advisor: Rafi Ahmed, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
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2009

Abstract

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The adaptive immune response has evolved multiple mechanisms for protection against invading pathogens. CD8 T cells mediate protection against intracellular pathogens such as viruses by recognizing and killing infected cells. Meanwhile, the B cell response provides antibody at sites of pathogen entry to opsonize or neutralize invading pathogens. Additionally, CD4 T cell responses are important for activating CD8 T cell and B cell responses. Although the role of CD4 T cells in initiating B cell responses is well established, less is understood about how CD4 T cells initiate or sustain CD8 T cell responses. This question has become increasingly important as we try to understand why CD8 T cell responses fail during chronic viral infections such as HIV, HBV and HCV. In these chronic infections there is often a lack of a CD4 T cell response, and this parallels functional exhaustion of the CD8 T cells. To better understand the interplay between CD4 T cell responses and CD8 T cell exhaustion we examined the effects of altering CD4 T cell and B cell responses during chronic LCMV infection of mice. We first examined the question of how transient removal of CD4 T cell help affects long-term CD8 T cell responses. Secondly, we determined whether improving CD4 T cell help would affect CD8 T cell exhaustion. A transient one-week blockade of CD4 T cell help via administration of αCD40L blocking antibody beginning at two weeks post infection had no direct effect on the function of CD8 T cells but resulted in reduced CD4 T cell function and decreased levels of humoral immunity. This decreased CD4 T cell response then resulted in greater CD8 T cell exhaustion and impaired viral clearance. On the other hand, adoptive transfer of virus specific CD4 T cells during chronic LCMV infection restored function in exhausted CD8 T cells and also enhanced antibody responses. This rescue was observed for several CD8 T cell epitopes, was synergized by αPD-L1 treatment, and resulted in improved viral control in treated animals. Thus, these studies provide insights into how CD4 T cells and B cells can affect CD8 T cell programming and how one might design therapies to combat chronic infections in humans.

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Chapter 1: Introduction

The adaptive immune response is critical for the effective clearance of invading pathogens as well as for providing immunological memory upon secondary challenge. In the cases of chronic or persisting infections, the immune response must also regulate itself to prevent tissue destruction and immunopathology. During the adaptive immune response, both cellular and humoral responses play important roles in providing immune effector function. Humoral responses are focused on antibody production, which can neutralize or opsonize free pathogens, while cellular immune responses are essential for the killing of intracellular pathogens. Both CD8 T cell and humoral responses are regulated by CD4 T cells, which provide antigen-specific signals to drive B cell and CD8 T cell proliferation and effector function. Antibody responses have been thought to be important for preventing viral infection, and long-lived neutralizing antibody is a hallmark of our most successful vaccines. More recent vaccine strategies for chronic viral infections have focused on raising strong CD8 T cell responses to kill infected cells. However, high levels of antigen can result in CD8 T cell exhaustion. Although down-regulation of the immune response is essential to prevent immunopathology, it may also prevent the successful clearance of chronic infections. Understanding the interplay between the different parts of adaptive immune response to regulate antigen load and alter CD8 T cell exhaustion will help us design vaccines and immunotherapy strategies to combat persistent viral infections.

Part I: Regulation of immune responses during chronic viral infection

LCMV as a model to study chronic viral infection

Lymphocytic choriomeningitis virus (LCMV) is a member of the *Arenaviridae* family of viruses. LCMV contains a bi-segmented single-stranded ambisense RNA genome. The S segment (3.4kb) of the genome encodes the nucleocapsid protein and the viral glycoprotein GP-C. The L RNA segment (7.2kb) contains the polymerase gene and the zinc-binding (Z) protein(1). Post-translational modification of the GP-C precursor yields the structural GP-1 and GP-2 proteins, which are assembled into tetrameric spikes on the virus surface(2). The GP-1 protein makes up the globular head containing the viral receptor binding domain and is the target of neutralizing antibody(3-5); while the GP-2 protein makes up the transmembrane stalk and contains the sites required for acid-dependent membrane fusion during viral entry(6). LCMV, as well as several other arenavirus family members, uses the ubiquitously expressed cell surface molecule α-dystroglycan for viral entry(3, 7). Differences in binding affinity of various strains of LCMV for α-dystroglycan has been correlated with changes in tissue tropism and disease outcome, high affinity binding leads to higher levels of replication, increased infection of antigen-presenting cells, and overall higher levels of immunosupression. Less pathogenic strains of LCMV are more likely to use alternative receptors for cell entry, but these receptors remain less well characterized(8, 9).

LCMV serves as an important tool for studying host-virus interactions as it is a naturally occurring pathogen in mice, and infection using different routes or viral strains

can result in differing disease outcomes. Peripheral inoculation of adult mice with the acutely replicating strain LCMV Armstrong induces a potent anti-viral CD8 T cell response that is capable of clearing virus within 5-7 days. However, intracranial inoculation of this same strain leads to CD8 T cell mediated fatal meningitis. Furthermore, mice infected in utero become lifelong carriers of virus without any pathological outcome, due to central tolerance within the thymus(10). Viruses isolated from the tissues of persistently infected mice have been shown to undergo genetic selection and inoculation with these purified viruses results in changes in tissue tropism and can lead to protracted viremia and long-term persistence of virus within the tissues(11). One example is the clone-13 strain of LCMV, which was isolated from the spleen of a mouse persistently infected with LCMV Armstrong. Although it differs from the Armstrong strain by only 2 amino acids, neither within a T cell epitope, infection with this virus results in a protracted 2-3 month viremia and severe immunosuppression(11, 12). Comparing immune responses following Armstrong and clone-13 infection offers important insights into how differing levels of viral replication influence CD8 T cell expansion and exhaustion. Importantly, LCMV does not code for immunomodulatory molecules that alter MHC presentation often associated with large DNA viruses, making this a uniquely attractive system to study the interaction between viral replication and the immune system(13).

Immune responses to acute LCMV infection

Infection of adult mice with LCMV Armstrong results in a CD8 T cell response that is composed of three distinct stages: naive, effector and memory. Following LCMV infection, naïve antigen specific CD8 T cells encounter antigen on professional antigen-presenting cells and undergo a rapid expansion and differentiate into highly activated effector CD8 T cells(14). These effector cells extravasate to peripheral tissues and control the infection, often by killing infected cells and producing effector cytokines. Antigen-specific effector CD8 T cells reach a peak number at day 8, then contract over the next three weeks, with approximately 90-95% of the antigen specific CD8 T cells undergoing apoptosis. However, a subset of these cells further differentiates and persists long-term as resting memory CD8 T cells(15, 16).

Extensive work by our lab and others has focused on identifying the signals required to drive CD8 T cell proliferation and memory formation. It has been demonstrated that only a brief encounter with antigen (24 hours or less) directs the formation of memory CD8 T cells(17-20). Type-I interferon has been shown to be necessary for the expansion of LCMV-specific CD8 T cells, and plays a critical role in their survival during the expansion phase(21). Interestingly, the magnitude of this dependence is altered by the inflammatory milieu associated with a given pathogen, and LCMV is uniquely sensitive to IFN-I signaling(22). Current research favors a "linear differentiation" model whereby the memory CD8 T cells that persist long-term following the CD8 T cell contraction are derived from precursors found within this expanded effector pool. These CD8 T cell memory precursors have been identified during the expansion phase by high expression of CD127 (IL-7Rα) and low expression of KLRG-1(23, 24). Further studies into this differentiation process have lead to the development

of the "decreasing potential model," whereby the memory subset of cells receives a shorter duration of antigen stimulation and/or inflammation(15). Inflammatory signals such as IL-12 have been implicated in up-regulating transcription factors such as Tbet and driving cells into an effector rather than memory cell fate(25). Although it remains unclear how the dynamics of this differential cell fate occurs, a recent study has suggested that following initial priming, individual effector CD8 T cells undergo divergent programming due to asymmetrical distribution of signaling molecules during cell division(26). These memory CD8 T cell precursors then undergo further differentiation during the contraction phase, down-regulating effector functions and up-regulating genes associated with the memory phenotype(27). Memory CD8 T cells following acute infection are characterized by the ability to survive long-term in the absence of antigen, proliferate in response to homeostatic signals, and to rapidly divide and produce effector cytokines upon rechallenge(27-33).

Following LCMV infection, the virus specific CD4 T cells undergo a naive, effector, and memory phase similar to CD8 T cells. This CD4 T cell response is reduced in size compared to the CD8 T cell response at each stage(34, 35). Activation of CD4 T cells also requires different co-stimulatory molecules compared to CD8 T cells, for example, CD40L (CD154) deficient animals mount a normal CD8 T cell response, but have severely diminished CD4 T cell responses following LCMV infection(35). The decreased size of the CD4 T cell response is not related to the initial precursor frequency or competition for antigen, but instead seems to be regulated by competition for signaling through IFNγ(36). This limiting of CD4 T cell burst size may be crucial, as vaccination with a CD4 T cell epitope resulted in fatal immunopathology following chronic LCMV

challenge(37). Effector CD4 T cells following LCMV infection make mostly IL-2 and the Th1 cytokine IFNγ; while lower frequencies of IL-4 producing cells are detected (38). Whether CD4 T cell help is essential for CD8 T cell priming and survival remains an unresolved question. Although previous studies have suggested a role for CD4 T cells in CD8 T cell maintenance(39, 40), other data has suggested that CD4 T cells are unnecessary for maintaining memory CD8 T cells after LCMV infection. Indeed, recent studies suggest that CD4 deficient mice may not completely clear LCMV infection, and this may partially explain the resulting CD8 T cell defects seen in LCMV infected CD4 knockout mice (D. Choo, unpublished observations).

LCMV Armstrong infection induces a potent antigen-specific humoral response in both Balb/c and C57BL/6 mice(41). This response is highly dependent on CD4 T cells and CD40:40L signaling; germinal centers, long-lived plasma cells, and memory B cells are absent in mice lacking CD4 T cells(42) and in CD40L deficient mice(43, 44). Following LMCV infection, there is an initial burst of antibody secreting cells in the spleen, followed by a germinal center reaction where LCMV-specific B cells undergo Ig class switching and somatic hypermutation. Approximately two weeks post infection, LCMV-specific antibody secreting cells are detected in the bone marrow, and these long-lived bone marrow plasma cells are responsible for maintaining the long-term antibody responses(41, 45). Memory B cell responses are also detectable in the spleen around two weeks post infection and reach a constant level around day 60 post infection(45, 46). These memory B cells can respond quickly upon antigen re-challenge, and may play a role in the maintenance of antibody secreting cells(45). Mice that cannot form germinal centers, such as mice lacking the CD4 T cell signaling molecules SAP(47) and

CD40L(43, 44), mount an initial antibody response, but fail to develop long-lived plasma cells in the bone marrow and have severely reduced antibody levels at later time points post infection. As seen in a number of viral infections, antibody of the IgG2a isotype predominates the response(48). In passive immunization studies, the Fc portion of the IgG2a antibody mediated protection from LCMV lethal challenge. This was complement-independent activity and did not correlate with neutralization in-vitro, as neutralizing antibodies of the IgG1 isotype were not protective(49). Interestingly, neutralizing antibodies are slow to develop after LCMV infection, and studies comparing the LCMV and vesicular stomatitis virus (VSV) glycoproteins suggested that the LCMV glycoprotein itself, and not the immune response to any viral backbone components make it a poor inducer of neutralizing antibodies(50).

Immune responses to chronic LCMV infection

LCMV clone-13 was isolated from mice persistently infected with LCMV Armstrong virus(12). It has 2 silent mutations and 2 amino acid changes from the parental Armstrong strain. There is a phenylalanine to leucine mutation at amino acid 260 of the glycoprotein, and a lysine to glutamine mutation at amino acid 1079 of the polymerase. In vitro analysis of these viruses demonstrated an enhanced infectivity of macrophages due to changes in the glycoprotein and enhanced levels of viral replication within the infected cell due to changes in the polymerase gene(51). In vivo studies with reassortant viruses demonstrated the mutation within the glycoprotein resulted in the establishment of the prolonged viremia similar to that seen during clone-13 infection(52).

Moreover, this leucine replacement within the glycoprotein is associated with higher α-dystroglycan binding and similar mutations are seen in a number of the LCMV strains that cause high levels of viral replication and immune suppression(9). Direct targeting of the killing of antigen-presenting cells as well as defects in dendritic cell activation has been demonstrated in these chronically infected mice(8, 53, 54). Infection of naïve mice with a high dose of the clone-13 strain of LCMV results in a protracted viremia that lasts approximately 2-3 months, with virus persisting in select tissues such as the kidney, brain and salivary glands(55). Moreover, in mice lacking CD4 T cells, infection with LCMV clone-13 results in persisting high levels of virus in the serum and tissues(56). Thus, the LCMV clone-13 model provides two useful models to study CD8 T cell responses during prolonged or persisting viral infection.

Chronic LCMV infections were initially characterized by the inability to detect antigen-specific cytotoxic T lymphocyte (CTL) activity following infection(57, 58). During chronic LCMV infection of CD4 deficient mice, CTLs can initially be detected during the first week of infection but eventually disappear. However, the ability to track antigen-specific T cell responses with MHC class I tetramers(59) has shown that CD8 T cells which lack effector function such as CTL killing and cytokine function persist during chronic LCMV infection(60). This functional impairment is increased during prolonged exposure to high antigen loads and seems to be worsened when CD4 T cell help is removed(56, 61). Recent studies comparing acute and exhausted CD8 T cells showed a unique gene pattern expressed during CD8 T cell exhaustion, and this phenotype developed over the course of infection(62). Chronic LCMV infection also results in increased frequencies of LCMV-specific CD8 T cells in the peripheral tissues

and alterations in the hierarchy of LCMV epitopes. Acute LCMV infection results in a number of dominant as well as subdominant CD8 T cell responses, and the size of the individual responses depends on several factors such as precursor frequency, efficiency of antigen processing, and MHC/TCR affinity for each epitope. The relative hierarchy of the CD8 T cell response seen at the peak of the response is maintained throughout memory(63). However, during clone-13 infection, the impairment of individual CD8 T cell responses is directly linked to the level of antigen, resulting in more severe impairment and deletion of the major epitopes (NP396, GP34), and a skewing of the response towards the slightly less dominant epitopes (GP33, GP276)(55, 64, 65). Exhaustion also shows a hierarchal pattern of loss of function with cells first losing the ability to kill infected targets and secrete IL-2 early during exhaustion, followed by loss of TNFα production, loss of IFNγ production, and finally possible deletion of the CD8 T cell. During the expansion at day 8 post clone-13 infection, antigen-specific CD8 T cells retain their ability to make IFNγ, but have a reduced percent of cells co-producing TNFα and the intensity of the cytokine is lower on a per cell basis compared to CD8 T cells in Armstrong infected animals(55). This exhaustion becomes more severe over the course of infection and these mice cannot clear virus from the tissues. Although there was a slight improvement in CD8 T cell function following suppression of serum viremia, these CD8 T cells did not differentiate into memory phenotype cells and did not persist in the absence of continued antigen stimulation(66).

During chronic LCMV infection, LCMV-specific CD4 T cells have been shown to also undergo exhaustion. LCMV-specific CD4 T cells initially lost their ability to make TNF, but retained low-levels of IFNγ production and failed to make IL-2(61).

Although there was a slight increase in the percent of CD4 T cells making TNF following resolution of serum virus (67), this did not result in an increased number and may be due to preferential survival of these cells (68). The loss of cytokine function by LCMV-specific CD4 T cells was seen across several CD4 T cell epitopes. Moreover, studies tracking LCMV-specific CD4 T cells in the transgenic SMARTA mouse demonstrated that LCMV-specific CD4 T cells proliferated, but produced less cytokine after clone-13 versus Armstrong infection. Additionally, mice vaccinated with CD4 T cell peptides and then challenged with LCMV, had enhanced CD4 T cell responses following infection with the acute Armstrong, but reduced responses following clone-13 infection(69). However, its interesting to note that when CD4 T cells and CD8 T cells are transferred into chronically infected hosts, the kinetics of exhaustion is much slower for the CD4 T cells(70). In addition, most of these studies have relied on detection of CD4 T cells by measuring function, or tracking transgenic CD4 T cells; the recent development of MHC class II tetramers for LCMV will hopefully give us a better understanding of CD4 T cell exhaustion and how it may differ from CD8 T cell exhaustion.

Mice lacking CD4 T cells or depleted of CD4 T cells prior to LCMV clone-13 infection cannot control viral replication and maintain high levels of persisting virus in their serum and tissues(42, 56, 71). This system also results a greater impairment of the CD8 T cell response, and very few antigen-specific CD8 T cells capable of making cytokine, including IFNγ, can be detected(56, 67). This greater functional impairment may be due to lack of CD4 T cell help, but these mice also lack a humoral response and that may contribute to an increased viral burden in these animals (42). B cell knockout animals and animals lacking CD40L also have high levels of persisting serum virus

following infection with chronic strains of LCMV(72, 73). However, B cell deficient mice have defects in CD4 T cell priming, and recent work has suggested that this may be critical for viral clearance in these mice (74). In immunotherapy models, both CD4 T cells and B cells have been shown to be capable of preventing exhaustion of transferred antigen-specific CD8 T cells(75). The effect of B cell transfers in immunotherapy settings depends slightly on the strain of LCMV(76), but suggests that B cells and CD4 T cells play a supporting role for CD8 T cell effector survival during persisting viral infection.

A number of different strategies to overcome CD8 T cell exhaustion have been examined using the LCMV infection model. Chronic LCMV infection induces expression inhibitory molecules on antigen-specific CD8 T cells including PD-1, LAG-3 and 2B4, and higher levels of exhaustion are correlated with expression of multiple molecules(77). Blockade of the PD-1 pathway results in improved CD8 T cell number and function in chronic LCMV infected mice(78). Blockade of PD-1 has also been shown to increase proliferation and function of HIV-specific CD8 T cell responses in vitro(79) and recent experiments in SIV infected rhesus macaques demonstrated improved CD8 T cell responses and enhanced viral control of SIV following PD-1 blockade in vivo(80). PD-1 blockade has also been shown to enhance CD8 T cell responses to LCMV following therapeutic vaccination(81). Other approaches have shown that blocking the immunosuppressive cytokine IL-10(82-84), or blockade of cell migration by treatment with FTY720 results in reduced CD8 T cell exhaustion(85). This latter study using FTY720 was particularly remarkable, as this treatment was also able to

mediate viral clearance after the establishment of chronic infection. Interestingly, CD4 T cells were required for this effect.

CD4 T cells and chronic infection in humans

CD4 T cell exhaustion and deletion is associated with poor CD8 T cell responses to both hepatitis C virus (HCV) and human immunodeficiency virus. Studies of HCV infected chimpanzees have shown that CD4 T cell responses are critical for viral control by CD8 T cells, despite the presence of memory CD8 T cells capable of clearing infection(86). In humans, strong sustained anti-HCV CD4 T cell responses correlate with spontaneous control and individuals who resolve infection have increased breadth of CD4 T cell responses compared to chronically infected individuals. CD8 T cell dysfunction parallels a loss of CD4 T cell help during HIV infection. The proliferative capacity of HIV-specific CD8 T cells has been shown to be enhanced upon co-culture with autologous CD4 T cells isolated during the acute stage of infection(87). Additionally, CD4 T cell help has been implicated in the persistence of long-lived CTLs during immunotherapy of cytomegalovirus (CMV). More recent studies with MHC class II tetramers have additionally suggested that skewing of CD4 T cell responses towards the Th1 or Th2 phenotype during HCV infection may play an important role in CD8 T cell function and disease outcome(88). Overall, a more robust understanding of CD4 T cell responses will provide us with insights into CD8 T cell function and exhaustion during chronic viral infection.

The first part of this thesis focuses on this interplay between B cell, CD4 T cell and CD8 T cell immune responses during chronic LCMV infection. Our first study examines the role CD4 T cells and B cells have on maintenance of CD8 T cells during the protracted viremia of LCMV clone-13 infection. Studies examining this question often use gene knockout or antibody depletion to completely remove the CD4 T cells, whereas we have transiently blocked CD4 T cell help by treatment with the αCD40L antibody MR-1. Mice treated for one week, beginning at two weeks post infection, mount normal early CD8 T cell responses but have impaired CD4 T cell function and reduced humoral immune responses. Mice followed longitudinally after termination of the blockade had long-term deficits in humoral immunity and impaired viral control compared to untreated clone-13 infected mice. This data suggests that changes in humoral immune responses affect viral levels and CD8 T cell exhaustion during chronic LCMV infection. In our second study, we looked at how restoring CD4 T cell signals could impact exhausted CD8 T cell number and function. Mice depleted of CD4 T cell help during the initial CD8 T cell priming harbor high levels of persisting viremia and high levels of CD8 T cell exhaustion. Naïve, LCMV-specific CD4 T cells transferred into these mice underwent proliferation in vivo and survived long-term post transfer. These LCMV-specific CD4 T cells enhanced proliferation and function of CD8 T cells to a wide range of LCMV epitopes. There was also emergence of LCMV-specific antibody following CD4 T cell transfer. This effect relied on antigen-specific CD4 T cell transfer and treated mice had enhanced control of LCMV virus. Combining the CD4 T cell therapy with αPD-L1 blockade further synergized the CD8 T cell rescue and viral

control. These data suggest that CD4 T cell immunotherapy may be a useful strategy to overcome CD8 T cell exhaustion.

Part II: Humoral immunity: germinal centers, long-lived plasma cells and memory B cells

Epidemiological studies have long suggested the presence of long-term protective immune responses in the absence of re-exposure. This has been shown for measles immunity on the Faroe Islands(89), yellow fever immunity in Virginia(90), and polio immunity in remote Eskimo villages in Alaska(91). Moreover, recent studies have shown long lived T cell and B cell memory after vaccination against smallpox(92-94). Although the relative importance of B cell and T cell immunity likely depends on the pathogen, the presence of high levels of neutralizing antibody provides a potent first line of defense against invading pathogens. Indeed, long-lived antibody responses have been a hallmark of our most successful vaccines developed to date. However, the regulation of these long-lived antibody responses is not completely understood. Murine models have provided some insights into germinal center formation and its importance in the formation of long-lived plasma cells and memory B cells. However, many questions remain to be addressed in understanding the mechanism of B cell memory fate decisions and in understanding how memory B cells and plasma cells are maintained for the life of the individual.

Differentiation and maturation of naïve B cells

B lymphocyte development initially occurs in the fetal liver and then after birth continues in the bone marrow. Developmental stages within the bone marrow include the successful re-arrangement of immunoglobulin (Ig) receptor genes and the subsequent expression of an IgM+ B cell receptor on the cell surface(95). The B cell receptor is subject to a negative selection step which deletes or re-arranges receptors on autoreactive B cells(96). Subsequently, the immature transitional B cells leave the bone marrow via the blood stream and are passively transported to the spleen where they undergo a final maturation step, co-expressing both IgM and IgD receptors, and the CD21 complement receptor on their surface. Approximately 1-2 x 10^7 immature B cells are generated daily in the adult murine bone marrow(97). However, only about 3% of these will successfully emigrate to the peripheral lymphoid tissues and differentiate into mature naïve B cells(98). Selection of these transitional B cells into the mature naïve B cell pool is dependent on weak signaling through the B cell receptor, and interaction with co-stimulatory molecules such as BlyS (BAFF)(98-101). The lifespan of a mature naïve B cell and the factors important in the homeostasis of the peripheral mature naïve B cell population remain active fields of research. Ligands and receptors within the same family as BAFF have been shown to be critical for the survival of mature naïve B cells(102).

Mature naïve B cell subsets in the spleen include the recirculating IgM^{low} IgD^{high} $CD21^{int}$ $CD23^{high}$ follicular B cells (FO) and the IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{low}$ $CD1d^{hi}$ marginal zone (MZ) B cells that reside at the red pulp junctions within the spleen.

Follicular B cells account for 80-90% of the splenic B cells and are important in most T-dependent B cell responses. Marginal zone B cells account for 5-10% of splenic B cells and are important in early bursts of plasma cells following infection, especially against pathogens in the blood. A third subset of B cells, the self renewing B1 B cells which reside in the peritoneal and pleural cavities, are primarily seeded by precursors that develop within the fetal liver(103). These cells provide T-independent antibody responses to common bacterial products and maintain the level of "natural antibodies" which are important in the early defense against bacterial and viral pathogens(104, 105).

Marginal zone and follicular zone B cells

Marginal zone (MZ) B cells have often been relegated to playing a role in T-independent immune responses; however, recent research has suggested that they may also be important for T-dependent responses. Compared to follicular B cells, MZ B cells express higher levels of MHC class II and the B7 co-stimulatory molecules on their surface. MZ B cells activated in vitro or in vivo serve as potent stimulators of CD4 T cells and likely play a role in initiating T cell responses in vivo(106). Adoptive transfer experiments using purified B cell subsets confirmed that MZ B cells differentiated into IgM and IgG antibody secreting cells (ASC) and were important in initiating pre-germinal center antibody responses. One study by Cerny and Song, which adoptively transferred MZ B cells into *scid* mice, demonstrated that MZ B cells were capable of entering germinal center reactions and undergoing somatic hypermutation(107). Critics, however, have suggested that the lack of intact secondary lymphoid organs in *scid* mice

may allow for T-B cell interactions that are not physiologically relevant in a normal host. In studies by Brink and colleagues, MZ B cells transferred into intact hosts significantly lagged in ability to be activated, recruited into the response and the germinal center reaction, and undergo somatic hypermutation when compared to follicular B cells(108). Thus MZ B cells play a role in the initial T cell activation phase and early plasma cell responses, but follicular zone B cells are likely to dominate the T-dependent germinal center reaction and memory responses and are therefore the focus of these studies.

CD4 T cells initiate B cell proliferation and differentiation

Following infection or immunization with a T-dependent antigen, dendritic cells within T cell areas of secondary lymphoid tissues activate antigen-specific CD4+ T cells. Interactions between the activated T cells with B cells initiate a primary round of B cell proliferation and differentiation. A subset of plasmablasts migrates to the red pulp where association with $CD11c^{high}$ dendritic cells appears to be critical for survival and differentiation to plasma cells. These short-lived plasma cells provide the initial burst of serum antibody, and usually have a half-life of 3-5 days. Short-lived plasma cells secrete both IgM+ and class-switched Ig, and are generally germ-line or lower affinity than the long-lived plasma cells that develop from the germinal center reaction (recent review (109)). Recent studies have suggested that the type of CD4 help required to initiate primary antibody responses to T-dependent antigens differs from that required to generate germinal centers, long-lived plasma cells and memory B cells. Mice deficient in the Slam-associated protein (SAP) produced normal levels of short-lived plasma cells and

serum antibody levels during primary responses to lymphocytic choriomeningitis virus (LCMV), but had severely reduced germinal center reactions, memory B cell numbers, and long-lived plasma cell numbers(110).

Despite the fundamental role CD4 T cells play in germinal center formation, only recently have studies begun to uncover the cellular and molecular mechanisms of CD4 T cell help. This area of research has been greatly enhanced by the characterization of a unique subset of CD4 T cells termed follicular B helper T cells (T_{FH})(111). Upon activation, CD4 T cells upregulate expression of the chemokine receptor CXCR5, and are able to migrate to B cell areas(112). Here, it is thought that these activated cells receive secondary signals for differentiation into T_{FH} cells, and although the accessory cell type remains undefined, signaling through OX40 and CD40 is thought to be critical. These specialized T_{FH} CD4 T cells express increased levels of CD40L, ICOS, and IL-10, molecules that positively regulate B cell differentiation. These cells are also an important source of IL-21, a potent stimulator of B cell proliferation, isotype switching, and Ig secretion in both human and murine B cells(113, 114).

The germinal center reaction

Following infection or immunization with a T-dependent antigen, proliferation and selection of helper CD4+ T cells with preferred TCR occurs over 3-5 days(115) and a subset of these cells up-regulate CXCR5 and migrate towards the T/B follicular borders(116). B cells within the follicle internalize soluble antigen and present it on surface MHC II molecules. Follicular B cells presenting cognate antigen then interact

with activated T_{FH} cells at the T/B cell border, which initiates a primary round of B cell proliferation and differentiation. A subset of B cells migrate back into the B cell area where they rapidly expand to form regions of clonally expanded B220+IgD- peanut agglutinin+ (PNA) B cells termed secondary follicles(117). Each germinal center is seeded by 3-4 different B cell clones(118). A subset of T_{FH} cells is also recruited into the secondary follicle and is required for germinal center formation and maintenance. Mice deficient in CD4 T cells, or signaling through CD40, CD28, ICOS pathways have severely impaired T-dependent responses and have reduced ability to form or maintain germinal centers (119, 120). Blocking CD40 or depleting CD4 T cells prevents germinal center formation and causes the collapse of existing germinal centers, whereas blocking of the CD28 pathway with an antibody that binds B7.2 only prevents the formation of new germinal centers and has no effect on existing germinal centers(119).

Although there is considerable research into the structure of the germinal center reaction, these studies have often relied on snapshots of cell localization within fixed tissue specimens. Recent developments in two-photon microscopy provide a powerful new tool for visualizing cellular interactions and movement within intact lymphoid follicles, refining our ability to study germinal centers as dynamic structures. Generally, it has been described that seven to ten days after initial priming, the secondary follicle polarizes into a dark and light zone and initiates a germinal center reaction. In the dark zone, B cell centroblasts express low levels of surface BCR and undergo intense proliferation with an estimated division rate of every 6-7 hours(117, 121). These dividing centroblasts also undergo mutations within the variable region of the Ig gene mediated by the activation-induced cytidine deaminase (AID) enzyme and the induction

of DNA-repair enzymes(122). Most of the resulting mutations result in cell death, while select mutations improve binding of BCR to cognate antigen. In the light zone, B cell centrocytes re-express surface BCR and undergo positive selection based on the ability of newly mutated BCRs to bind original antigen and consequently receive survival signals from follicular dendritic cells (FDCs) or CD4 T cells(117). Positively selected centrocytes can undergo further diversification by re-entering the centroblast pool, or can be directed to differentiate and enter the long-lived plasma cell or memory B cell pool. After three weeks post primary immunization, germinal center reactions have subsided and stable levels of high-affinity antibody can be measured in the serum. Recent studies using two-photon microscopy have both confirmed and questioned the established model whereby distinct areas of light and dark zones differentiate the centroblasts undergoing proliferation and hypermutation from the centrocytes undergoing selection(123-125). However, there is an enrichment of uniquely differentiated FDCs and CD4 T cells in the light zone, and there remains strong evidence that CD4 T cells are important in the selection of GC B cells. Further studies using the 2-photon microscopy technique to analyze the migration of B cells and T lymphocytes within the germinal center reaction will likely improve our understanding of this process by which high affinity B cells are selected for differentiation into the long-lived memory B cell or plasma cell pool.

Follicular dendritic cells in the germinal center

Follicular dendritic cells (FDCs) play an important, but controversial role during the germinal center process. Textbook descriptions cite an importance for antigen

complexes retained on FDCs within the light zone for enhanced selection of high affinity receptors following somatic hypermutation. Follicular dendritic cells found in the light zone display a unique pattern of markers and exhibit higher complexity of processes suggesting a differentiation state different from FDCs found in the dark zone or a primary B cell follicle. However, many researchers have questioned the need for the FDC network for affinity selection. Mice lacking lymphotoxin-α (Ltα) cannot form germinal centers in the spleen and lack lymph nodes or peyers patches. Although immunization with low-dose antigen resulted in reduced affinity maturation, high doses of antigen induced high affinity IgG1 responses similar to that seen in a wild-type mouse(126). Similarly, mice lacking lymphotoxin-β (LTβ) form abortive germinal center reactions and have reduced affinity maturation, however maturation continues after the collapse of the germinal center(127). Some skeptics of these studies suggest that precursor or alternate types of cells may provide signals similar to FDCs and that such derived systems do not well mimic the intact response. In support of these results, other researchers have shown that increased levels of immune-complexes on FDC does not result in reduced stringency of affinity maturation(128). Suggestions that antigen competition is important in the selection of high affinity cells in vivo comes from experiments where low affinity transgenic B cells can form germinal centers and enter memory pool when higher affinity B cells are not present(129). Thus immune complexes may enhance B cell responses and affinity maturation in conditions of limiting or low-affinity antigen whereas in the case of antigen depot-forming adjuvants or high affinity antigens may bypass this need. In bone marrow chimera experiments where radiation-resistant cells lacked CD21/CD35, and thus mice had reduced immune complex trapping,

sustained IgG responses were diminished. Aside from this debate, FDCs are an important source of chemokines for the trafficking of B cells during the initiation of the germinal center reaction. FDCs are an important source of the CXCL13 chemokine, an important chemokine for B cell and T cell motility within the germinal center reaction. Moreover, FDCs likely provide essential signals for B-cell survival, proliferation and differentiation in vitro(130) and FDC derived IL-6 has been suggested to be critical for plasma cell differentiation(131). New studies using two-photon microscopy will hopefully give us new insight into the roles of FDCs during the initiation and maintenance of secondary follicles and germinal centers in vivo.

Post germinal center fate

How post-germinal center fate decisions are determined remains an open field of investigation. There is evidence that a high-affinity BCR favors plasma cell differentiation; single cell analysis of the plasma cell versus memory B cell pool showed a selection towards higher affinity variants in the plasma cell fraction(132). Secondly, in mice constitutively expressing higher levels of the anti-apoptotic molecule Bcl-2, researchers observed enhanced survival of germinal center and memory B cells, but no increased survival of high affinity plasma cells in the bone marrow(133). Other data suggests a more instructive model of plasma cell versus memory B cell differentiation; CD40 signaling favors a memory phenotype, while OX40L signaling favors a plasma cell fate(134). Cytokines, such as IL-2 and IL-10 have been shown to drive plasma cell differentiation in the absence of CD40 signaling(135), while IL-4 directs cells to a

memory B cell fate(131). IL-6 induction of the cyclin-dependent kinase inhibitor p18 is also required for plasmablast formation and antibody secretion(136, 137).

Long lived plasma cells

Although there is an initial burst of serum antibody by short-lived plasma cells in the spleen, it is long-lived plasma cells in the bone marrow that are responsible for the long-term maintenance of serum antibody(138, 139). These long-lived plasma cells or their precursors, plasmablasts, originate within the germinal center reaction (140). Upon plasma cell differentiation, there is increased steady-state levels of heavy and light chain Ig mRNA, possibly due to both increased levels of mRNA transcription and improved mRNA stability(141, 142). The transcription factors XBP-1(143) and Blimp-1(144) are required for differentiation of both long and short-lived plasma cells(145), while the *Ikaros* family member, *Aiolos,* has recently been shown to be uniquely essential for the formation of long-lived plasma cells(146). Many of the prototypical B cell markers are down regulated on plasma cells, including MHC II, B220, CD19, CD21 and CD22. The prominent identifying marker expressed on plasma cells is the proteoglycan syndecam-1 (CD138), which recognizes extracellular matrix and growth factors(147). The plasma cells in the spleen also upregulate expression of CXCR4 whose ligand CXCL12 is present in the splenic red pulp, lymph nodes, and medullary cords of the bone marrow, thus facilitating migration of plasma cells away from the follicles into these long-lived niches(148). Although the exact combination of signals that make up this survival niche

are not known, ex vivo studies have suggested a role for bone marrow stromal cells, IL-6, SDF-1, TNFα and BCMA in plasma cell survival(149, 150).

It is now generally accepted that plasma cells survive long-term in the absence of antigen restimulation. LCMV immune mice irradiated 50 days post infection to destroy radiation-sensitive (active cell-cycling) memory B cells but not the radiation resistant (non-cycling) plasma cells estimated the half life of LCMV-specific plasma cells to be approximately 94 days in the bone marrow and 172 days in the spleen. Another study tracked proliferation by BrdU incorporation and found long-lived plasma cell survival without cell division(151). However, in the LCMV experiments there was a slight decline in the long-lived plasma cell numbers, and it has been suggested that memory B cells may differentiate into plasma cells either through non-specific TLR signals or CD4 T cell signals to replenish the long-lived plasma cell pool(152, 153).

Memory B cells

Memory B cells re-circulate within secondary lymphoid tissues and are primed to proliferate and differentiate into high-affinity antibody secreting cells (ASC) following secondary exposure. Although CD27 has served as a good marker for memory B cells in humans, the panel of markers to differentiate mouse memory B cells remains more complicated(154). Studies by Rajwesky and colleagues demonstrated that memory B cells could persist long-term in the absence of specific antigen stimulation. In their studies, they engineered mice where the BCR specificity coded by an Ig transgene could be switched at a memory time point(155). Additionally, memory B cell numbers are maintained in mice lacking immune complexes, albeit at lower numbers than control

animals(156). However, the signals required for the maintenance of memory B cells in the spleen remain unknown. Naïve B cells require signaling through the TNF family member BAFF and it is possible that such signals are also required for memory B cell maintenance. It is not known if memory B cells undergo homeostatic proliferation such as that driven by IL-7 and IL-15 for memory CD8 T cells.

Memory B cells often express the IgG isotype, and it has been proposed that upon re-exposure, the unique signaling within the membrane-spanning region of IgG drives increased accumulation of progeny cells and plasmablasts during the secondary response(157). Indeed, human memory B cells expressing isotype switched Ig genes have been shown to enter their first division sooner and lead to increased plasmablast formation per division in vitro(158, 159). It is unclear whether memory B cells re-enter germinal center reactions during secondary immune responses.

Maintenance of serological memory

Although continued re-exposure to antigen is likely responsible for maintaining high levels of serum antibody to a number of important human pathogens, it is less clear how serum antibody levels are regulated in the absence of antigen re-exposure. Although a subset of long-lived plasma cells persists following vaccination, memory B cells have been suggested to be important to replenish this long-lived population. It has been proposed that bystander activation, by either non-specific activated CD4 T cells or microbial products, such as LPS or CpG, drives the differentiation of memory B cells into plasma cells. Lanzavecchia and colleagues have shown that in vitro stimulation of

human memory B cells with microbial products that signal through toll-like receptors can induce differentiation into plasma cells in vitro*(153)*. Indeed, human memory B cells express higher levels of the toll-like receptors (TLRs) such as TLR9, which recognizes unmethlyated CpG DNA(160). However, a recent study taking advantage of the hCD20 transgenic mice to deplete B cells but not plasma cells from mice has shown no differences in antibody titers following B cell depletion(161). These experiments have not yet been repeated in other systems and it remains to be seen how well the mouse system translates to human immunology. Given the importance of maintaining serological memory for the life of an individual, it is likely that memory B cells and long-lived plasma cells both play a role in providing long-term antibody responses.

In the following studies, we have developed an assay to measure circulating memory B cells in human peripheral blood. We have shown that the cells detected in this assay are of the canonical memory CD27+CD19+ phenotype. We then used this assay to detect memory B cells following vaccination for anthrax or smallpox. We also measured anthrax-specific memory B cells in people who were exposed to anthrax during 2001. This assay, which is highly adaptable to any bacterial, protein, or viral antigen, provides a useful tool to better understand the size and maintenance of memory B cells following vaccination or antigen exposure.

Chapter 2: Transient CD40:CD154 blockade inhibits B cell responses and prevents viral control during chronic LCMV infection

Abstract:

CD4 T cells play an essential role in the development of B cell responses to LCMV infection. CD4 T cells are also critical for the maintenance of CD8 T cell responses during chronic LCMV infection. Previous studies have shown that the CD40:CD40 Ligand signaling is essential for CD4 T cell help to B cells, but not for CD8 T cell priming following acute LCMV infection. However, mice deficient in CD154 (CD40 Ligand) are unable to clear persisting strains of LCMV, similar to the phenotype seen in CD4 T cell and B cell knockout mice. In this study we transiently blocked CD4 T cell help by administration of anti-CD154 (αCD40L, MR-1) antibody. By blocking CD154 for one week beginning at day 15-post infection, we specifically inhibited germinal center and B cell responses but did not alter initial CD8 T cell priming. Following treatment with αCD154, we saw a significant decline in germinal center B cells, antigen-specific antibody secreting cells, and memory B cells, but no changes in CD8 T cell number or function. This transient blockade resulted in a long-term deficit in LCMV-specific antibody and long-lived plasma cells in the bone marrow. Importantly, inhibiting B cell and CD4 T cell responses early post infection resulted in reduced viral control and increased CD8 T cell exhaustion. This data suggests that short-term inhibition of CD4 T cell responses and reduced B cell responses can have long-term effects on viral control and CD8 T cell exhaustion during chronic LCMV infection.

Introduction:

Previous studies have suggested an essential role for CD4 T cell help in the priming of CD8 T cells during persistent viral infections. Mice lacking CD4 T cells or depleted of CD4 T cells prior to LCMV infection cannot control chronic LCMV infection(56, 71). CD4 T cell help has also been shown to be critical for CTL maintenance and recovery from hepatitis C virus (HCV) in humans and non-human primates(86, 162). Likewise, T helper cell function has been positively correlated with CTL activity in human immunodeficiency virus (HIV) infected humans(163). However, the mechanisms by which CD4 T cell help maintains functional CTL during persisting virus remains less understood. Previous studies examining the role of CD4 T cells during LCMV infection have relied on CD4 deficiency during the entire CD8 T cell priming, which may result in different signals during the early activation of CD8 T cells. In this study, we used an antibody blockade of the CD40:CD40 Ligand pathway to explore whether removing CD4 T cell help after initial CD8 T cell priming effects the ongoing CD8 T cell function and ability to control LCMV virus.

CD40 Ligand (CD40L) or CD154 is a member of the TNF family of molecules and binds to TNFR family member CD40 on B cells, playing an essential role in T-dependent humoral immune responses(164). Patients suffering from X-linked hyper-IgM syndrome have mutations in their CD40L gene(165). Although it is primarily expressed at high levels on CD4 T cells, up-regulation of CD154 is also seen on activated CD8 T cells. CD154 expression has also been noted on non-T cells including mast cells,

basophils, eosinophils, NK cells, monocytes, dendritic cells and thrombocytes. In addition to B cells, CD40 is expressed by endothelial cells, fibroblasts, epithelial cells and dendritic cells(166). For dendritic cells, interactions between CD40 and CD154 are thought to be critical for the maturation of these cells, or "licensing" of these cells to provide secondary co-stimulatory signals to T cells during CD8 T cell priming(167).

Expression of CD154 on CD4 T cells is rapidly up-regulated following activation of T cells with anti-CD3, and CD28 engagement further augments and stabilizes this expression(168). Early expression is independent of protein synthesis, but maximal expression follows up-regulation of mRNA levels and protein synthesis.

Initial studies showed an essential role for co-stimulation via the CD40:CD154 in the priming of cytotoxic CD8 T cells(169). However, infection models have suggested that this role may be redundant if antigen-presenting cells are directly activated by viral pathogens. Studies of LCMV-infected CD154 deficient mice have shown that the CD40:CD154 pathway is not essential for initial CD8 T cell priming, but loss of this pathway results in lower CTL memory(43, 44). B cell responses in CD154 knockout mice are severely impaired, with only a minor, T-independent short-lived antibody response followed by lack of formation of germinal centers, long-lived antibody secreting cells, and memory B cells(43, 170). A lack of CD4 T cell proliferation and poor cytokine function in the CD40L knockout mice suggested that Th cells were not sufficiently primed in mice lacking CD154 and thereby led to a defect in the formation of long-lived CTLs(73, 170, 171). However, other studies show that CD4 T cells primed in CD40 deficient mice provided good B cell help when transferred into CD40 sufficient mice and challenged with DNP-LCMV(172). Infection of CD154 deficient mice with more

chronic strains of LCMV show a failure to control virus replication and CTL dysfunction suggesting a role for CD154 in priming of CTLs during persistent viral infections(73, 170). Interestingly, infection of CD154 knockout mice with other viral pathogens such as VSV result in similar profound B cell defects(43, 172) but more pronounced defects in CTL priming(173) suggesting that the need for CD40L signaling may be highly dependent on the tropism and level of replication during viral infection.

CD40 cross-linking activates proliferation, differentiation and Ig production in mature B cell subsets(164). During germinal center reactions, CD40 engagement rescues B cells from apoptosis, allowing isotype switching, selection, and differentiation of memory B cells. Engagement of CD40 has also been shown to induce the re-expression of telomerase activity of memory B cells and may play an essential role in the long lifespan of these cells(174, 175). Treatment with the αCD40L antibody has been shown to inhibit plasma cell generation during autoimmune models of arthritis, systemic lupus erythematosus (SLE), and experimental autoimmune encephalomyelitis (EAE)(176-179). Additionally, for lupus nephritis and EAE, αCD40L antibody treatment has been shown to ameliorate ongoing disease. Treatment using αCD40L antibody in humans with SLE has resulted in decreases in levels of anti-double-stranded DNA antibodies and decreases in the SLE disease activity index(180).

In this study we wanted to more closely examine the role of CD4 T cell help during the later stages of CD8 T cell and B cell priming. Because initial experiments demonstrated an inability to deplete CD4 T cells in LCMV clone-13 infected mice, we instead blocked CD4 T cell help by administration of the αCD154 (MR-1) antibody. Transient αCD154 antibody treatment was carried out for one week beginning at two

weeks post infection. This time point was chosen because it is after initial T cell priming but prior to the peak germinal center response, thereby arresting the development of long-lived plasma cells and memory B cells. Following one week of αCD154 treatment, we saw no differences in the CD8 T cell function, but reduced IFNγ by antigen-specific CD4 T cells. Transient blockade of the CD40:CD154 pathway resulted in abrogation of the B cell response despite the continued presence of virus in these mice. Moreover, this inhibition of CD4 T cell help resulted in poor viral control, with mice unable to resolve serum virus by day 75-post infection. Mice that could not initially control serum viremia had a long-term elevated viral burden and greater CD8 T cell exhaustion. These experiments emphasize an important role for the B cell response and for CD4 T cell help to prevent CD8 T cell exhaustion during persisting viral infections such as LCMV clone-13.

Materials and Methods:

Mice and infections:

4-6wk old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). LCMV stocks were grown in BHK cells and titered on Vero E6 cells as previously described (Ahmed et al., 1984). 4-6 week old mice were infected with $2x10^6$ pfu of LCMV clone-13 i.v. or $2x10^5$ pfu i.p. of LCMV Armstrong virus. All mice were used in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines.

CD4 T cell depletion and αCD40L antibody blockade

CD4 T cells were depleted by administration of 250μg i.p. of GK1.5 (BioExpress) on day 15 and 17 post infection. The αCD40L (MR-1) antibody was provided by Dr. Chris Larsen, Emory University. 250μg of MR-1 antibody was administered i.p. on days 14, 16, 18, and 20 post infection.

Tissue harvest and lymphocyte isolation

Lymphocytes were isolated from the spleen as previously described(55). Bone marrow was harvested from 2 femurs by flushing with 10ml RPMI/1% FCS. The total number of antibody secreting cells in the bone marrow was determined by the correction factor for percent of total bone marrow estimated by femurs as previously described (Slifka and

Ahmed 1995). Pieces of tissues for viral titer were weighed and snap frozen in DMEM-1% FCS for viral titer. Serum and tissue LCMV titers were determined by plaque assays using the Vero E6 cell line as described previously (Ahmed et al., 1984).

Antibodies and flow cytometry

All antibodies were obtained from BD Biosciences except for PNA (Vector). All surface stains were done in PBS+1%FCS on ice. B cell stains were pre-incubated with anti-CD16/32 (BD Biosciences). MHC Class I tetramers were generated and used as previously described(55). For the intracellular cytokine stimulations, $1x10^6$ splenocytes were incubated for 5 hours with 2μg/ ml of peptide in the presence of Brefeldin A (GolgiPlug, 1μl/ml). Intracellular cytokine staining was performed using the BD Pharmingen fix/perm kit per the manufacturer's instructions. All samples were read using the BD FACS Calibur and Cell Quest Software and analyzed using Flowjo (Treestar) software.

ELISPOT

LCMV and total IgG ELISPOTS were performed on spleen and bone marrow lymphocytes as previously described (Slifka and Ahmed, 1996). 96-well ELISPOT plates (Millipore) were coated overnight with either anti-mouse IgG (Caltag) or 100μl of LCMV infected BHK lysate diluted in PBS. Plates were then washed with PBS/Tween-20(0.2%) followed by PBS and then blocked with RPMI/10% FCS for 2 hours. Single cell suspensions from the spleen or bone marrow were plated in three-fold dilutions in RPMI/10% and incubated for 5 hours at 37° C. Plates were washed with PBS followed

by PBS/Tween(0.2%) and incubated overnight with biotinylated goat anti-mouse IgG (Caltag) diluted in PBS/1%FCS/Tween (0.2%). Plates were then washed in PBS/Tween and incubated one hour with horseradish peroxidase-Avidin D (Vector). Plates were again washed with PBS/Tween followed by a final wash with PBS. The enzyme chromagen substrate, 3-Amino-9-Ethyl-carbazole (AEC) (MP Biomedical) was diluted in N,N-dimethylformamide (Sigma) at 20mg/ml and stored at 4° C for one month. This AEC stock solution was diluted in 0.1M sodium acetate buffer (pH 5.0) to a final concentration of 0.3mg/ml and filtered with a 0.2μm filter prior to use. Following final wash, 100ul of the enzyme chromogen substrate was added and allowed to incubate for approximately 8 minutes, or until spots could be visualized and background coloring began to become significant. The reaction was stopped by running plates under tap water. Spots were allowed to dry and then were enumerated manually using a stereoscope or by using an automated plate reader and CTL Immunospot software (Cellular Technology).

Quantification of memory B cells

Virus-specific memory B cells were measured by modification of a described limiting dilution method (Slifka and Ahmed, 1996). Graded doses of splenocytes were cultured for 6 days in a flat-bottomed 96-well dish in the presence of $1x10^6$ irradiated (1,200 rad) feeder splenocytes, 0.4 μg of R595 lipopolysaccharide (Alexis Biochemicals) and 20 μl of concanavalin A (ConA) conditioned supernatant supplemented with freshly added 50 μM β-mercaptoethanol, in a total volume of 200 μl. The ConA conditioned supernatant was prepared by culturing $1.25x10^6$ mouse splenocytes with 2.5μg/ml of ConA and

20ng/ml of PMA for 48 hours in 20ml of DMEM/5%FCS. After 48 hours, the supernatant was then collected, cellular debris removed by centrifugation, and the supernatant stored at –80°C until use. Three-fold dilutions of splenocytes were tested in replicates of 12 wells each. After 6 d of polyclonal activation, cells were washed and transferred to LCMV-antigen-coated 96-wellMultiscreen-HA filter plates (Millipore) and ASC ELISPOTs were performed as described above. We calculated the frequency of LCMV-specific memory B cells by standard limiting-dilution single-hit kinetics.

ELISA

LCMV-specific and total IgG ELISAs were performed as previously described(46). Flat bottom 96-well plates (Nunc) were coated with either LCMV lysate or an anti-Ig (αIgG+M+A, Caltag) antibody overnight at room temperature. Plates were then washed with PBS/0.5%Tween-20 and blocked with PBS/10% FCS/0.2%Tween-20 for two hours. Plates were washed (PBS/0.5%Tween-20) and the serum samples are diluted into serial three-fold dilutions in PBS/1%FCS/0.02%Tween across the rows of the plate and incubated 90 minutes at room temperature. The plates were again washed (PBS/0.5%Tween-20) and incubated with a secondary goat anti-mouse horseradish peroxidase conjugated antibody (Caltag) diluted in PBS/1%FCS/0.2%Tween-20. Plates were incubated for 90 minutes at room temperature and then washed in PBS/0.5%Tween-20. Plates were then developed using the o-phenylenediamine dihydrochloride (OPD) chromagen substrate (Sigma) diluted in citrate buffer (pH 5.0), and the reaction quenched by adding 1M HCL after 15 minutes. The absorbance was immediately read at the 405

nm wavelength in a Biorad 3550 plate reader. LCMV-specific antibody titers were determined by an endpoint titer 0.1 OD > background (naïve serum) well.

Statistical analysis

Statistical analysis was performed using the two-tailed unpaired Student's t test using Prism (GraphPad Software Inc.)

Results:

The clone-13 strain of LCMV was isolated from persistently infected carrier mice and differs from the parental Armstrong strain in only 2 amino acids, one in the viral polymerase and one in the glycoprotein. Previous in vitro experiments demonstrated that these mutations result in 1) an enhanced infectivity of macrophages and 2) enhanced viral replication within the macrophages, mapping to the mutation in the glycoprotein, and polymerase each, respectively. In vivo experiments with reassortant Armstrong and clone-13 viruses have shown that the single amino acid change in the viral glycoprotein results in a protracted viremia similar to the clone-13 strain(52). We first wanted to address whether the persistence of virus changed the kinetics and magnitude of the humoral response following clone-13 infection when compared to the response seen following acute LCMV Armstrong infection.

Chronically infected mice mount a humoral response with kinetics similar to the acute infection

C57BL/6 mice were inoculated with either the Armstrong or clone-13 strain of LCMV and B cell responses were monitored in the spleen and bone marrow. Infection with either strain of LCMV resulted in a rapid proliferation of LCMV-specific antibody secreting cells (ASC) in the spleen that peaked approximately one-week post infection. This response contracted by three weeks post infection and a population of long-lived ASC was maintained at a steady level for the life of the animal. LCMV-specific ASC

were first detected in the bone marrow approximately two weeks post infection and were maintained at steady levels similar to acutely infected controls **(Figure 1a)**.

We next examined the isotypes of antibody formed after either acute or chronic LCMV infection. We collected serum from mice infected with either Armstrong or clone-13 and compared the isotype of LCMV-specific antibody by ELISA. In agreement with earlier studies, LCMV Armstrong infection induced primarily an IgG2a antibody response. Interestingly, clone-13 infected mice had high levels of IgG2a antibodies in their serum similar to acutely infected animals, but these mice also had a substantially higher IgG1 response compared to acutely infected controls **(Figure 1b)**.

Infection with the LCMV clone-13 strain is associated with increased immune pathology and destruction of fibroblastic reticular cells in the spleen, so we wanted to examine whether germinal centers form normally in these chronically infected mice. We examined the kinetics of germinal center formation by flow cytometry and saw similar kinetics of germinal centers by PNA and FAS co-staining B cells. Chronically infected mice had a higher peak frequency of PNA+FAS+ B cells and had slightly more cells staining at late time-points **(Figure 1c)**. This slight elevation in PNA and FAS staining correlated with only very small germinal centers by immunofluorecense, and these mice had background levels of staining by two months post infection (data not shown). Memory B cells are responsible for secondary immune responses and may play a role in the replenishment of long-lived plasma cells. One-month post infection, Armstrong and clone-13 mice had similar levels of LCMV-specific memory B cells in the spleen **(Figure 1d)**.

Antibody depletion of cell populations is ineffective during the acute stages of LCMV clone-13 infection

Earlier studies in our lab have shown a critical role for CD4 T cells during LCMV infection by depleting CD4 T cells prior to LCMV clone-13 infection(56). However, we wanted to examine the role CD4 T cells play after the initial CD8 T cell priming and what role CD4 T cells played in maintaining the B cell response during peak germinal center formation during LCMV clone-13 infection. CD4 T cells in LCMV clone-13 infected mice undergo exhaustion similar to that seen by CD8 T cells(61). In agreement with these earlier studies, CD4 T cells isolated at day 15 post clone-13 infection made less IFNγ and TNFα following in vivo stimulation compared to CD4 T cells from acutely infected mice **(Figure 1e)**.

Therefore, we were interested in seeing if these CD4 T cells continue to provide signals needed for CD8 and B cell priming despite their exhausted phenotype. We first attempted to address this question by depleting CD4 T cells beginning at two weeks post clone-13 infection. We chose to deplete around two weeks post infection because this will allow the initial CD8 T cell priming to occur and although it is after initial formation of germinal centers, long-lived plasma cells and memory B cells have not yet reached peak levels. Mice were administered αCD4 (GK1.5) on days 15 and 17 post infection and then were sacrificed one week later on day 22 post infection. Surprisingly, we saw minimal reductions in the percent of CD4 T cells in the spleens of the three clone-13 mice treated with αCD4, but almost complete depletion of CD4 T cells in infection matched Armstrong animals **(Figure 2)**. This inability to deplete is not specific to the CD4 T cell subset, as both CD8 T cells and B cells (CD20 transgenic) did not deplete

during the early stages of clone-13 infection. We can deplete CD4 T cells from these mice during the later stages of clone-13 infection, as mice begin to control serum viremia (E.J. Wherry, and W.T. Langley, unpublished observations).

To better understand the role CD4 T cells play in B cell and CD8 T cell responses during the later stages of priming we decided to block the CD40:CD154 (CD40L) pathway. Mice lacking CD40L fail to form germinal centers and make long-lived plasma cells or memory B cells during acute LCMV infection(43, 172). Here we wanted to examine what role simply transiently blocking the ongoing B cell response would have on the long-term B cell response during chronic LCMV infection.

αCD154 (MR-1) treatment reduces spleen cellularity and alters B lymphocyte subsets in the spleen of LCMV clone-13 infected mice

We first wanted to examine the overall effects of αCD154 treatment in clone-13 infected mice. Mice infected with LCMV clone-13 were treated with αCD154 at days 14, 16, 18, and 20 post infection, and the spleens were analyzed at day 23-post infection **(Figure 3a)**. Mice infected with the clone-13 strain of LCMV and treated with αCD154 had an approximately 2-fold reduction in lymphocytes recovered from the spleen at day 23-post infection ($p=0.007$). When we looked at specific lymphocyte subsets in the spleen we saw no reduction in the percent of CD8 T cell or CD4 T cells. There was a slight decrease in the percent of memory CD8 T cells (CD44 high) but this was not statistically significant ($p=0.283$). Mice treated with αCD154 did have a reduction in the total numbers of B cells ($p=0.004$), and this loss of B cells was more pronounced in the naïve IgD- B cell subset ($p=0.0002$) **(Figure 3b)**. Analysis of the LCMV-specific CD8 T

cell responses showed no differences in the number of tetramer positive cells for either the GP33 or GP276 epitope of LCMV (data not shown) or in the ability of LCMV-specific CD8 T cells to make IFNγ following in vitro peptide stimulation (p=0.18 for GP33 and p=0.40 for GP276) **(Figure 3c)**. We did observe a significant loss in the number of LCMV-specific CD4 T cells making IFNγ following stimulation with the GP61 peptide **(Figure 3d)**. Therefore, although αCD154 treatment did reduce the CD4 T cell and B cell response, there was no direct effect on the number or function of LCMV-specific CD8 T cells.

αCD154 treatment of clone-13 mice results in abrogation of humoral immunity in chronically infected mice

We next examined the role CD40:CD154 blockade had on the B cell response during chronic LCMV infection. Clone-13 infected mice were treated with αCD154 on day 14, 16, 18, 20, and the B cell responses in the spleen and bone marrow were analyzed at day 23 post infection. Immediately following αCD154 treatment at day 23, there was a dramatic reduction in the number or LCMV-specific plasma cells in the spleen, and a slight reduction in the number of ASC the bone marrow **(Figure 4a)**. Mice treated with αCD154 also had a lower frequency of germinal center B cells as measured by PNA and FAS staining in the spleen **(Figure 4b)**. These mice also had reduced accumulation of memory B cells in the spleen compared to untreated clone-13 infected controls **(Figure 4c)**. This data confirms a role for CD40:CD154 signaling for the maintenance of germinal centers and blockade of this pathway resulted in decreased accumulation of

LCMV-specific antibody secreting cells and memory B cells in chronically LCMV-infected mice.

Transient CD154 blockade results in long-term reduction of LCMV-specific antibody

Despite the similar kinetics of the B cell response, it is important to note that during LCMV clone-13 infection there is a persisting viremia of approximately 2-3 months. We wanted to ask whether there was a low level of B cell priming during this prolonged viremia. Mice transiently treated with αCD154 from day 14-20 were then left untreated and LCMV-specific antibody response was followed longitudinally in the serum. LCMV-specific antibody titers remained lower in animals that had received the single week of CD154 blockade, up to 75 days post infection **(Figure 5a)**. This was not due to non-specific killing of plasma cells as mice with reduced LCMV-specific antibody had similar levels of total antibody in their serum at the same time-point (day 48 post infection) **(Figure 5b)**.

This reduction of LCMV-specific antibody levels following CD154 blockade correlated with a reduced number of long-lived plasma cells in the spleen of treated mice examined at 4 months post infection **(Figure 5a)**. Therefore, although this was a transient blockade of the B cell response, it resulted in long-term reductions in humoral immunity in the αCD154 treated mice.

Reduced viral control and increased CD8 T cell exhaustion following transient CD154 blockade

B cell responses have been shown to play a critical role in the clearance of chronic LCMV strains(181). We therefore wanted to examine whether a reduction in the B cell response resulted in a reduced ability to control viremia in the αCD154 treated mice. Mice treated with αCD154 had similar levels of virus immediately following cessation of treatment at day 25-post infection, but had significantly increased levels of serum virus at day 35 ($p=0.002$) and day 45 ($p=0.007$) post infection **(Figure 6a)**. Mice treated with αCD154 showed impaired ability to clear serum virus; at day 75 post infection 5/6 untreated mice had completely suppressed serum virus, compared to only 1/6 of the αCD154 treated mice.

To further examine this reduced viral control following αCD154 treatment, mice were sacrificed at 4 months post infection and the viral titers were examined in several tissues. Two of the three mice examined had persisting virus in the serum. In addition, the mouse that had suppressed serum virus still had detectable levels of virus in the spleen. Moreover, mice treated with αCD154 had higher levels of viral loads in the kidney, a long-term reservoir of virus during LCMV clone-13 infection, compared to untreated controls **(Figure 6b)**. Therefore, mice transiently treated with αCD154 have lower levels of viral control in both the serum and tissues long-term after the blockade.

We next examined the CD8 T cell response at 4 months post infection. Mice treated with αCD154 did not have significantly fewer numbers of LCMV-specific tetramer positive cells for the GP33 or GP276 epitope in the spleen, liver or lungs **(Figure 6c)**. However, there was increased CD8 T cell exhaustion, with a slight reduction in the CD8 T cells making IFNγ cells to several CD8 T cell LCMV epitopes. More importantly, there was a severe reduction in the number of IFNγ+ CD8 T cells that

were also making TNFα. The total number of IFNγ+TNFα+ cells for either the GP33 or GP276 epitope was severely reduced in mice receiving αCD154 **(Figure 6d)**. Thus, reductions in B cell responses and CD4 T cell responses had a long-term impact on the ability to control serum viremia and prevent CD8 T cell exhaustion.

Discussion:

The magnitude of the primary CD8 T cell response during acute LCMV infection is not altered in mice lacking B cells or CD4 T cells(182). However, this primary CD8 T cell response may not be sufficient to completely clear virus, and the subsequent exhaustion of the CD8 T cells can lead to reappearance of circulating virus(73). Furthermore, it has been shown that mice lacking CD4 T cells and B cells fail to clear persisting strains of LCMV(56, 72). However, many of these previous studies have been done in gene-knockout animals or mice that are depleted of CD4 T cells prior to infection. Although CD4 T cell help is not thought to be required for initial CD8 T cell priming, it is hard to rule out a role for early signals by the CD4 T cells during the programming of CD8 T cells in that model. In this study, we wanted to provide CD4 T cell help during the initial priming of CD8 T cells but then transiently remove help during the B cell priming. Mice were treated with the αCD40L (CD154) antibody MR-1 for one week starting at two weeks post infection. Mice transiently treated with αCD154 antibody had intact cytokine production by LCMV-specific CD8 T cells, but had abrogated B cell responses and poor cytokine production by antigen-specific CD4 T cells. The B cell response remained lower in the treated mice long-term after cessation of antibody blockade and these mice had long-term deficits in viral control. This resulted in higher levels of CD8 T cell exhaustion and increased viral burden in these animals, suggesting that CD4 T cell and B cell responses play an important role for in controlling viral replication and preventing CD8 T cell exhaustion during chronic viral infection.

The CD40-CD154 pathway has been shown to be essential for B cell priming by CD4 T cells, but does not alter initial CD8 T cell priming. Mice deficient in CD154 fail

to produce germinal centers, memory B cells and long-lived plasma cells when infected with LCMV or vesicular stomatitis virus (VSV) (43, 44). This is not dependent on CD40 interactions on the antigen presenting cells, as CD4 T cells primed in CD40 deficient hosts are capable of providing help for antibody production to the T-dependent antigen LCMV-DNP (172). However, CD4 T cells primed in CD154 deficient animals do have defects in cytokine function and proliferation. Interestingly, mice infected with VSV have defects in cytokine production by CD8 T cell cells, and co-infection with LCMV rescues these CD8 T cells. Thus, there may be deficits in signaling to CD8 T cells in CD154 deficient mice that are not detected in the acute LCMV-infection model(173).

In this study, CD4 T cells were initially primed in the presence CD40:CD154 signaling; however, we measured reduced cytokine function in LCMV-specific CD4 T cells one week after αCD154 antibody treatment. Continued signaling through the CD40:CD154 pathway may be required to maintain CD4 T cell function during a protracted viral infection such as LCMV clone-13. On the other hand, this inability to detect cytokine production could also be due to direct deletion of antigen-specific CD4 T cells. However, this study used only a transient blockade of one week, and serum virus persists following the termination of antibody therapy. Thus, it is possible that new CD4 T cells could be primed in these animals following termination of the blockade. Indeed, transgenic LCMV-specific CD4 T cells (SMARTA) transferred into clone-13 infected mice at day 22 post infection become activated and secrete cytokines when examined two weeks post transfer (R.D. Aubert, unpublished observation). Recent studies by our lab also have also shown that LCMV-specific CD4 T cells can be primed and influence ongoing CD8 T cell and B cell responses in mice with high levels of CD8 T cell

exhaustion (R.D. Aubert, unpublished observations). Therefore, although we cannot rule out a direct role for the lack of CD4 T cell help in CD8 T cell exhaustion, this data suggests that the transient removal of CD4 T cell help is not likely solely responsible for the long-term deficits in viral control seen in this study.

Although B cell responses were thought to be non-essential for the priming of CD8 T cells and the clearance of LCMV Armstrong infection, recent studies have shown low levels of persistent virus in these mice. B cell deficient mice are unable to suppress the acute LCMV-Traub strain(73), and B cell responses have been shown to be essential for clearance of chronic strains of LCMV such as clone-13 and WE. Additionally, B cells and CD4 T cells both prevent exhaustion of CD8 T cells transferred into LCMV infected carrier mice(183). Early LCMV infection studies showed that administration of LCMV-specific antibody protected newborn mice from lethal LCMV challenge. Although neutralizing antibody is not detected until approximately one month post infection, anti-LCMV antibodies have been shown to mediate viral reduction by Fc activity, with the IgG2a isotype being the crucial determinant of in vivo protection(49). In addition, experiments with LCMV-WE have shown that passive transfer of hyperimmune serum was capable of controlling viremia and preventing loss of CTLs in CD154 knockout mice. However, this study started treatment immediately following infection, and may have resulted in a lower initial viremia which would not naturally occur since early antibody responses peak approximately one week post infection. Here, we see a similar role for B cells and antibody in the preventing CD8 T cell exhaustion and in mediating viral control during LCMV clone-13 infection. When the B cell response was inhibited during the ongoing germinal center reaction, levels of plasma

cells were reduced in the spleen, and there is was subsequent increased level of serum virus by day 35-post infection. These mice had difficulty suppressing serum virus and continued to harbor higher levels of virus in the serum and tissues up to 4 months post infection. These mice had no differences in the numbers of LCMV-specific CD8 T cells in the tissues at 4 months post transfer but those CD8 T cells exhibited higher levels of exhaustion with greater loss of IFNγ and almost complete loss of cells capable of co-producing IFNγ and TNFα.

In our study, we were able to transiently block CD4 T cell help through the CD40:CD154 pathway during the latter stages of CD8 T cell and B cell priming. This blockade did not directly affect the CD8 T cells, but lead to deficits in CD4 T cell function and reduced germinal center activity, resulting in a lack of accumulation of memory B cells and long-lived plasma cells. Immediately following the blockade we saw no differences in virus titer, but the mice transiently treated with αCD154 antibody had reduced ability to control serum virus. This resulting protracted viremia resulted in greater CD8 T cell exhaustion and a long-term inability to control LCMV clone-13 virus. Here we have shown that CD8 T cells primed in a CD4 sufficient environment cannot control virus without cooperation from CD4 T cells and antibody. This work suggests that continued focus on vaccines that induce both strong humoral and cellular immune responses will be critical to control of chronic infections.

Chapter 2 Figure Legends:

Figure 2-1: Kinetics of humoral immune response during acute and chronic LCMV infection. Mice were infected with either the acute (Armstrong) or chronic (clone-13) strain of LCMV and humoral immune responses were compared at each time-point. (A) The kinetics of antibody secreting cells was measured in the spleen and bone marrow by ELISPOT. (B) Comparison of antibody isotypes at day 45-post infection. (C) Kinetics of germinal centers as measured by PNA+FAS+ staining in the spleen during LCMV infection. (D) LCMV-specific memory B cells measured at one-month post infection. (E) Cytokine production by LCMV-specific CD4 T cells (GP61 epitope) at day 15 post infection. Percentage in parenthesis is percent of $IFN\gamma^+$ cells co-producing TNFα. (n= 3-10 mice per group for all B cell assays and cytokine plots are one representative mouse from several experiments).

Figure 2-2: Antibody depletion of cell populations ineffective during the acute stages of LCMV clone-13 infection. Mice infected with either LCMV Armstrong or Clone-13 15 days prior were given 250μg αCD4 (GK1.5) on day 15 and 17 post infection. CD4 and CD8 T cell percentages in the spleen were measured on day 22-post infection.

Figure 2-3: αCD154 (MR-1) treatment reduces spleen cellularity and alters B lymphocyte subsets in the spleen of LCMV clone-13 infected mice. (A) Experimental design: mice were treated with αCD154 (MR-1) for one week starting at day 15 post infection and the spleens were analyzed day 23 post infection. (B) Overall cellularity of

the spleen and altered frequency of lymphocyte subsets in the treated versus untreated clone-13 infected mice. (C) Total number of CD8 T cells making IFNγ when re-stimulated with the two immunodominant LCMV epitopes (GP33 and GP276). (D) Reduced IFNγ production by LCMV-specific CD4 T cells. (n=4 mice/group). ns=not significant, *p<0.05, **p<0.01, ***p<0.005 all analyzed by student t-test.

Figure 2-4: αCD154 treatment of clone-13 mice results in abrogation of humoral immunity in chronically infected mice. B cell responses were analyzed in the spleen and bone marrow following αCD154 blockade. Mice were treated day 15-20, and analyzed day 23-post infection. (A) Antibody secreting cells in the spleen and bone marrow measured by ELISPOT (B) Germinal center B cells measured by PNA+FAS+ staining in the spleen (line indicates background staining measured in an uninfected mouse). (C) LCMV-specific memory B cells in the spleen. Limit of detection is 100 MBCs/spleen (n=3 untreated, n= 4 αCD154 treated mice). All statistical analysis performed using student t-test.

Figure 2-5: Transient CD154 blockade results in long-term reduction of LCMV-specific antibody. (A) LCMV-specific antibody was measured longitudinally in the serum following αCD154 treatment. (n=6-8 mice/ group) (B) Total IgG levels in the serum at day 48-post infection as measured by ELISA. (C) Antibody secreting cells in the spleen and bone marrow were measured at 4 months post infection measured by ELISPOT analysis (n=3mice/group). **p<0.01, ***p<0.005, analyzed by student t-test.

Figure 2-6: Reduced viral control and increased CD8 T cell exhaustion following transient CD154 blockade results in long-term reduction of LCMV-specific antibody. (A) Serum viral titers measured longitudinally following αCD154 blockade (n=6-8 mice/group) *$p<0.05$, **$p<0.01$, no statistical significance at day 24 ($p=0.2$) (B) Tissue titers in the spleen and kidney 4 months post infection (n=3 mice/group). Dotted line for (A) and (B) indicate limit of detection. (C) Number of tetramer positive cells in the tissues for the immunodominant LCMV epitopes GP33 and GP276 at 4 months post infection. (D) Cytokine production by CD8 T cells at 4 months post infection. Parenthesis indicate percentage $IFN\gamma^+$ cells co-producing TNFα. Summarized graph is the total number of $IFN\gamma^+$ cells co-producing TNFα for either the GP33 or GP276 epitope.

Figure 2-1

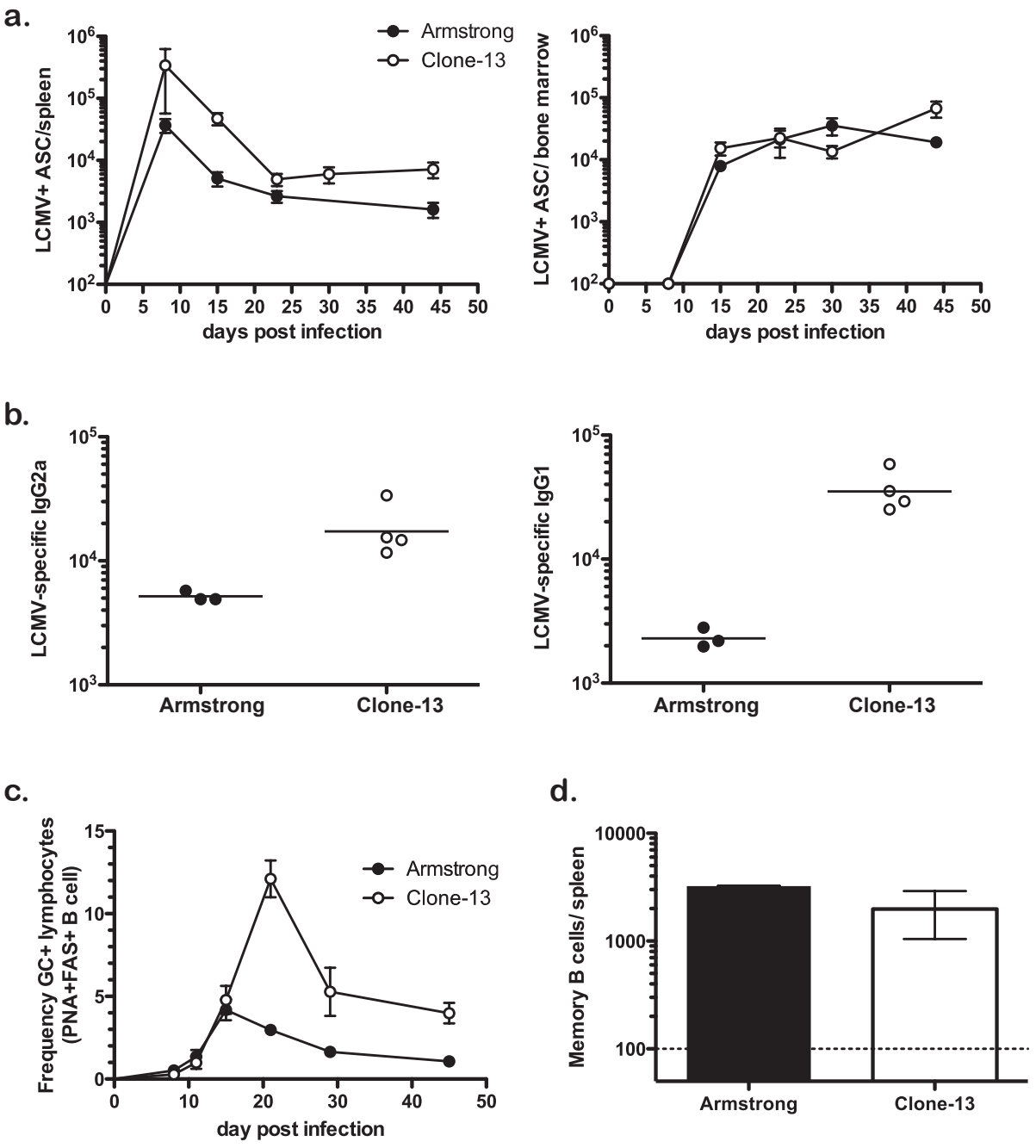

a.
LCMV+ ASC/spleen
Armstrong
Clone-13
days post infection
LCMV+ ASC/ bone marrow
days post infection
b.
LCMV-specific IgG2a
Armstrong
Clone-13
LCMV-specific IgG1
Armstrong
Clone-13
c.
Frequency GC+ lymphocytes (PNA+FAS+ B cell)
Armstrong
Clone-13
day post infection
d.
Memory B cells/ spleen
Armstrong
Clone-13

Figure 2-1

e.

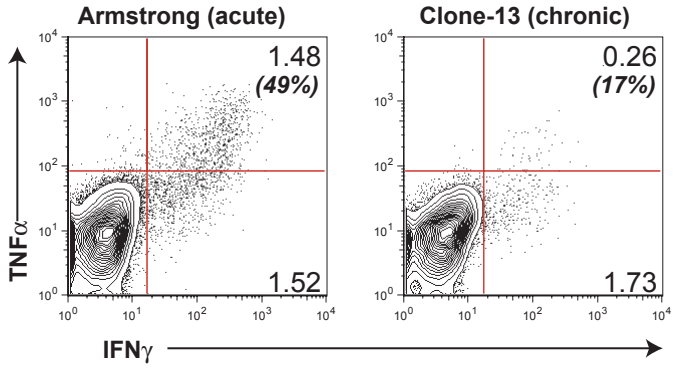

Armstrong (acute)
Clone-13 (chronic)
1.48
(49%)
1.52
0.26
(17%)
1.73
TNFα
IFNγ

Figure 2-2

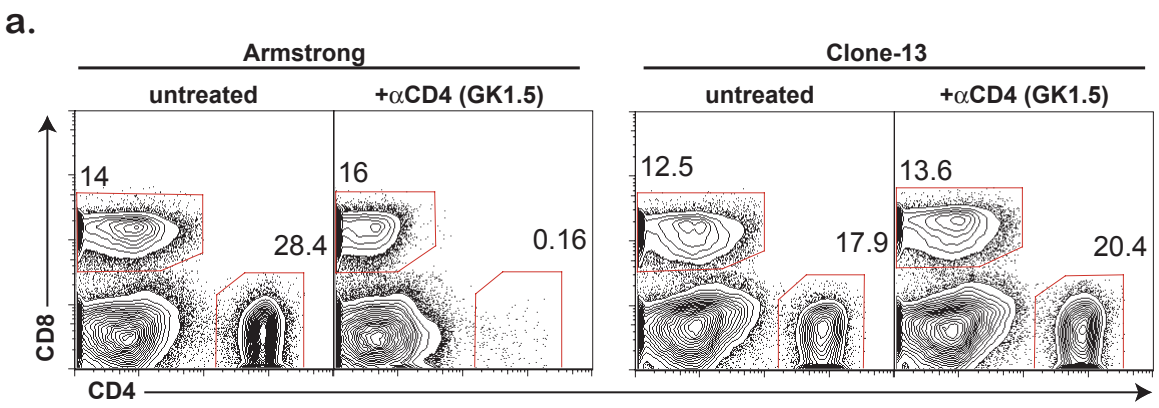

a.
Armstrong
Clone-13
untreated
+αCD4 (GK1.5)
untreated
+αCD4 (GK1.5)
14
28.4
16
0.16
12.5
17.9
13.6
20.4
CD8
CD4

Figure 2-3

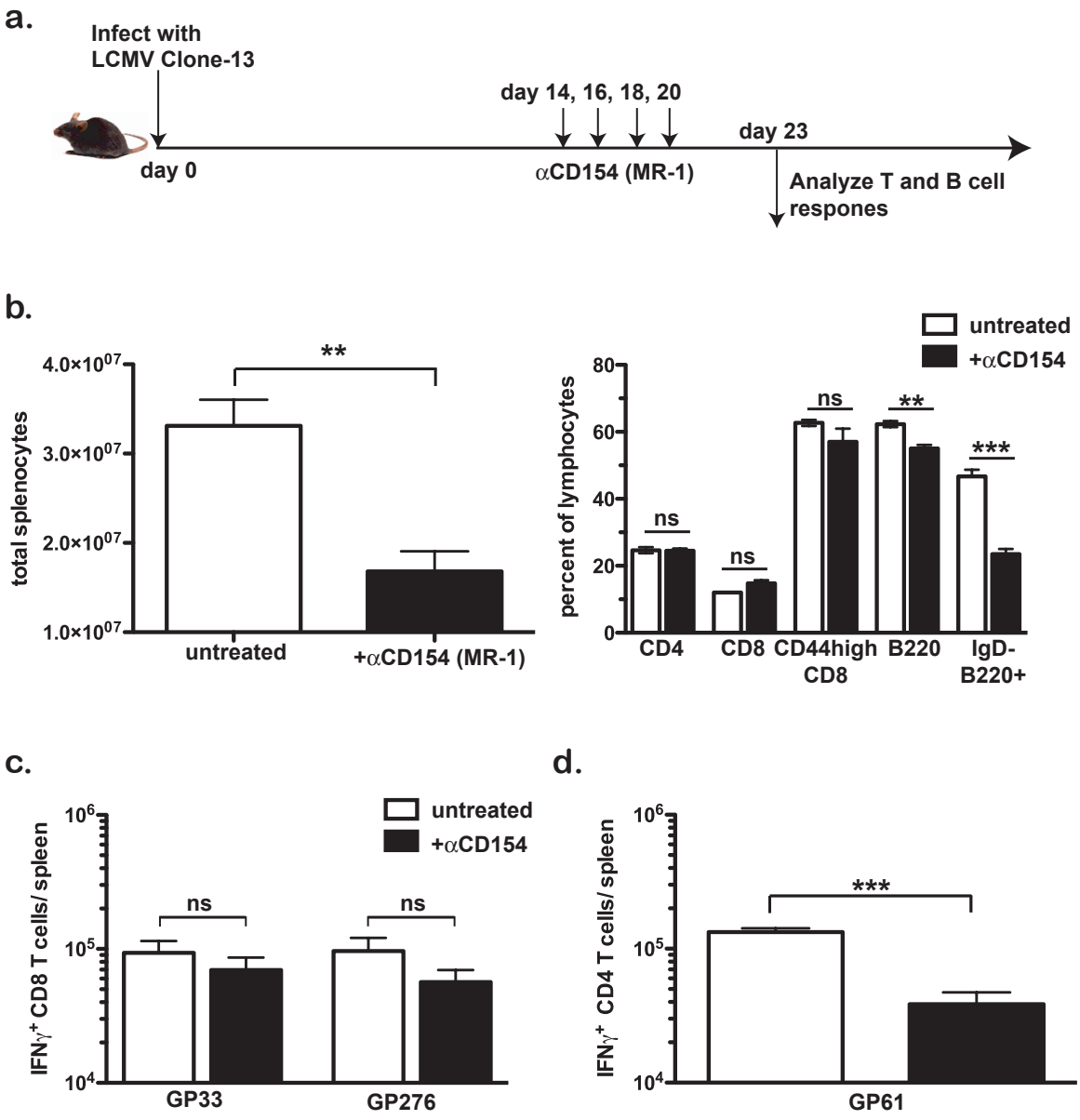

a.
Infect with
LCMV Clone-13
day 0
day 14, 16, 18, 20
αCD154 (MR-1)
day 23
Analyze T and B cell
respones
b.
total splenocytes
4.0×10^07
3.0×10^07
2.0×10^07
1.0×10^07
**
untreated
+αCD154 (MR-1)
untreated
+αCD154
percent of lymphocytes
80
60
40
20
0
ns
ns
ns
**
***
CD4
CD8
CD44high CD8
B220
IgD- B220+
c.
IFNγ+ CD8 T cells/ spleen
10^6
10^5
10^4
untreated
+αCD154
ns
ns
GP33
GP276
d.
IFNγ+ CD4 T cells/ spleen
10^6
10^5
10^4
***
GP61

Figure 2-4

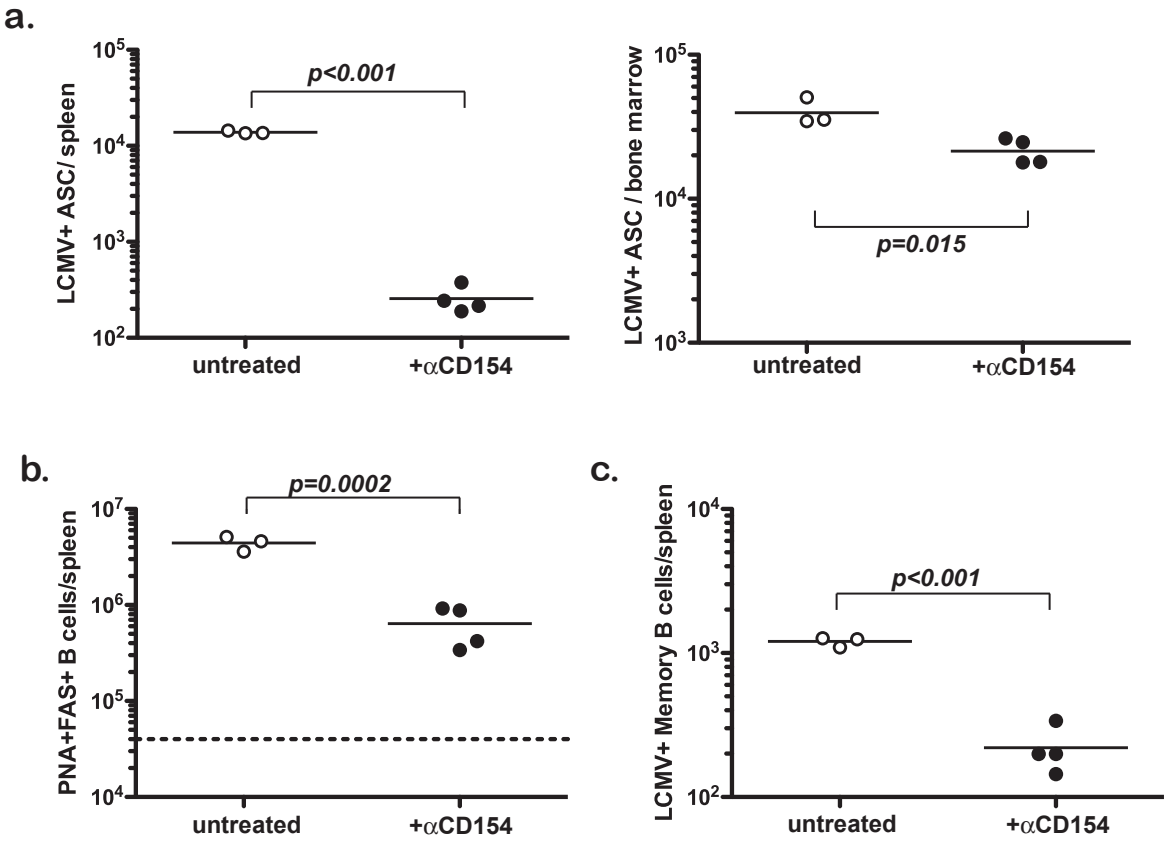

a.
LCMV+ ASC/ spleen
10^5
10^4
10^3
10^2
p<0.001
untreated
+αCD154
LCMV+ ASC / bone marrow
10^5
10^4
10^3
p=0.015
untreated
+αCD154
b.
PNA+FAS+ B cells/spleen
10^7
10^6
10^5
10^4
p=0.0002
untreated
+αCD154
c.
LCMV+ Memory B cells/spleen
10^4
10^3
10^2
p<0.001
untreated
+αCD154

Figure 2-5

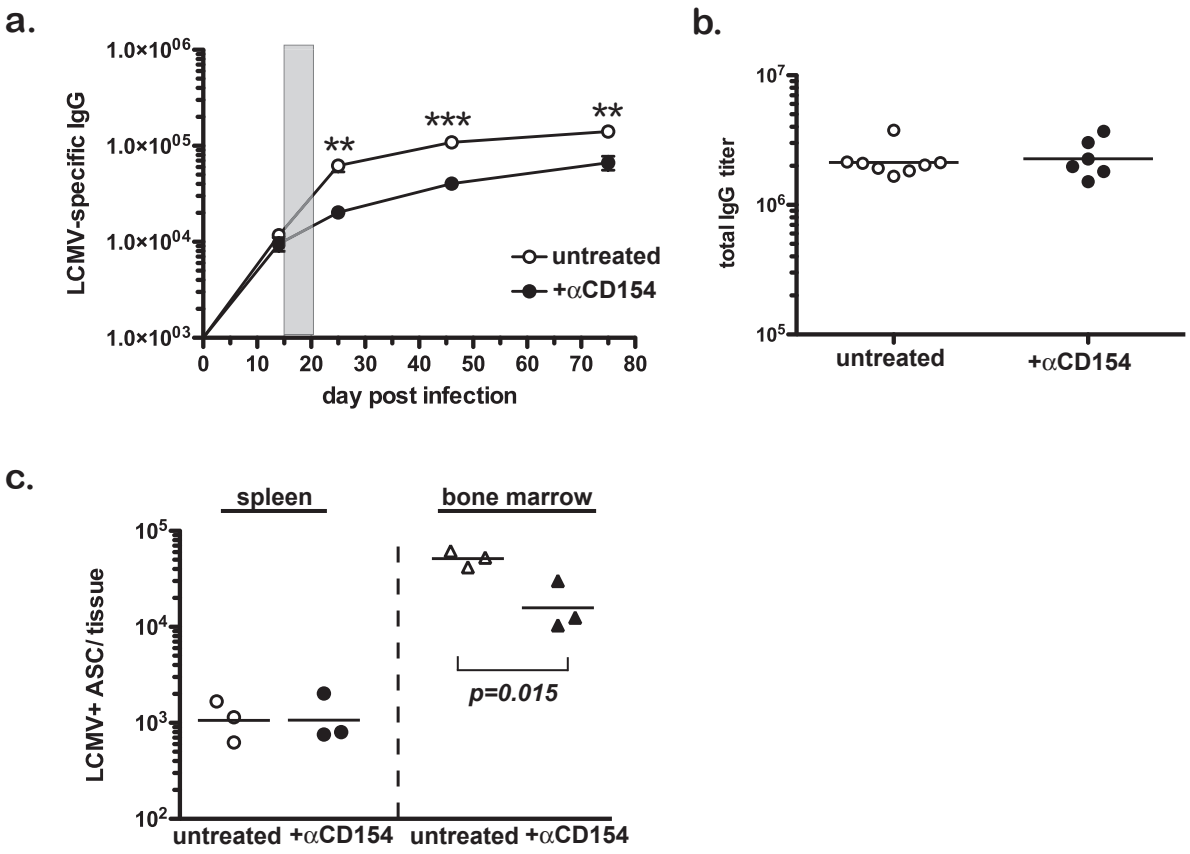
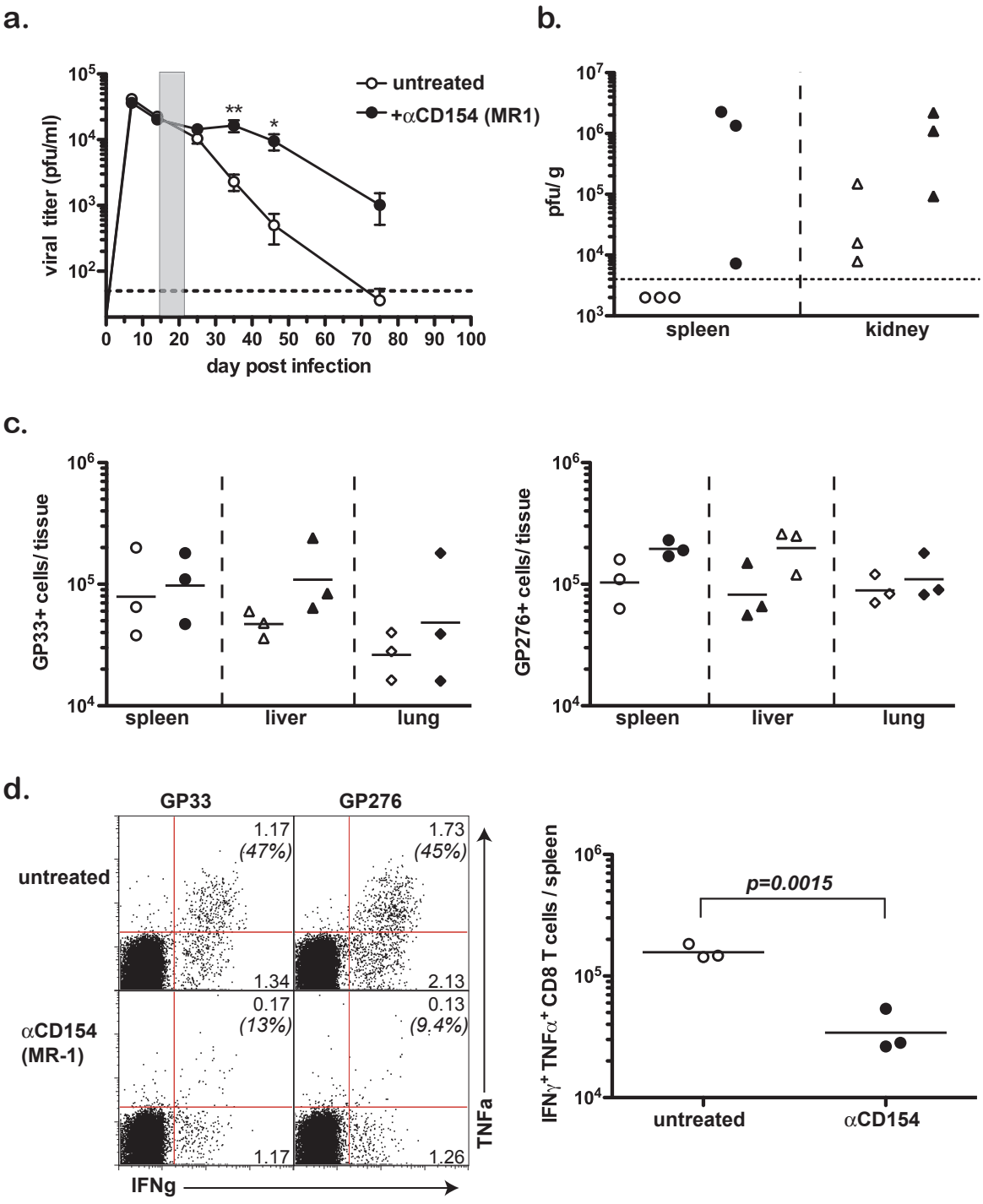

a.
LCMV-specific IgG
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
0 10 20 30 40 50 60 70 80
day post infection
**
***
**
untreated
+αCD154
b.
total IgG titer
10^7
10^6
10^5
untreated
+αCD154
c.
spleen
bone marrow
LCMV+ ASC/ tissue
10^5
10^4
10^3
10^2
p=0.015
untreated +αCD154 untreated +αCD154

Figure 2-6


a.
b.
c.
d.
untreated
+αCD154 (MR1)
viral titer (pfu/ml)
day post infection
pfu/ g
spleen
kidney
GP33+ cells/ tissue
GP276+ cells/ tissue
liver
lung
GP33
GP276
1.17 (47%)
1.73 (45%)
1.34
2.13
0.17 (13%)
0.13 (9.4%)
1.17
1.26
αCD154 (MR-1)
IFNg
TNFa
p=0.0015
IFNγ+ TNFα+ CD8 T cells / spleen

Chapter 3: CD4 T cell help rescues exhausted CD8 T cells

Abstract:

CD4 T cell help has been shown to be important in the maintenance of CD8 T cells capable of clearing persistent viral infections in both mice and humans. CD4 immunotherapy, therefore, may be a compelling approach to overcome CD8 T cell exhaustion during chronic infections. Here, we demonstrated that naïve antigen-specific CD4 T cells transferred into mice chronically infected with lymphocytic choriomeningitis virus were capable of being primed *in vivo*. These CD4 T cells divided, produced cytokine, and persisted long-term. Furthermore, these CD4 T cells had a profound effect on the exhausted CD8 T cells. Following CD4 T cell transfer there was a rapid expansion of LCMV-specific CD8 T cells in both lymphoid and non-lymphoid tissues. The expanding CD8 T cell population has enhanced effector function, and the rescue was observed across a wide-range of major and minor LCMV-specific epitopes. The LCMV-specific antibody response was also improved following CD4 T cell transfer and this improved immune response resulted in reductions in serum viral titer. Combining the CD4 T cell therapy with a blockade of the PD-1/PD-L1 pathway resulted in enhanced CD4 T cell function in-vivo and further synergized the rescue of LCMV-specific CD8 T cells. Overall, this data suggests that adoptive transfer of antigen-specific CD4 T cells could provide a useful strategy to improve overall CD8 T cell and B cell function during chronic viral infection.

Introduction:

Effector CD8 T cell responses are important in controlling viral infection. Work from our lab and others has shown that CD4 T cells are required to maintain CD8 T cell responses during persistent lymphocytic choriomeningitis virus (LCMV)(56, 60, 71, 184) and mouse gamma-herpesvirus infections(184). CD4 T cells have also been shown to be critical for maintenance of CD8 T cell responses in hepatitis C virus (HCV) infected chimpanzees(86) and T cell help has been positively correlated with CTL activity capable of triggering spontaneous recovery from acute HCV infections(162). Moreover, experiments have shown that chronic exposure to antigen often leads to impaired CD4 T cell function and this dysfunction parallels decreased CD8 T cell function(61). Impaired CD4 function has been implicated in the diminished proliferative capacity of human immunodeficiency virus (HIV)-specific CD8 T cell response(163), and longitudinal clinical studies have shown CD4 T cell dysfunction during relapsing HCV infection(88).

Direct immune transfer of CTLs has been shown to be successful in targeting tumors(185) and preventing human cytomegalovirus (CMV) disease in bone marrow transplant recipients(186, 187). However, targeting of CD4 T cells may also be helpful in the context of replicating pathogens such as HIV and HCV that often undergo CTL escape(188, 189). Previous studies transferring LCMV-specific CD4 T cells into chronically infected mice show a low frequency of viral escape to the CD4 T cell epitope(70). CD4 T cell help has also been suggested to be important for long-term CTL survival post transfer(186). Moreover, restoring function in CD4 T cells may provide signals that can enhance B cell responses, and a balanced CD8 T cell and B cell response

may be critical for combating the high viral loads and preventing viral escape during HCV and HIV infection.

By transiently depleting CD4 T cells at the time of infection, mice are unable to mount a robust CD8 T cell response to the chronic strain of LCMV clone-13(56). These mice have extremely low-levels of LCMV-specific CD8 T cells detectable by tetramer and the antigen-specific CD8 T cells make small amounts of IFNγ, but no TNFα or IL-2. There is no detectable antigen-specific CD4 T cell response and these mice fail to produce LCMV-specific antibody. In this study, we examined whether restoration of CD4 T cell help by direct transfer of antigen-specific CD4 T cells can improve CD8 T cell and B cell responses during an ongoing chronic infection. Transferred LCMV-specific CD4 T cells proliferated and produced cytokine *in vivo*. Following CD4 T cell transfer there was elevated numbers of tetramer positive cells in the tissues and enhanced cytokine produced by exhausted CD8 T cells. The CD4 rescue was antigen-specific and also led to the emergence of an LCMV-specific antibody response. Short-term treatment with αPD-L1 resulted in improved function of the transferred cells and resulted in synergistic improvements in the CD8 T cell rescue and viral control. Overall, these results suggest that adoptive transfer of CD4 T cells in conjunction with antibody immunotherapy may provide a promising strategy to overcome chronic viral infections such as HIV and HCV and to enhance immune responses to established tumors.

Materials and Methods:

Mice and infection

4-6wk old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). SMARTA transgenic mice, with CD4 T cells specific for the gp61-80 (GLNGPDIYKGVYQFKSVEFD) epitope of LCMV(190), were provided by Dr. Charles Surh (Scripps Research Institute) and were backcrossed onto Thy1.1 or Ly5.1 congenic mice strains and maintained at Emory University. LCMV clone-13 stocks were grown in BHK cells and titered on Vero E6 cells as previously described(12). 6-wk old mice were infected with $2x10^6$ pfu of LCMV clone-13 i.v. For depletion of CD4 T cells prior to infection, mice were given 500μg of anti-mouse CD4 (GK1.5; BioExpress) i.p. on days –2 and 0 prior to infection as previously described. (Matloubian et al., 1994). All mice were used in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines.

CD4 T cell isolation and in-vivo antibody blockade

Splenocytes were isolated from SMARTA transgenic mice. Untouched CD4 T cells were selected by incubation with biotinylated antibody cocktail (CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119), followed by anti-biotin magnetic bead selection of non-CD4 T cells on MACS columns per manufacturer's recommendations (Miltenyi). Post column purity was approximately 95% CD4 T cells and contaminating CD8 T cells were <0.01% following selection. Purified CD4 T cells were diluted in PBS

and $4x10^6$ cells were injected i.v. into uninfected (naïve) B6 mice, or into mice infected with LCMV clone-13 approximately 2-3 months prior to transfer. For CFSE labeling, $10x10^6$ purified SMARTA CD4 T cells were incubated with 2.5μM 5-(and –6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described. For αPD-L1 blockade, 200μg of rat anti-mouse PD-L1 antibody (10F.9G2) was administered i.p. 5 times over fifteen days at three-day intervals beginning at one day post SMARTA transfer.

Tissue harvest and lymphocyte isolation

Lymphocytes were isolated from tissues including the spleen, liver, lung and blood as previously described(55). Livers and lungs were perfused with cold PBS prior to removal for lymphocyte isolation. Bone marrow was harvested from 2 femurs by flushing with cold RPMI/1%FCS. Pieces of tissues for viral titer were weighed and snap frozen in DMEM-1% FCS for viral titer. Serum and tissue LCMV titers were determined by plaque assays as described previously (Ahmed et al., 1984).

Antibodies and flow cytometry

All antibodies were obtained from BD Biosciences except for PNA (Vector) and PD-1 (Biolegend). All surface stains were done in PBS+1%FCS on ice. B cell stains were pre-incubated with anti-CD16/32 (BD). MHC Class I tetramers were generated and used as previously described(55). For the intracellular cytokine stimulations, $1x10^6$ splenocytes were incubated for 5 hours with 2μg/ ml of peptide in the presence of Brefeldin A (GolgiPlug, 1μl/ml). Intracellular cytokine staining was performed using the

BD Pharmingen fix/perm kit per the manufacturer's instructions. All samples were read using the BD FACS Calibur and Cell Quest Software or BD LSRII and Diva Software and analyzed using Flowjo (Treestar) software.

ELISA

LCMV-specific and total IgG ELISAs were performed as previously described(46). Briefly, 96-well plates (Nunc) were coated with either LCMV lysate or an anti-Ig (αIgG+M+A, Caltag) antibody overnight at room temperature. Plates were then washed with PBS/0.5%Tween-20 and blocked with PBS/10% FCS/0.2%Tween-20 for two hours. Plates were washed (PBS/0.5%Tween-20) and the serum samples are diluted into serial three-fold dilutions in PBS/1%FCS/0.02%Tween across the row of the plate and incubated 90 minutes at room temperature. The plates were again washed (PBS/0.5%Tween-20) and incubated with a secondary goat anti-mouse horseradish peroxidase conjugated antibody (Caltag) diluted in PBS/1%FCS/0.2%Tween-20. Plates were incubated for 90 minutes at room temperature and then washed in PBS/0.5%Tween-20. Plates were then developed using the o-phenylenediamine dihydrochloride (OPD) chromagen substrate (Sigma) diluted in citrate buffer (pH 5.0), and the reaction quenched by adding 1M HCL after 15 minutes. The absorbance was immediately read at 405 nm wavelength in a Biorad 3550 plate reader. LCMV-specific antibody titers were determined by an endpoint titer 0.1 OD > background (naïve serum) well.

Statistical analysis

Statistical analysis was performed using the two-tailed unpaired Student's t test or the Mann-Whitney test (where noted) using Prism software analysis (GraphPad Software Inc.).

Results:

To examine the ability of CD4 T cell help to influence CD8 T cell exhaustion we used a stringent model of CD8 T cell exhaustion wherein we get lack of viral control and very high levels of CD8 T cell dysfunction. Naïve mice are depleted of CD4 T cells by administration of the gk1.5 antibody prior to infection with the clone-13 strain of LCMV. CD4 T cell numbers in these mice return to normal numbers over 2-3 weeks, but no antigen-specific CD4 T cells can be detected by intracellular cytokine analysis. These chronically infected mice demonstrate high levels of viremia and high levels of CD8 exhaustion, with very few LCMV-specific CD8 T cells capable of making IFNγ, TNFα, or IL-2 (56).

Naïve transgenic CD4 T cells undergo rapid antigen-driven activation and proliferation following transfer into chronically infected hosts.

Initial studies examined the fate of naïve LCMV-specific CD4 T cells when transferred into LCMV chronically infected mice. SMARTA transgenic mice have CD4 T cells specific for the gp61-80 (GLNGPDIYKGVYQFKSVEFD) epitope of LCMV(190). LCMV-specific CD4 T cells were isolated from congenic Thy1.1 SMARTA transgenic mice by negative selection of CD4 T cells, resulting in >95% CD4 T cells and less than 0.01% CD8 T cells **(Supplemental 1)**. Purified LCMV-specific CD4 T cells were then labeled with CFSE and $4x10^6$ cells were transferred i.v. into either uninfected (naïve) or chronically infected Thy1.2 recipients. Chronically infected recipients were infected with LCMV approximately 2-3 months prior, but maintained

high levels of persisting virus in the serum (~10^4 pfu/ml) as determined by plaque assay. The frequency, rate of cell division, and up-regulation of activation markers on the Thy1.1 transferred cells was examined at day 2.5 post transfer in the spleen and non-lymphoid tissues **(Figure 1a).** The SMARTA CD4 T cells transferred into the infected recipient underwent rapid antigen-driven proliferation and up-regulated CD44, whereas CD4 T cells transferred into naïve animals remained undivided and mostly of the naïve, $CD44^{low}$ phenotype. By day 2.5 post transfer, all of the transferred CD4 T cells had divided, with most undergoing approximately 4-7 divisions as determined by CFSE dilution **(Figure 1b)**. Dividing CD4 T cells resulted in expanded numbers of SMARTA CD4 T cells recovered in the spleen, liver and lungs on day 2.5 post transfer in the infected recipients, but not in the uninfected mice **(Figure 1c)**.

Naïve transgenic CD4 T cells persist and maintain cytokine effector function long-term post transfer

The peak expansion of the SMARTA CD4 T cells in the peripheral blood occurred approximately one-week post transfer, with the transferred CD4 T cells subsequently undergoing a slow contraction and persisting long-term in chronically infected animals **(Figure 2a)**. To examine long-term the maintenance and functional capabilities of transferred cells, SMARTA CD4 T cells were isolated from various lymphoid and non-lymphoid tissues at one and 4 months post transfer. High frequencies of SMARTA CD4 T cells were isolated from the spleen, liver and bone marrow at 4 months post transfer **(Figure 2b)**. One-month post transfer, the SMARTA CD4 T cells made mostly IFNγ, with only small amounts of TNFα and IL-2 (data not shown).

SMARTA CD4 cells isolated from the spleen 4 months were able to produce IFNγ, TNFα and IL-2 upon *ex vivo* re-stimulation, demonstrating that the CD4 T cells did not permanently lose the ability to make cytokine post transfer **(Figure 2c)**.

Transfer of CD4 T cell help enhances LCMV-specific CD8 T cell responses

We next wanted to determine whether transferred CD4 T cells would influence exhausted CD8 T cell responses. We monitored LCMV-specific CD8 T cell responses longitudinally in the peripheral blood following SMARTA CD4 T cell transfer. Mice not receiving CD4 T cells had low levels of LCMV-specific CD8 T cells as measured by GP33 and GP276 tetramer staining in the blood. However, mice receiving a single transfer of naïve antigen-specific CD4 T cells had elevated levels of tetramer which could be measured around two weeks post transfer. This increased tetramer response was long-lasting and could be detected in the PBMCs at 2 months post transfer, whereas untreated animals continued to maintain very low frequencies of tetramer positive cells **(Figure 3a)**. To look more closely at the CD8 T cell response, we next examined the LCMV-specific CD8 T cells within the lymphoid and non-lymphoid tissues. Mice given LCMV-specific CD4 T cells had elevated frequencies of LCMV-specific CD8 T cells for both the GP33 and GP276 epitope in the spleen, liver, lung and bone marrow 35 days post CD4 T cell transfer **(Figure 3b)**. The total number of CD8 T cells specific for the GP33 and GP276 epitope of LCMV was also elevated in the tissues of mice receiving LCMV-specific CD4 T cells compared to untreated control mice at 35 days post transfer. This increase in the LCMV-specific CD8 T cell response was statistically significant in the tissues as determined by the Mann-Whitney test **(Figure 3c)**.

More importantly, the exhausted CD8 T cells during late stages of chronic LCMV infection make very low levels of IFNγ in response to re-stimulation with cognate peptide. Therefore, we were interested in determining whether this increase in CD8 T cell numbers also resulted in increased function by LCMV-specific CD8 T cells following the transfer of CD4 T cell help. On day 7 post transfer, the CD8 T cells made significantly more IFNγ following stimulation with either the GP33 or GP276 LCMV-specific peptide **(Figure 3d)**. Therefore, transfer of SMARTA CD4 T cells enhanced both the number and function of exhausted CD8 T cell responses in chronically infected recipients.

Rescue of CD8 T cell function requires transfer of antigen-specific CD4 T cell help

To determine whether the rescue of the CD8 T cells relies on antigen-specific interactions, we isolated and transferred either naïve SMARTA or naïve OT-II transgenic CD4 T cells into our chronically infected hosts. We then looked at CD4 T cell numbers and LCMV-specific CD8 T cell responses post transfer. Mice that received no cells, or the non-specific OT-II cells had low frequencies of CD8 T cell making IFNγ, while the mice receiving SMARTA CD4 T cells showed increased frequencies of IFNγ positive CD8 T cells **(Figure 4a)**. Overall levels of virus in mice receiving OT-II cells remained similar to untreated mice, while mice receiving SMARTA cells had reduced viral titers 30 days post transfer **(Figure 4b)**. This increase in function by LCMV-specific CD8 T cells was not limited to the GP33 and GP276 epitopes; there was increased IFNγ production for a wide range of CD8 T cell epitopes in the mice receiving the SMARTA

CD4 T cells compared to untreated mice or mice receiving the non-specific OT-II CD4 T cells **(Figure 4c).**

Enhanced B cell responses following CD4 T cell transfer

B cells responses are important in resolution of chronic LCMV strains(72, 191) and have been shown to be important in preventing exhaustion of CTLs following immune transfer into LCMV carrier mice(75, 183). Mice depleted of CD4 T cells prior to infection with clone-13 also fail to mount an LCMV-specific antibody response. We therefore wanted to test whether LCMV-specific CD4 T cells could provide help to the B cells and initiate a humoral response post transfer. Staining of splenocytes 35 days post transfer showed higher percentages of germinal center B cells as detected with the markers PNA and FAS on B cells in the spleen **(Figure 5a)**. Increased levels of LCMV-specific antibody could be detected in the serum of treated mice two months post transfer **(Figure 5b)**. Therefore transferred SMARTA CD4 T cells were able to provide signals critical to both CD8 and B cell responses to LCMV previously not provided in this "helpless" model of CD8 T cell exhaustion.

CD4 T cell therapy synergizes with αPD-L1 blockade to enhance the function of exhausted CD8 T cells

Previously, our lab had shown that blocking the PD1/PD-L1 pathway enhanced CD8 T cell responses during chronic viral infection(78) and enhanced responses following therapeutic vaccination(81). However, how the PD-1/PD-L1 pathway regulates CD4 T cell responses during chronic viral infection remains less understood.

As expected, naïve SMARTA CD4 T cells transferred into chronically infected mice upregulate PD-1 by two weeks post transfer **(Figure 6a)**. We therefore wanted to determine whether (1) blocking the PD-1 pathway would influence the proliferation and function of the transferred CD4 T cells and (2) determine if αPD-L1 and CD4 T cell transfer could synergize to rescue exhausted CD8 T cells. Chronically LCMV-infected mice were given naïve SMARTA CD4 T cells and then treated with the blocking αPD-L1 antibody for two weeks starting day 1 post transfer. We monitored CD4 T cell numbers and function as well as examining CD8 T cell rescue following the co-treatment of CD4 T cells with PD-1 antibody blockade.

The total number of SMARTA CD4 T cells recovered in the spleen, liver and lung of the mice did not change following αPD-L1 treatment. We measured the total numbers of SMARTA cells at days 2.5, 7 and 15, and there were no difference in SMARTA CD4 T cells recovered with or without PD-1 blockade in the spleen **(Figure 6b)**. There was also no difference in the recovery of SMARTA CD4 T cells following αPD-L1 treatment in the liver and lungs. However, the αPD-L1 therapy enhanced the functionality of the transferred cells, with a greater percent making IFNγ on day 15 post transfer **(Figure 6c)**. Similar improvements in cytokine production were seen for TNFα and IL-2, with fewer transferred cells making these cytokines at day 15 post transfer (data not shown). Therefore, although blockade of PD-1 did not affect the number of SMARTA CD4 T cells recovered, it could enhance the ability of these transferred cells to produce effector cytokines in the chronic LCMV-infected host.

Secondly, we wanted to see if the blockade of the PD-1/PD-L1 pathway could synergize with the CD4 T cell help to enhance function of exhausted CD8 T cells. The

number and function of the exhausted CD8 T cells were examined two weeks following CD4 T cell transfer in mice treated with αPD-L1. All mice treated with αPD-L1, SMARTA CD4 T cells or both αPD-L1 and SMARTA CD4 T cells had elevated frequencies of tetramer positive cells in the spleen, liver and lung (data not shown). More significantly, we wanted to measure the functionality of the LCMV-specific CD8 T cells following the SMARTA transfer and combined αPD-L1 blockade. The αPD-L1 treatment or SMARTA T cell transfer alone enhance IFNγ production in LCMV-specific CD8 T cells, and there was a slightly enhanced rescue in the dual treated mice **(Figure 6d)**. Importantly, the dual treated mice had a greater percent of IFNγ cells that were also making TNFα at day 15-post treatment **(Figure 6e)**. This increase in dual functional CD8 T cells was significantly increased compared to mice given either SMARTA CD4 T cells or αPD-L1 treatment alone. These dual treated mice also had an enhanced viral clearance compared to mice given SMARTA CD4 T cells alone (data not shown). Therefore, the blockade of inhibitory signals such as the PD-1/PD-L1 pathway synergizes with the transfer of CD4 T cells to improve CD8 T cell responses during established chronic LCMV infection.

Discussion:

Recent work in our lab has shown that exhausted CD8 T cells express a unique pattern of genes and that the exhausted phenotype develops longitudinally over the course of chronic LCMV infection(62). CD4 T cells play a critical role in the programming of CD8 T cells during chronic LCMV, and depletion of CD4 T cell help during the initial T cell priming results in high viral loads and the persistence of exhausted CD8 T cells(56). In this study, we wanted to determine whether the restoration of CD4 T cell signals could affect the ability of CD8 T cells to function following the establishment of this exhausted phenotype. Transfer of antigen-specific CD4 T cells, but not irrelevant CD4 T cells resulted in enhanced numbers and function of LCMV-specific CD8 T cells. These transferred CD4 T cells also resulted in the production of LCMV-specific antibody and lead to enhanced viral control in the blood and tissues of chronically LCMV infected mice. These results suggest CD4 T cell immunotherapy may alter CD8 T cell exhaustion in patients with chronic viral infections.

The naïve CD4 T cells transferred into chronically LCMV infected mice quickly divided and made cytokine at day 2.5 post transfer. These cells made mostly IFNγ, with small amounts of TNFα and IL-2. Importantly, these cells maintained IFNγ production and recovered the ability to make IL-2 at 5 months post transfer. Earlier studies looking at the transfer of SMARTA CD4 T cells into chronically infected LCMV-Docile carriers suggested that the CD4 T cells became exhausted, and completely lose IL-2 production(70). Additionally, in that study, CD4 T cell help capable of triggering

antibody production was seen at early (day 15) but not later (> day 55) time points post SMARTA transfer. In this study we do not see complete loss of IL-2, and the emergence of LCMV-specific antibody occurs between one to two months post transfer. Differences between this and the previous study include the LCMV strain used and the way persistence of virus was established. Recovery of IL-2 by the transferred CD4 T cells likely requires the overall reductions in viral load that we see at late time points post transfer. The delay we see in the development of LCMV antibody may be attributed to overall immuno-suppression and inflammation, disrupted splenic architecture for germinal center establishment, or specific suppression of circulating mature B cells during the chronic LCMV infection. However, the enhancement of both the CD8 T cell and B cell responses are likely critical for long-term viral control, and further studies are needed to address the relative role of B cell and CD8 T cell responses in this model.

Previous studies have shown immune suppression in chronically LCMV infected mice due to a number of factors, including viral targeting and killing of antigen presenting cells(53, 192), down-regulation of co-stimulatory molecules on dendritic cells(54), suppression of bone marrow progenitors(54), and immune modulation by cytokines such as IL-10(82-84). In our model, immune-suppression likely occurs; mice that have reduced viral loads show recovery of CD4 T cell cytokine function at later time points post transfer. However, the mice undergoing slower viral clearance do have continued CD4 T cell proliferation and higher SMARTA CD4 percentages at two months post transfer, suggesting that high viral load does not prevent the establishment of CD4 T cell help post transfer. Importantly, CD4 T cell transfer lead to enhanced CD8 T cell responses despite the immunosuppressive environment present during an established

chronic infection. All mice receiving SMARTA CD4 T cells have increased CD8 T cell function and viral loads reduced at one months post transfer. In addition, this increased function was to a wide-range of LCMV-specific CD8 T cell epitopes and resulted in lower viral titers in all of the treated mice.

The most promising work looking at the effect of CD4 T cells on human CD8 T cells showed that CD8 T cells from chronic HIV infected patients have enhanced proliferation when co-cultured *in vitro* with CD4 T cells isolated from the patient at early time points post infection(87). CD4 T cell help has also been shown to be important in maintaining long-lived CTLs during immunotherapy of CMV patients(186). However, studies with therapeutic vaccination of patients with HBV antigens have also suggested that antigen-specific CD4 T cells may preferentially differentiate into Th2 type effectors(193); thus additional signals driving CD4 T cells to Th1 differentiation may be important to consider during CD4 T cell therapies. Studies of CD4 T cells in patients with chronic HIV and HCV infection suggest that IL-2 production correlates with strong CTL responses and viral control(87, 162). Additionally, direct treatment of LCMV infected mice with IL-2 enhanced CD8 T cell function and viral control(194), thus making it an attractive therapy strategy. In CD8 T cell therapies, tumor-infiltrating lymphocytes engineered to make IL-2 have had success in clinical trials(195). However we must also consider implications of IL-2 and regulatory T cell differentiation and function when designing treatments targeting CD4 T cells. Other potential targets that could be included during immunotherapy transfers include blocking immunosuppressive cytokines such as IL-10 and TGFβ; or targeting of T cell regulatory pathways such as

PD-1 and LAG-3 which have both been shown to control CD8 T cell exhaustion(62, 77, 78).

In this study we combined the CD4 T cell therapy with blockade of the PD-1/PD-L1 pathway. Using the same model of helpless CD8 T cell exhaustion, PD-1 blockade and PD-1 blockade combined with therapeutic vaccination enhanced IFNγ effector function and reduced overall viral loads(78, 81). Although both CD4 T cell transfer and PD-1 blockade enhanced IFNγ effector function, the combined therapy used in this study greatly increased the percent of cells that co-produced both IFNγ and TNFα. This combination of polyfunctional CD8 T cells and LCMV-specific antibody following CD4 T cell transfer resulted in long-term viral control. Studies examining CD8 T cell exhaustion during LCMV have shown differential exhaustion for the immunodominant LCMV epitopes(60), and interestingly, the two therapies had differing effects on their ability to rescue GP33 versus GP276-specific CD8 T cell responses. Importantly, by combining the CD4 T cell help with a transient blockade of PD-1, we were able to measure long-term improvements in CD8 T cell function to a wide range of epitopes and lower viral loads not only in the serum, but also the kidney, which often harbors high levels of persisting virus.

Therapies aimed at eliminating persistent viral infections and tumors have often focused on enhancing CTL number and function directly through targeting CD8 T cells. Our study suggests that targeting the CD4 T cells may also have positive effects on overcoming established CD8 T cell dysfunction. Transferred CD4 T cells persist long-term post transfer and provide help to enhance both ongoing B cell and T cell responses.

Thus CD4 T cell immunotherapy may be an alternate strategy to overcome CD8 T cell dysfunction and enhance immune responses to patients with chronic viral infections.

Chapter 3 Figure Legends:

Figure 3-1: Naïve transgenic CD4 T cells undergo rapid antigen-driven activation and proliferation following transfer into chronically infected hosts.

(A) LCMV-specific CD4 T cells were isolated from Thy1.1 SMARTA transgenic mice, labeled with CFSE, and transferred into either uninfected (naïve) or chronically infected Thy1.2 recipients. LCMV chronically infected mice had been infected 2-3 months prior and had high levels of persistent virus (as determined by serum plaque assay). Frequency of transferred cells, division rates and activation markers were analyzed in the spleen, liver and lung at day 2.5 post transfer. (B) Frequency of SMARTA CD4 T cells recovered in the spleen, liver and lung at day 2.5 post transfer in uninfected (left side) versus chronically infected (right side) recipients. Second plot shows division rates for transferred cells (gated on Thy1.1) by CFSE dilution and CD44 expression in each of the tissues for one representative mouse per group. (C) The total number of recovered cells in the uninfected versus infected recipients, summarizes 2-3 mice/group from one of two independent experiments.

Figure 3-2: SMARTA CD4 T cells expand, contract and persist long-term in vivo.

(A) LCMV-specific CD4 T cells transferred into chronically infected mice were measured by congenic markers Thy1.1 or Ly5.1 long-term post transfer in the PBMCs. Kinetics of SMARTA-specific CD4 T cells in the PBMC is summarized from several experiments, with one representative graph shown for days 1, 15, and 35-post transfer. (B) Transferred CD4 T cells could be detected in the spleen, lung and bone marrow at 4

months post transfer. (C) SMARTA CD4 T cells isolated from the spleen remain functional 4 months post transfer, number is percent of transferred SMARTA CD4 T cells (Ly5.1+) making cytokine following *ex vivo* stimulation with the GP61-80 (GLNGPDIYKGVYQFKSVEFD) peptide; one representative mouse (n=5).

Figure 3-3: Transfer of CD4 T cell help enhances LCMV-specific CD8 T cell responses.

(A) LCMV-specific CD8 T cell responses were monitored in the PBMCs following SMARTA T cell transfer by staining for the LCMV-specific GP33 and GP276 tetramer. n=4 untreated and 6-8 treated mice for each time-point. Data plots showing LCMV-specific tetramer staining (gated on CD8 T cells) for one representative mouse/group. (B) CD8 T cell responses to two immunodominant LCMV epitopes (GP33 and GP276) were measured in the lymphoid and non-lymphoid tissues on day 35 post transfer. (C) Summarized tetramer numbers (n=4 untreated, 7 treated mice) in the various tissues day 35 post transfer, *$p<0.05$, ** $p<0.01$ as determined by Mann-Whitney test. (D) Mice treated with SMARTA CD4 T cells also have a higher frequency of CD8 T cell making IFNγ at 7 days post transfer for both the GP33 and GP276 LCMV epitopes (n=8 mice/group, combined from two independent experiments). $p=0.0086$ for GP33 and $p=0.034$ for GP276 as determined by student t test.

Figure 3-4: Rescue of CD8 T cell function and enhanced viral control requires transfer of antigen-specific CD4 T cells.

Either antigen-specific SMARTA or non-specific OT-II CD4 T cells were transferred into chronically LCMV infected mice and viral titers and cytokine analyzed 35 days post transfer. (A) Number of CD8 T cells make IFNγ (combined numbers of IFNγ+ to 4 LCMV-specific epitopes: GP33, GP276, GP118 and NP 205) (B) Viral titer at day 35 post transfer (n=4 no treatment, 3 OT-II, 7 SMARTA). (C) Percent of cells making IFNγ for individual LCMV epitopes. All statistics analyzed using student t-test.

Figure 3-5: Transfer of naïve SMARTA CD4 T cells leads to an elevated B cell response in chronically infected mice.

(A) Frequencies of germinal center B cells were measured by flow cytometry (PNA+FAS+) in the spleen on day 35-post SMARTA CD4 T cell transfer. Individual mice showing representative PNA and FAS staining, gated on B220+ cells. (B) LCMV-specific antibody, as measured by ELISA is elevated in the treated mice at 2 months post transfer (n= 4 untreated, and 6 SMARTA treated mice). All statistics analyzed using student t-test.

Figure 3-6: CD4 T cell therapy synergizes with αPD-L1 blockade to enhance the function of exhausted CD8 T cells.

(A) PD-1 expression on SMARTA CD4 T cells following transfer into chronically infected hosts at day 15-post transfer. Histogram is gated on Thy1.1 cells and compared to the expression of PD-1 on naïve $CD44^{low}$ CD4 T cells. (B) Number of SMARTA CD4 T cells isolated from the spleen at days 2.5, 7 or 15 post transfer (n=3-5 mice/group). (C) Percent of transferred SMARTA CD4 T cells making IFNγ at day 15 post transfer (n=4

mice/group). (D) CD8 T cell function following αPD-L1 treatment for two weeks post SMARTA CD4 T cell transfer. Number of CD8 T cells making IFNγ to 4 different LCMV epitopes for: untreated (white), αPD-L1 treated (light grey), SMARTA treated (dark grey), or SMARTA+αPD-L1 (black) treated mice. Summarized number of CD8 T cells co-producing IFNγ and TNFα for 6 LCMV epitopes following treatment. Statistics analyzed using student t test: * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Supplemental Figure 3-1:

(A) CD4 T cells were isolated from naïve SMARTA transgenic mice by selection of non-CD4 T cells using the MACS (Miltenyi) magnetic column. Post-column purification was approximately 95% CD4 T cells and the contaminating CD8 T cells were <0.01%. (B) No outgrowth of donor (Thy1.1) CD8 T cells from the naïve SMARTA transgenic mice were detected in the chronically infected recipients at one-month post transfer.

Figure 3-1

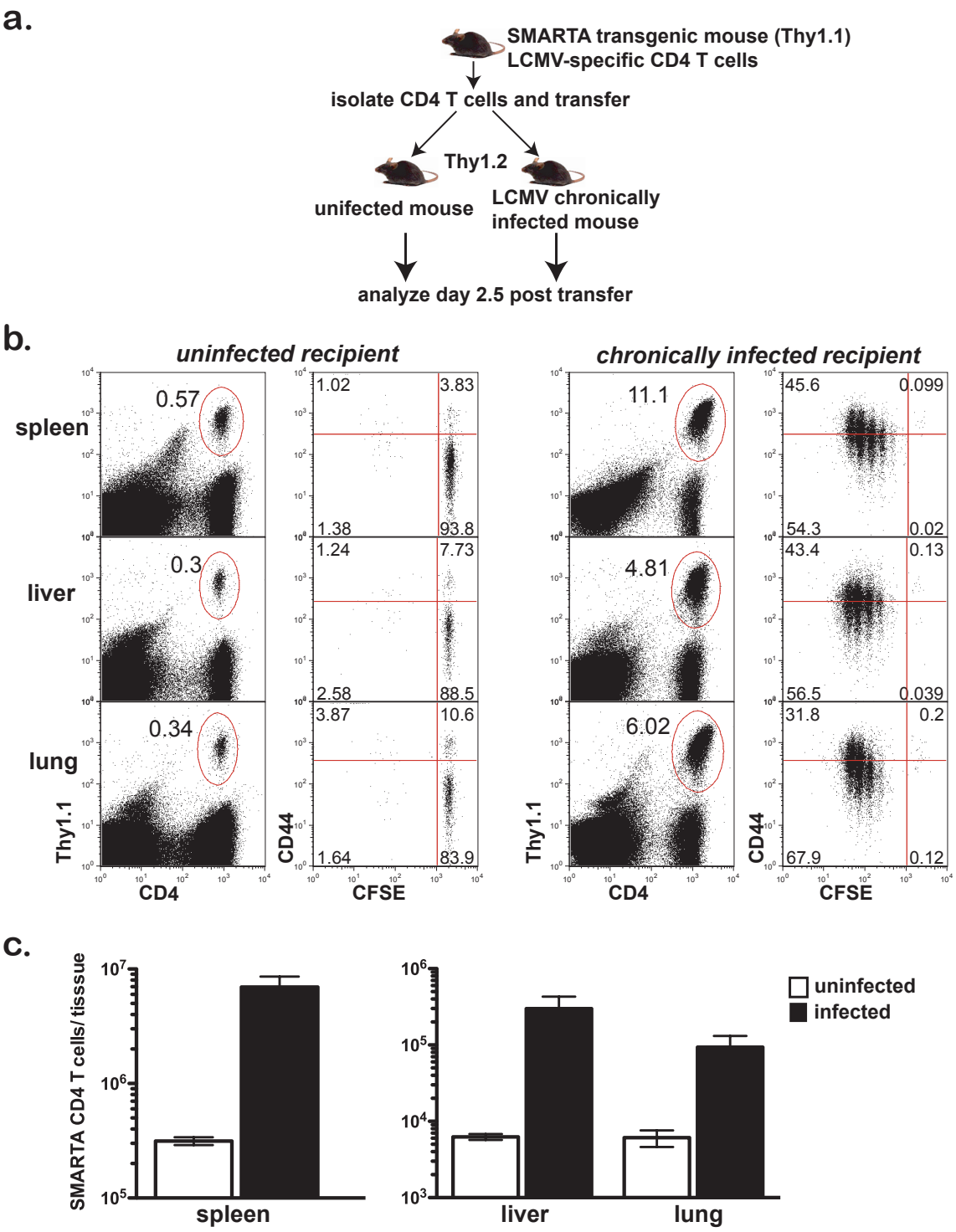

a.
SMARTA transgenic mouse (Thy1.1)
LCMV-specific CD4 T cells
isolate CD4 T cells and transfer
Thy1.2
unifected mouse
LCMV chronically infected mouse
analyze day 2.5 post transfer
b.
uninfected recipient
chronically infected recipient
spleen
liver
lung
0.57
0.3
0.34
11.1
4.81
6.02
1.02
3.83
1.38
93.8
1.24
7.73
2.58
88.5
3.87
10.6
1.64
83.9
45.6
0.099
54.3
0.02
43.4
0.13
56.5
0.039
31.8
0.2
67.9
0.12
Thy1.1
CD4
CD44
CFSE
c.
SMARTA CD4 T cells/ tisssue
spleen
liver
lung
uninfected
infected

Figure 3-2

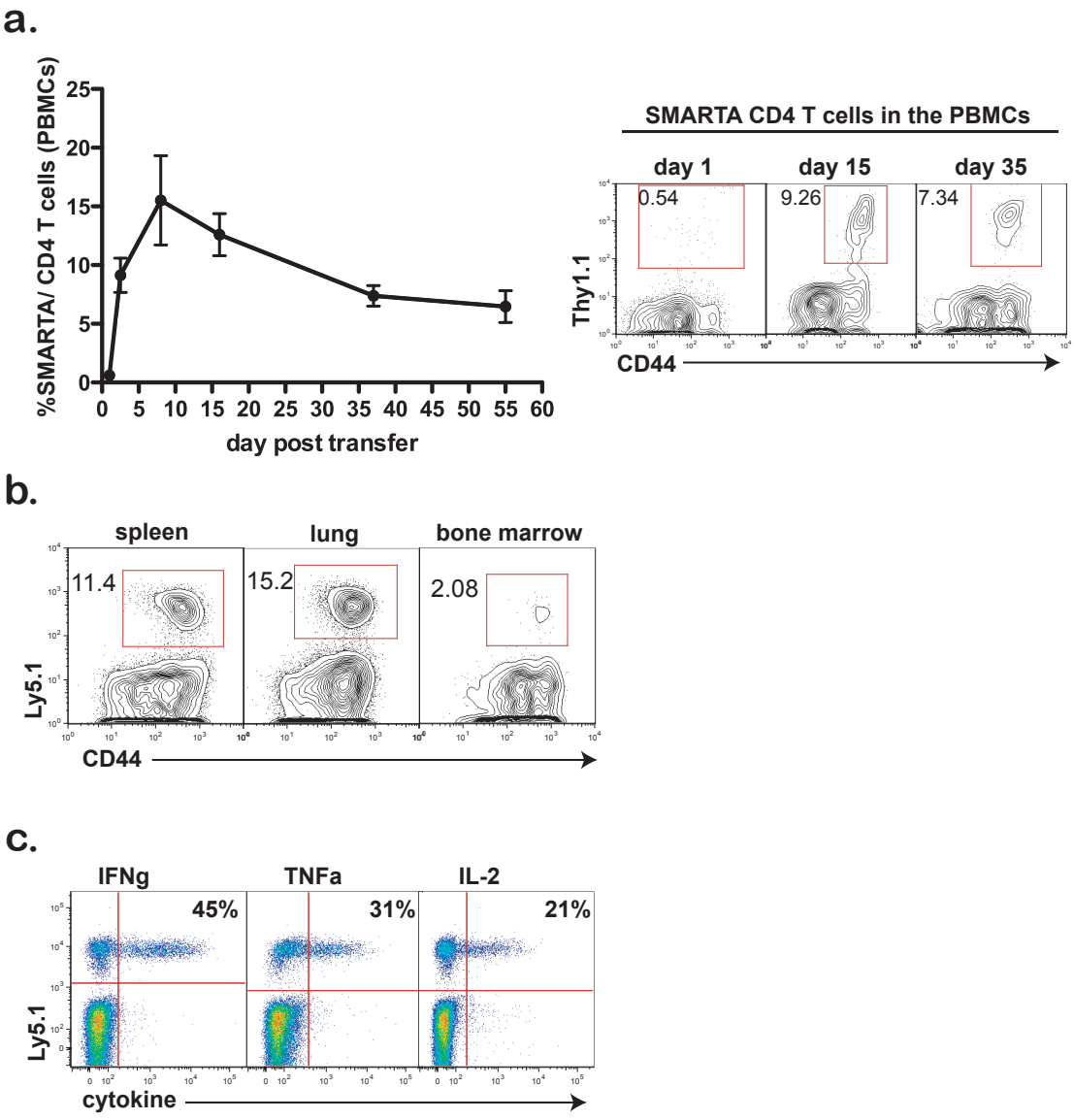

a.
%SMARTA/ CD4 T cells (PBMCs)
day post transfer
SMARTA CD4 T cells in the PBMCs
day 1
day 15
day 35
0.54
9.26
7.34
Thy1.1
CD44
b.
spleen
lung
bone marrow
11.4
15.2
2.08
Ly5.1
CD44
c.
IFNg
TNFa
IL-2
45%
31%
21%
Ly5.1
cytokine

Figure 3-3

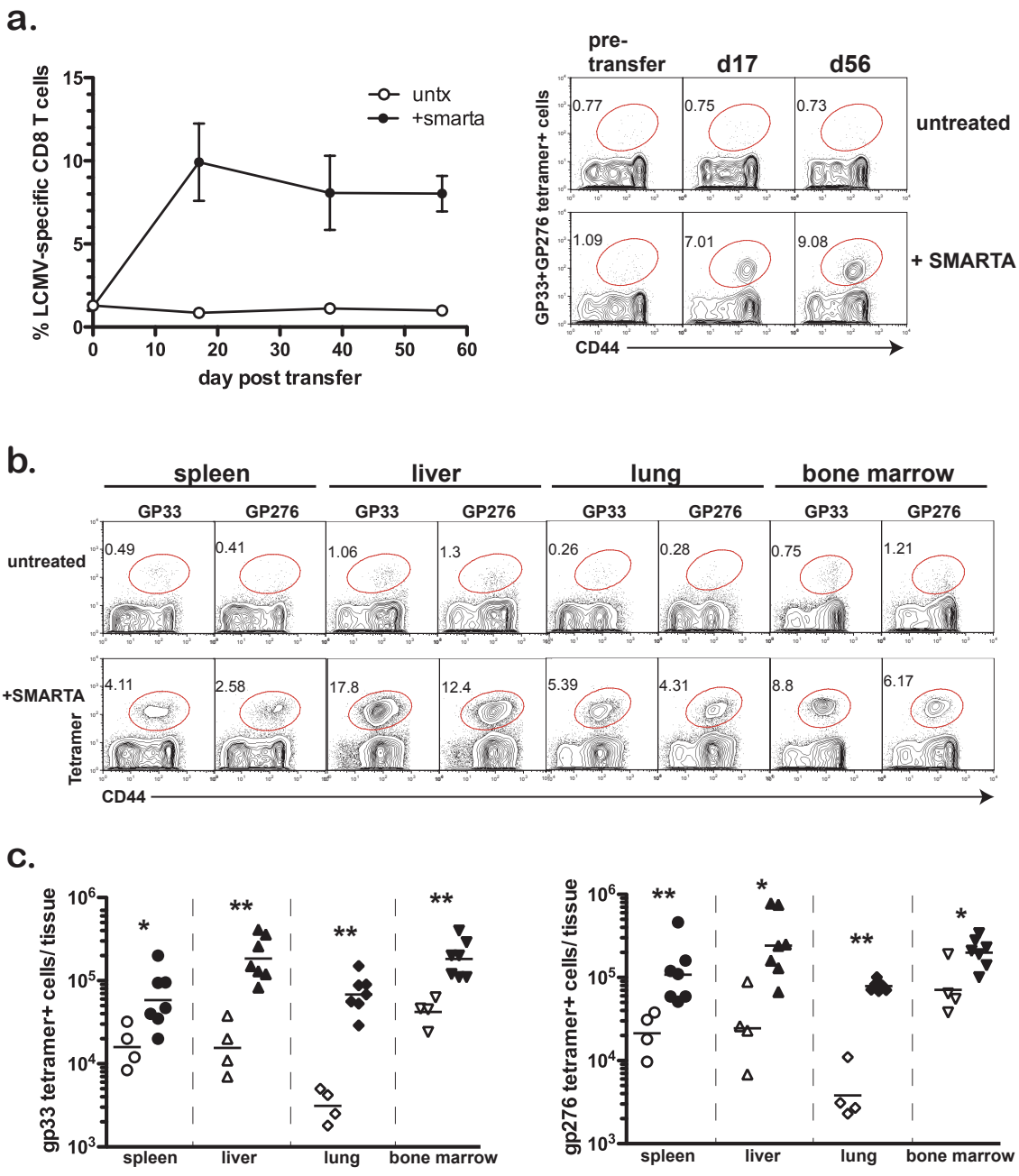

a.
% LCMV-specific CD8 T cells
day post transfer
untx
+smarta
pre-transfer
d17
d56
GP33+GP276 tetramer+ cells
0.77
0.75
0.73
untreated
1.09
7.01
9.08
+ SMARTA
CD44
b.
spleen
liver
lung
bone marrow
GP33
GP276
untreated
0.49
0.41
1.06
1.3
0.26
0.28
0.75
1.21
+SMARTA
4.11
2.58
17.8
12.4
5.39
4.31
8.8
6.17
Tetramer
CD44
c.
gp33 tetramer+ cells/ tissue
gp276 tetramer+ cells/ tissue
spleen
liver
lung
bone marrow

Figure 3-3

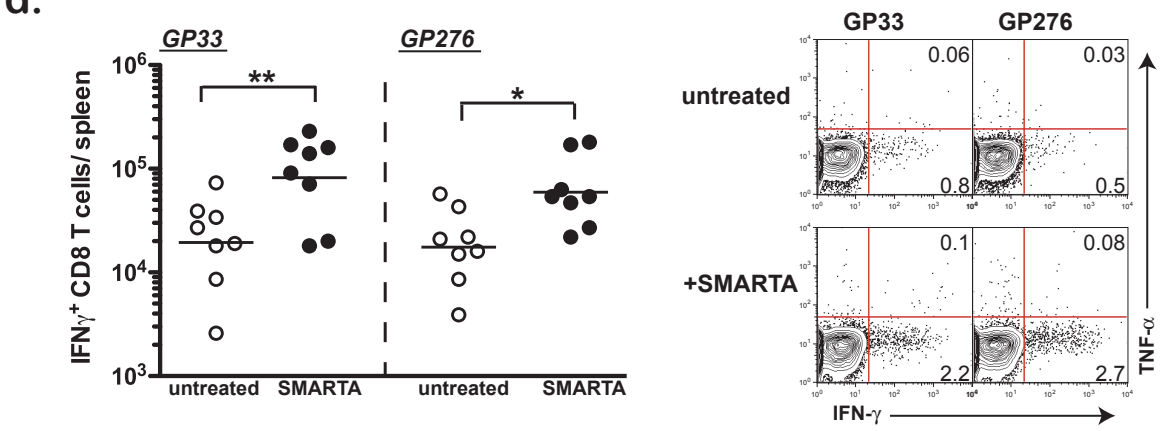

d.
GP33
GP276
IFNγ+ CD8 T cells/ spleen
10^6
10^5
10^4
10^3
**
*
untreated
SMARTA
untreated
SMARTA
GP33
GP276
untreated
+SMARTA
0.06
0.03
0.8
0.5
0.1
0.08
2.2
2.7
IFN-γ
TNF-α

Figure 3-4

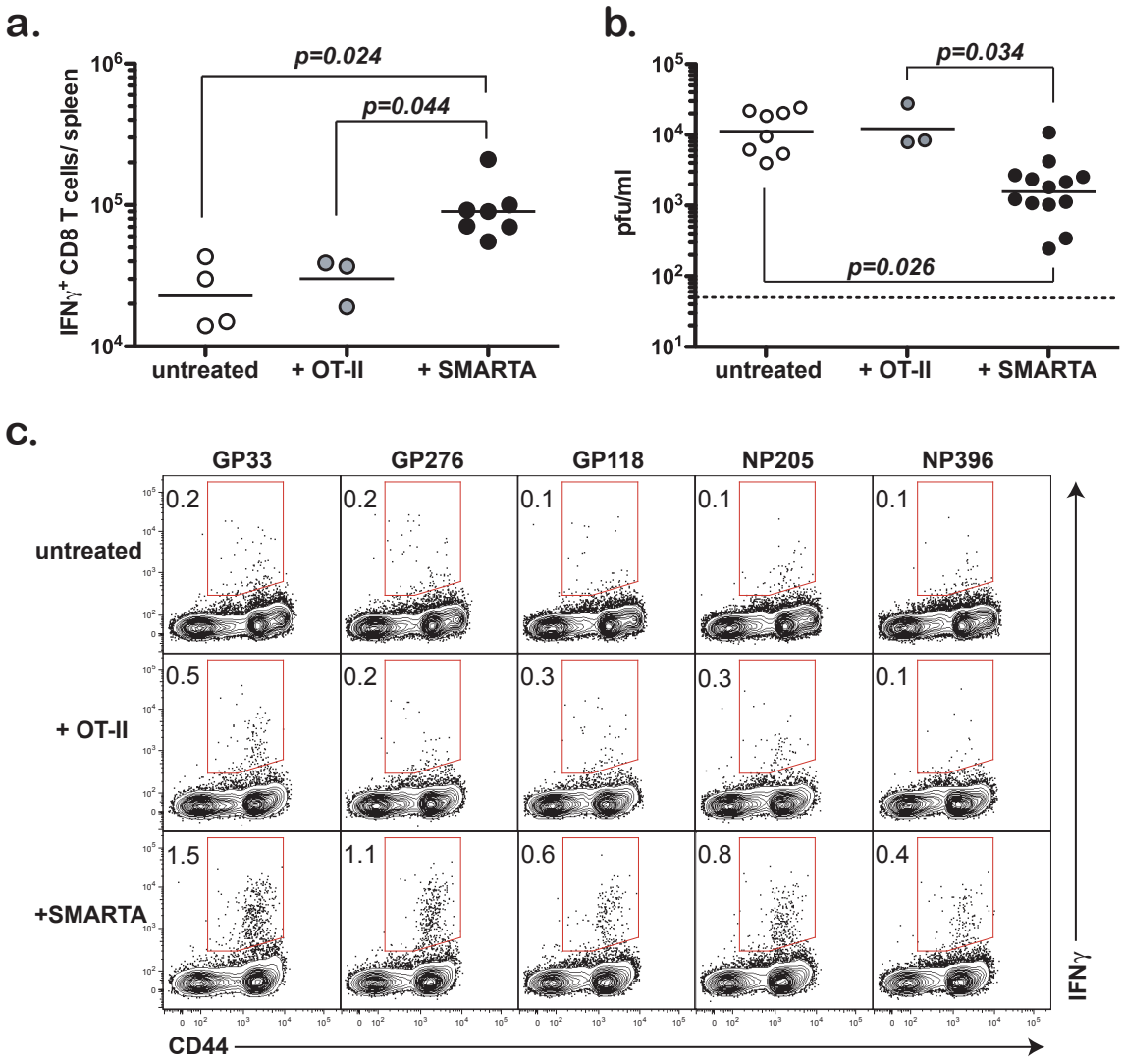

a.
p=0.024
p=0.044
IFNγ+ CD8 T cells/ spleen
untreated
+ OT-II
+ SMARTA
b.
p=0.034
p=0.026
pfu/ml
untreated
+ OT-II
+ SMARTA
c.
GP33
GP276
GP118
NP205
NP396
untreated
+ OT-II
+SMARTA
0.2
0.2
0.1
0.1
0.1
0.5
0.2
0.3
0.3
0.1
1.5
1.1
0.6
0.8
0.4
CD44
IFNγ

Figure 3-5

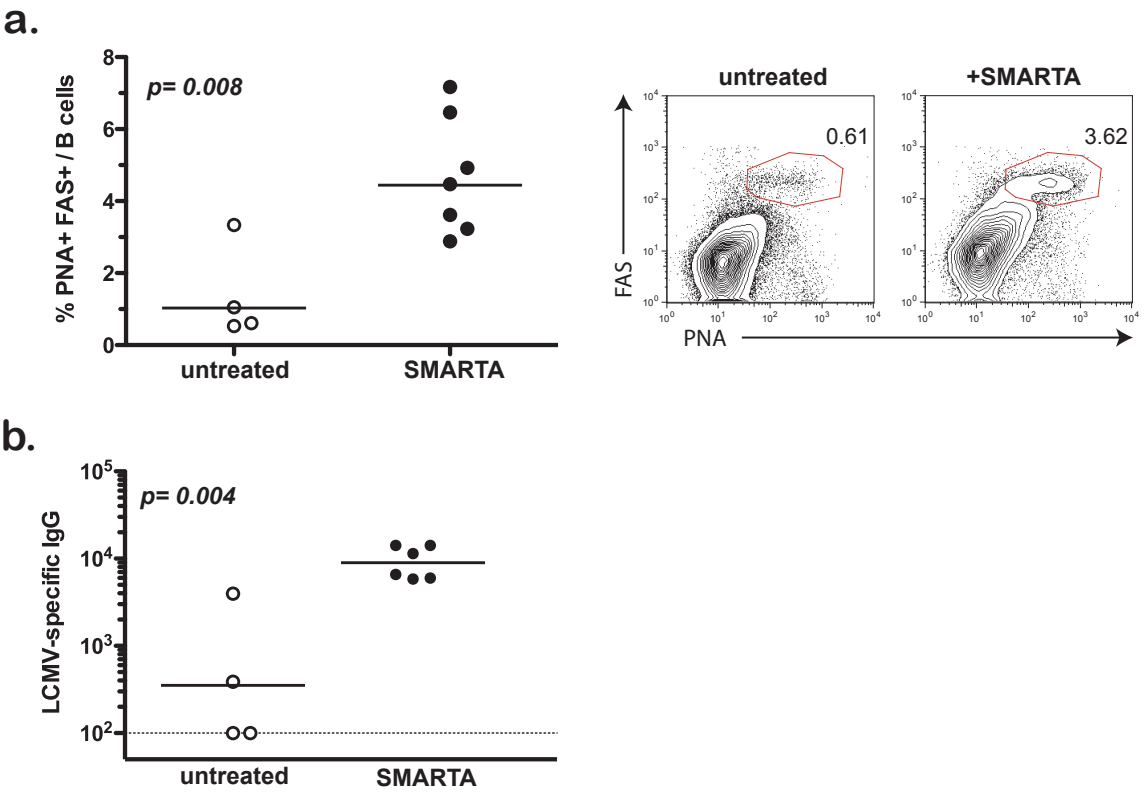
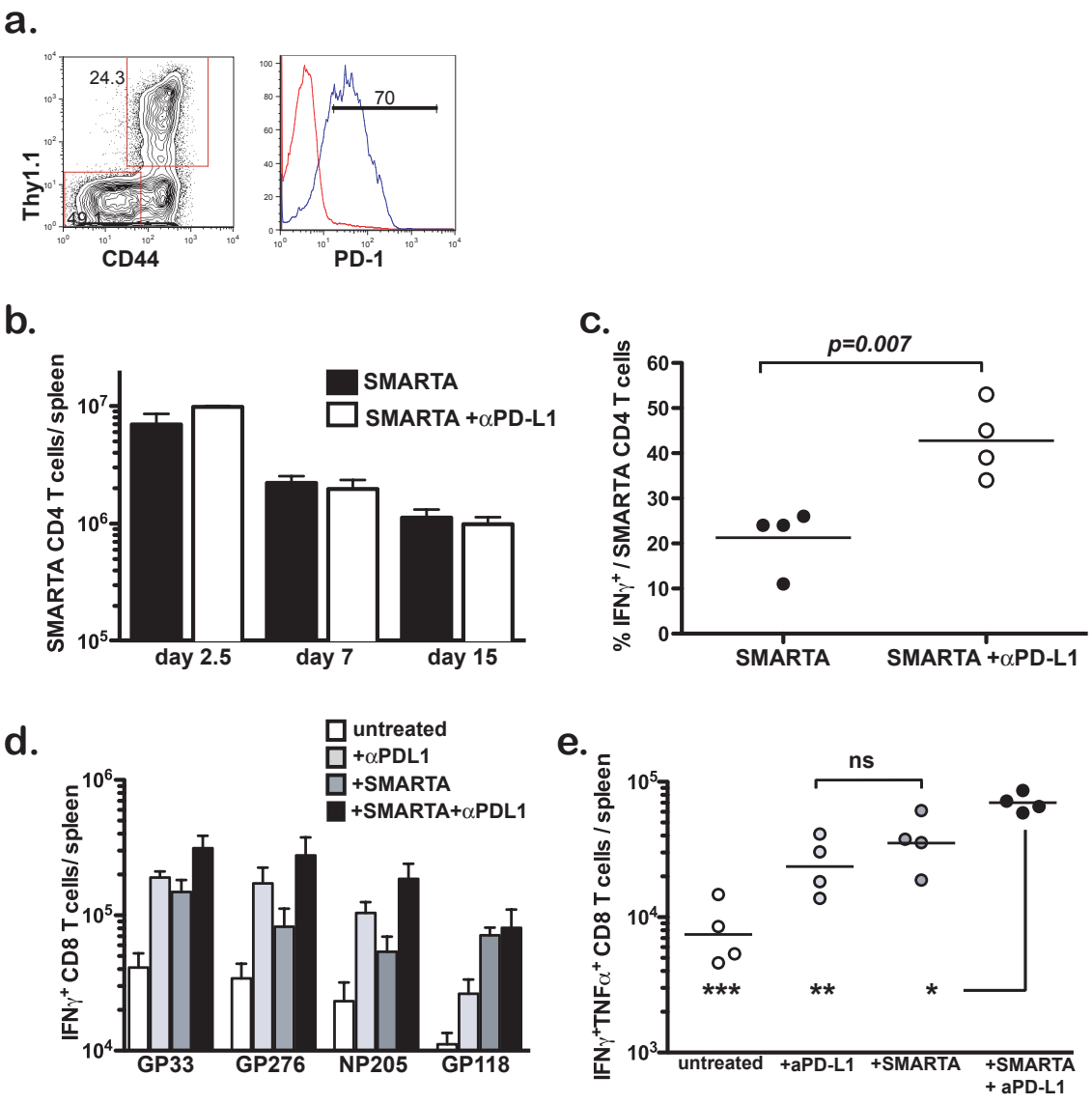
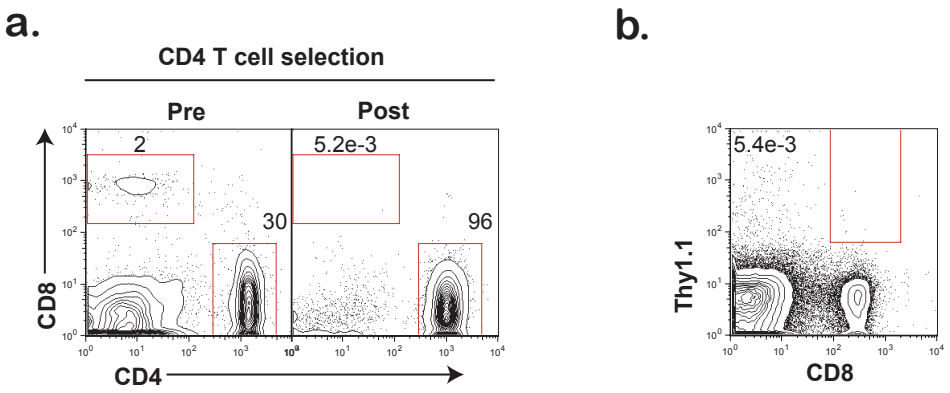

a.
% PNA+ FAS+ / B cells
p= 0.008
untreated
SMARTA
untreated
+SMARTA
0.61
3.62
FAS
PNA
b.
LCMV-specific IgG
p= 0.004
untreated
SMARTA

Figure 3-6


a.
24.3
49.1
Thy1.1
CD44
70
PD-1
b.
SMARTA
SMARTA +αPD-L1
SMARTA CD4 T cells/ spleen
day 2.5
day 7
day 15
c.
p=0.007
% IFNγ+ / SMARTA CD4 T cells
SMARTA
SMARTA +αPD-L1
d.
untreated
+αPDL1
+SMARTA
+SMARTA+αPDL1
IFNγ+ CD8 T cells/ spleen
GP33
GP276
NP205
GP118
e.
ns
IFNγ+TNFα+ CD8 T cells / spleen
***
**
*
untreated
+aPD-L1
+SMARTA
+SMARTA
+ aPD-L1

Supplemental Figure 3-1



Chapter 4: Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system

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Journal of Immunological Methods 286 (2004) 111–122

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Research Paper

Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system

Shane Crotty[a,b,1], Rachael D. Aubert[a,1], John Glidewell[a], Rafi Ahmed[a,*]

[a] *Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Road, Rm G-211, Atlanta, GA 30322, USA*

[b] *La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, USA*

Received 17 November 2003; accepted 16 December 2003

Abstract

In the interest of better understanding the role of human memory B cells in protection against disease, we developed an assay to quantitate antigen-specific memory B cells in human blood. This assay utilizes a 6-day polyclonal stimulation of PBMC followed by an antigen-specific ELISPOT for the detection of memory B cells that have differentiated into antibody secreting cells (ASC) in vitro. We have used this assay to demonstrate that the anthrax vaccine (AVA; BioThrax) elicits a substantial population of protective-antigen (PA) specific memory B cells, and these B cells satisfy the canonical surface phenotype of human memory B cells: $CD19^+CD20^+Ig^+CD27^+$. These anti-PA antigen-specific memory B cells are IgG^+ and represent up to 2% of circulating IgG^+ B cells. Furthermore, these results confirm that vaccine-elicited memory B cells reside in the $CD27^+$ B cell population. This ELISPOT-based system has been designed in a generalized manner, such that the assay can be rapidly adapted to detect human antigen-specific memory B cells of any given specificity. This method should be useful for quantitatively assessing the potency of vaccines and the longevity of B cell immunological memory to various vaccines or infectious diseases.

Keywords: Memory B cell; Vaccine; Anthrax; Human; Immune memory; Pokeweed mitogen; Smallpox

1. Introduction

Memory B cells and long-lived plasma cells are responsible for the long-term humoral immunity elicited by most vaccines (Crotty and Ahmed, in press). Long-lived plasma cells are responsible for the continuous maintenance of serum antibody levels (Slifka et al., 1998; Manz et al., 2002; Crotty et al., 2003b). Memory B cells are responsible for driving the rapid anamnestic antibody response that occurs after re-exposure to antigen, which is important for eliminating the pathogen and toxic antigens not cleared by pre-existing circulating antibodies. Memory B cells may also play a role in replenishing the pool of long-lived plasma cells to maintain long-term antibody levels in the absence of pathogen (Slifka et al., 1998; Bernasconi et al., 2002). Though memory B cells are important for long-term humoral immunity, it

* Corresponding author. Tel.: +1-404-727-4700; fax: +1-404-727-3722.

E-mail addresses: shane@liai.org (S. Crotty), ra@microbio.emory.edu (R. Ahmed).

[1] These authors contributed equally to this work.

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doi:10.1016/j.jim.2003.12.015

is unclear how well memory B cells are generated after different vaccinations, how large antigen-specific pools of memory B cells are, and how long they are maintained in the absence of re-exposure to antigen. It is also unclear whether memory B cell and serum antibody levels correlate well for most antigens, and it is therefore important to be able to track memory B cells as an independent parameter of antigen-specific immune memory.

There are two immune memory patterns that have been observed for the majority of licensed human vaccines: (1) the vaccine elicits long-term stable antibody production, or (2) the vaccine elicits antibody levels that decline over a period of a few years. Several human vaccines elicit stable long-term (>40 years) antibody responses: the smallpox vaccine (el-Ad et al., 1990; Crotty et al., 2003a; Hammarlund et al., 2003; Manischewitz et al., 2003), the yellow fever vaccine (Plotkin and Orenstein, 1999), and both the live (Plotkin and Orenstein, 1999) and inactivated (Bottiger et al., 1998) poliovirus vaccines. For those exceptionally potent vaccines, it would be quite valuable to know whether they elicit stable long-term memory B cell responses. And if they do, it would be valuable to characterize and understand the role of memory B cells in the protective immunity provided by these vaccines. Antibodies are usually the measured correlate of protection for a vaccine, but the actual protection may be mediated by circulating antibodies in combination with other arms of the adaptive immune system, such as memory B cells and memory T cells. Therefore, it is important to quantify memory B cell responses to fully assess the armamentarium of protective immune responses elicited by these very effective vaccines. In addition, memory B cells may be central players in the long-term maintenance of antibody levels and it is crucial to understand the mechanism of this process in the context of vaccines that elicit lifelong antibody production, so that we can design better vaccines in the future.

The second pattern mentioned above is observed in numerous other vaccine situations, including AVA (Anthrax Vaccine Adsorbed, now being remarketed as BioThrax): circulating antibody levels drop to low levels within a few years post-vaccination. The hepatitis B (HBV) vaccine is a well-characterized example known for antibody titers that drop over several years. Antibodies against HBsAg are the defined correlate of protection for the HBV vaccine, and the minimum protective level has been established as 10 mIU/ml (Plotkin and Orenstein, 1999). When serum anti-HBsAg antibody levels drop below that level, booster immunization is recommended. However, many individuals with low or undetectable levels of HBsAg fail to obtain booster immunizations, and some fraction of that population subsequently becomes exposed to HBV. Interestingly, many of those individuals are still protected from HBV infection. Why? It has been proposed that this protection could be due to either memory T cells or memory B cells. Memory B cells are an appealing explanation since HBV surface antigen-specific antibodies are the correlate of protection for the vaccines, and memory B cells will rapidly differentiate into anti-HBsAg antibody secreting cells upon virus exposure (West and Calandra, 1996). Of course, it is entirely reasonable to expect that memory T cells would also play a role in protection from HBV.

As mentioned above, antibodies are frequently correlates of protective immunity. Indeed, for all human vaccines for which the correlate of protection is known, that correlate is antigen-specific antibody. This is particularly true of bacterial vaccines, and in this regard the AVA anthrax vaccine functions like a classic bacterial toxin vaccine. Anthrax protective antigen (PA) is the shared protein component of both anthrax toxins (lethal toxin and edema toxin). Passive immunization with anti-PA antibodies is sufficient to protect guinea pigs from a lethal anthrax challenge (Reuveny et al., 2001; Welkos et al., 2001; Kobiler et al., 2002). This demonstrates the clear protective value of anti-PA antibodies. PA protein is a major component of the AVA vaccine. High levels of anti-PA antibodies elicited by vaccination are correlated with protection in primates given a lethal inhalation challenge with anthrax (Ivins et al., 1996, 1998). However, in some experiments some vaccinated monkeys did not have significant levels of anti-PA antibodies at the time of challenge (>1 year post-vaccination), but yet were still protected (Ivins et al., 1996). This indicates that circulating anti-PA neutralizing antibodies are sufficient, but not necessary, for protection. What is this alternative source of protection? Memory B cells are an excellent candidate for this alternative source of

protection. Memory B cells may be a particularly crucial component of protective immunity after levels of circulating anti-anthrax antibodies wane at some point post-vaccination. The memory B cells are programmed to rapidly respond to antigen exposure, proliferating and differentiating into antibody secreting cells quickly after detecting the infectious agent. This would result in a rapid replenishment of the toxin neutralizing antibody levels post-exposure. If this memory B cell response occurs rapidly enough (and it may occur within the first 3–5 days of exposure), the vaccinated individual should be protected from the anthrax toxins by the newly synthesized toxin-neutralizing antibodies. Therefore, one of our goals is to understand memory B cell responses after anthrax vaccination.

In the interest of better understanding human memory B cells, we developed an ELISPOT-based assay to quantitate antigen-specific memory B cells in human blood. Here we report the characteristics of this technique and demonstrate the ability of this technique to detect antigen-specific memory B cells after anthrax vaccination and other antigen exposures. We have also demonstrated that the detected B cells satisfy the canonical surface phenotype of human memory B cells: $CD19^{+}CD20^{+}Ig^{+}CD27^{+}$. Importantly, this human memory B cell assay can be readily adapted to almost any antigen of interest and should be a valuable tool for numerous investigators.

2. Materials and methods

2.1. Study participants

All AVA-vaccinated individuals were normal healthy volunteers employed at the Centers for Disease Control (CDC) and vaccinated due to potential occupational exposure. Informed consent was obtained. All AVA-vaccinated individuals had received the full schedule of five subcutaneous immunizations prior to inclusion in this study. Initial date of vaccination for the individuals ranged from 1971 to 1999, and all had received a booster immunization within the past 2 years. None had been boosted within 2 months of the dates the blood samples were obtained. Note that these individuals were not part of the AVRP human clinical trial. Smallpox vaccine-immunized individuals were normal healthy adult volunteers.

Anthrax-exposed individuals were part of a CDC study tracking immune responses in the individuals exposed to anthrax in the 2001 bioterrorism attacks (Quinn et al., manuscript submitted).

2.2. Cell and serum isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using 10-ml Vacutainer cell preparation tubes (CPT) (Becton Dickinson, San Diego, CA) with sodium citrate, following the instructions of the manufacturer, except washes were done using PBS + 2% FCS. PBMC were resuspended in R-10: RPMI-1640 + 10% fetal calf serum (FCS, heat inactivated) (HyClone, Logan, Utah) and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (200 mM), and freshly added B-mercaptoethanol (β-me)(50 mM). Fresh cells were used in all assays. The memory B cell assay has since been validated on frozen cells, with similar results (Crotty et al., unpublished data).

2.3. Antibodies and reagents

Concanavalin A, phytohemagglutinin (PHA), and *Staphylococcus aureus*, Cowan (SAC) were purchased from Sigma (St. Louis, MO). CD20 FITC (clone 2H7), CD27 PE (clone M-T271), and CD3 APC were all purchased from PharMingen (San Diego, CA). Recombinant PA was provided via the AVRP. Vaccinia virus (VV) antigen was produced by first growing VV_{WR} stocks on HeLa cells in T175 flasks, infecting at a multiplicity of infection of 0.5 (MOI = 0.5). Cells were harvested at ~ 60 h, when the cells were not yet floating, and virus was isolated by rapid freeze-thawing the cell pellet 3 × in a volume of 2.3-ml RPMI + 1% FCS. Cell debris was removed by centrifugation. Clarified supernatant was frozen at − 80 °C as virus stock. VV_{WR} stocks were titered on Vero cells (~ 2×10^8 PFU/ml). VV antigen preparation for antibody ELISA and B cell ELISPOT use was done by UV inactivating stock VV_{WR} with trioxsalen/psoralen (4′ aminomethyl-trioxsalen HCl; Calbiochem)(Tsung et al., 1996). 1×10^8 PFU/ml VV_{WR} in 0.1% BSA and 10 μg/ml trioxsalen were incubated for 10 min at room temperature and then

UV-inactivated with 2.25 J/cm^2 (10 min in a Fisher UV Crosslinker UVXL-1000). This resulted in a >10^8-fold reduction in PFU. UV-inactivated virus was then used at a 1:5 (ELISPOT) or 1:25 (ELISA) dilution in PBS with BSA supplemented to a final concentration of 0.1%.

2.4. Memory B cell assay

PBMC were plated in 24-well dishes at 5×10^5 cells/well in R-10 supplemented with an optimized mix of polyclonal mitogens: 1/100,000 pokeweed mitogen extract (PWM) (made at Emory University, Atlanta, GA), 6 μg/ml fully phosphothioated CpG ODN-2006 (Hartmann et al., 2000), and 1/10,000 fixed *S. aureus*, Cowan (SAC) (Sigma). Six to twenty-four wells were cultured per individual. A negative control well was cultured in R-10 alone. Cells were cultured for 6 days at 37 °C, 6–8% CO_2. Substantial lymphocyte blasting and clustering could be observed in the culture wells from days 3 to 6. Note that experiments done for Figs. 2 and 3 were done without CpG oligonucleotide supplement. Addition of CpG improves the overall culture growth and ASC numbers by $\sim 1.5–2.0\times$.

In preparation for the ELISPOT, 96-well filter plates (Millipore, MAHA N4510) were coated with recombinant anthrax protective antigen (PA) at a concentration of 1 μg/ml. Keyhole limpet hemocyanin (KLH, 2.5 μg/ml) (Pierce Biochemicals) was used as a non-anthrax antigen control. To detect all IgG secreting cells, a separate plate was coated with 10 μg/ml goat anti-human Ig (or 4 μl/ml) (Caltag Laboratories, Burlingame, CA). Plates were washed and blocked with RPMI-1640 plus 1% bovine serum albumin (BSA, fraction V; Sigma) for 2–4 h at 37 °C prior to use.

The cultured PBMC were washed thoroughly, plated onto the ELISPOT plates, and incubated at 37 °C for 5 h. Plates were then washed with phosphate buffered saline (PBS, pH 7.2) followed by PBS containing 0.05% Tween-20 (PBST). Plates were then incubated overnight in 1 μg/ml mouse anti-human pan IgG Fc biotin conjugated antibody (Hybridoma Reagent Laboratory #HP6043B, Baldwin, MD) in PBST + 1% FCS. Plates were washed and then incubated with 5 μg/ml HRP-conjugated avidin-D (Vector Laboratories, Burlingame, CA) in PBST–1% FCS. Plates were again washed and then developed using 3 amino-9 ethyl-carbazole (AEC, Sigma). Developed plates were counted by inspection or using a plate reader (Cellular Technology, Cleveland, OH). Data are best represented as the frequency of antigen-specific B cells as a percentage of the total IgG$^+$ memory B cells per million PBMC. Standard growth, as determined by total IgG$^+$ spots per 5×10^5 cells cultured, was 5–15,000 IgG$^+$ spots. A Good Laboratory Practices-like (GLP-like) standard operating procedure (SOP) of this assay is available upon request.

2.5. Pokeweed mitogen (PWM)

PWM was first identified as a mitogenic lectin for lymphocytes in 1964 (Farnes et al., 1964) and can be isolated as a crude saline extract from the roots of *Phytolacca americana* (common names: pokeweed, pokesalad, or pigeonberry), generally harvested during the summer months (Borjeson et al., 1966). Pokeweed mitogen was produced at Emory according to classic techniques. Two kilograms of *P. americana* roots were harvested in August 2002. The roots were washed extensively, chopped into small pieces, and puréed in a counter top blender. Roots were then solubilized in 2 l of sterile PBS. Cold saline extraction was performed by leaving the solution overnight at 4 °C. The liquid phase was then siphoned off as the middle phase of the separated slurries. Approximately 1 liter of extract was obtained in this manner. The extract was purified and sterilized by ultracentrifugation at 27,000 × *g* for 1.5 h. The clarified extract was then aliquoted and frozen at − 80 °C. PWM_{emory} was compared to an old stock of PWM from Gibco (no longer available) and four ICN lots (ICN, Irvine, CA) (PWM available via Sigma is produced by ICN). PWM_{emory} (hereafter simply referred to as PWM) exhibited activity greater than or equal to that of commercially available PWM, and we found enormous variability in the commercially available material, leading us to produce and use our own PWM for consistency. We recommend that individual lots of PWM from ICN be titrated for activity across a dilution range from 1:1000–1:1,000,000 (ICN, Irvine, CA).

2.6. ELISA

Direct ELISA was done using Linbro/Titertek flat-bottomed 96-well plates (ICN, Costa Mesa, CA)

coated overnight with 0.1 μg/ml PA. Plates were washed and culture supernatant samples were added to the plate and serially diluted (twofold dilutions) in PBS + 0.2%Tween-20 + 1% FCS. Plates were incubated 90 min at room temperature. Plates were washed and then incubated with 0.25 μg/ml mouse anti-human pan IgG Fc biotin conjugated antibody (Hybridoma Reagent Laboratory #HP6043B) in PBS + 0.2% Tween-20 + 1% FCS for 90 min at room temperature. Plates were washed and then incubated for 60 min with 1.0 μg/ml HRP-conjugated avidin-D (Vector Laboratories) in PBS + 0.2% Tween-20 + 1% FCS. Plates were again washed and then developed using 0.4 mg/ml *O*-phenyleneiamine (OPD) in citrate buffer (pH 5.0) with 0.01% H_2O_2. Reaction was quenched with 1 M HCl after 15 min. Plates were read immediately at OD_{490}, with an OD_{595} baseline, in a BioRad 3550 plate reader.

2.7. Purification and culture of CD27$^+$ and CD27$^-$ B cell populations

For the B cell sorting experiments, approximately 250×10^6 PBMC were isolated from 11 CPT blood tubes drawn from a single AVA-vaccinated donor. These PBMC were initially enriched for B cells by positive selection using anti-CD19 paramagnetic beads and MACS columns (MiltenyBiotech, Germany), using the manufacturer's recommended protocol, except the buffer used was PBS plus 0.5% FCS plus 2 mM EDTA. Both the positive selected cell fraction and the flowthrough (non-binding) cells were retained for further use. The B cell depleted fraction ($CD19^-$) was run over a second positive selection column and the resulting flow through was $<0.5\%$ B cells. Following the MACS purification, there were approximately $6–7 \times 10^6$ $CD19^+$ B cells eluted from the column and 120×10^6 $CD19^-$ cells in the flow through fraction. The CD19 positively selected cell fraction was further fractionated by flow cytometric cell sorting (FACSVantage; Becton Dickinson) to obtain $CD27^+$ vs. $CD27^-$ B cell populations (all $CD19^+CD20^+$). Following cell sorting, approximately 1×10^6 $CD27^+$ B cells and 1.5×10^6 $CD27^-$ B cells were recovered.

Unsorted PBMCs (1×10^5) were cultured in 1 ml of R-10 containing PWM, SAC and β-me as described in the previous experiments. To mimic the natural percentage of approximately 5% B cells in the unmanipulated PBMCs and to provide T cell help to sorted B cells, 10,000 of either the $CD27^+$ or $CD27^-$ B cell fraction were remixed with 450,000 of the B cell depleted fraction. The ''reconstituted'' well contained 10,000 each of the $CD27^+$ and $CD27^-$ B cell fraction. For the ''T cell only'' well, 450,000 of the B cell depleted fraction were cultured without any added B cells.

3. Human memory B cell assay development

Several years ago, our laboratory developed an assay to track the development and maintenance of antigen-specific memory B cells in a murine model (Slifka and Ahmed, 1996; Crotty et al., 2003b). This assay utilizes an ex vivo 6-day stimulation of splenocytes to induce quiescent memory B cells to differentiate into plasma cells/antibody secreting cells (ASC) which can then be readily measured by ELISPOT analysis, using plates coated with the viral antigen of interest (Crotty et al., 2003b). The mouse memory B cell assay was initially done using specific antigen as part of the ex vivo stimulation (Slifka and Ahmed, 1996), but was later modified to utilize a polyclonal stimulation to make the assay easier to use and standardized (Crotty et al., 2003b). Using techniques based on our mouse memory B cell assay, we have now developed a human memory B cell assay. To develop the human memory B cell assay, we screened a variety of mitogens for maximal activity inducing polyclonal proliferation of human IgG^+ memory B cells and their differentiation into antibody secreting cells (Fig. 1). PBMC were isolated from normal human volunteers, and total PBMC were stimulated in culture for 5–7 days. After culture, cells were transferred to ELISPOT plates coated with anti-IgG antibody, and IgG secreting cells were detected in a short-term (5 h) assay. Pokeweed mitogen (PWM) combined with fixed *S. aureus* (Cowan strain, SAC) and a CpG oligonucleotide (ODN-2006) was the best combination identified (Fig. 1A). Analysis of cultures harvested at various time points indicated that maximal numbers of ASC were present at day 6 of stimulation (Fig. 1B), though robust results were also observed at day 5.

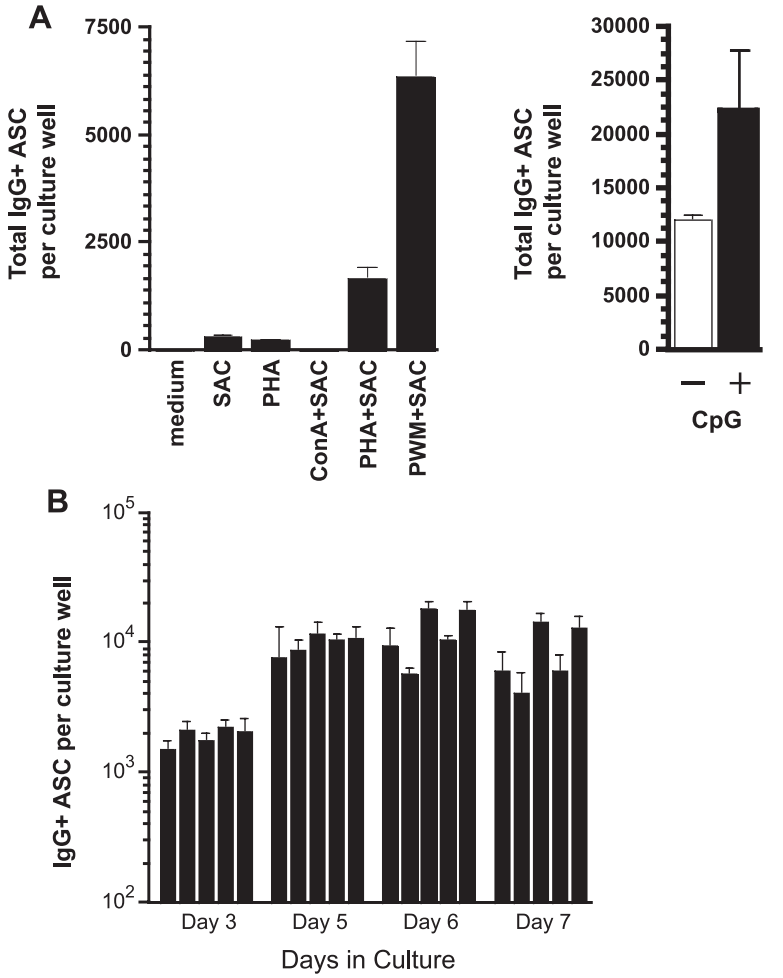

Fig. 1. Human memory B cell assay. (A) Memory B cell growth and differentiation under varied stimulation conditions. PBMC from healthy donors were cultured for 5 days with varying concentrations of polyclonal mitogens. After 5 days, these cells were washed and plated onto ELISPOT plates coated with α-human Ig antibodies, and antibody secreting cells (ASC) were detected using an α-IgGγ secondary antibody. The left graph shows a comparison of several mitogens. "Medium", medium alone. SAC, 1/10,000. Phytohemagglutinin (PHA), 2.0 μg/ml. Concanavalin A (ConA), 3 μg/ml. PWM, 1/100,000. Of the mitogens tested, PWM and SAC provided the best proliferation and differentiation of memory B cells into ASC. Error bars were calculated as the S.E.M. of three samples. On the right is a comparison of ASC production in PWM + SAC supplemented (+) or not supplemented (−) with phosphothiolated CpG oligonucleotide. Addition of CpG resulted in a ~1.5–2.0× increase in the total IgG^+ ASC observed. (B) Kinetics of memory B cell expansion and differentiation into antibody secreting cells (ASC). PBMC were stimulated with PWM and SAC and assayed for IgG ASC by ELISPOT on days 3, 5, 6, and 7. Five individuals are shown. For each individual, six replicate wells were stimulated. Error bars show the full growth range among the six replicates. Maximal IgG^+ ASC were detected on days 5 and 6 post-stimulation.

We then adapted the assay to function in an antigen-specific manner by utilizing ELISPOT plates coated with recombinant Protective Antigen (PA) of anthrax, and we used the assay to test for the presence of anti-anthrax memory B cells in the blood of individuals who received the licensed anthrax vaccine, AVA. Memory B cell responses to the anthrax PA protein were detected in AVA-vaccinated individuals (Fig. 2A–B). PA-specific responses were not seen in unvaccinated individuals (Fig. 2A–B). The absence of responses to an irrelevant protein (KLH) is shown as a negative control for all subjects

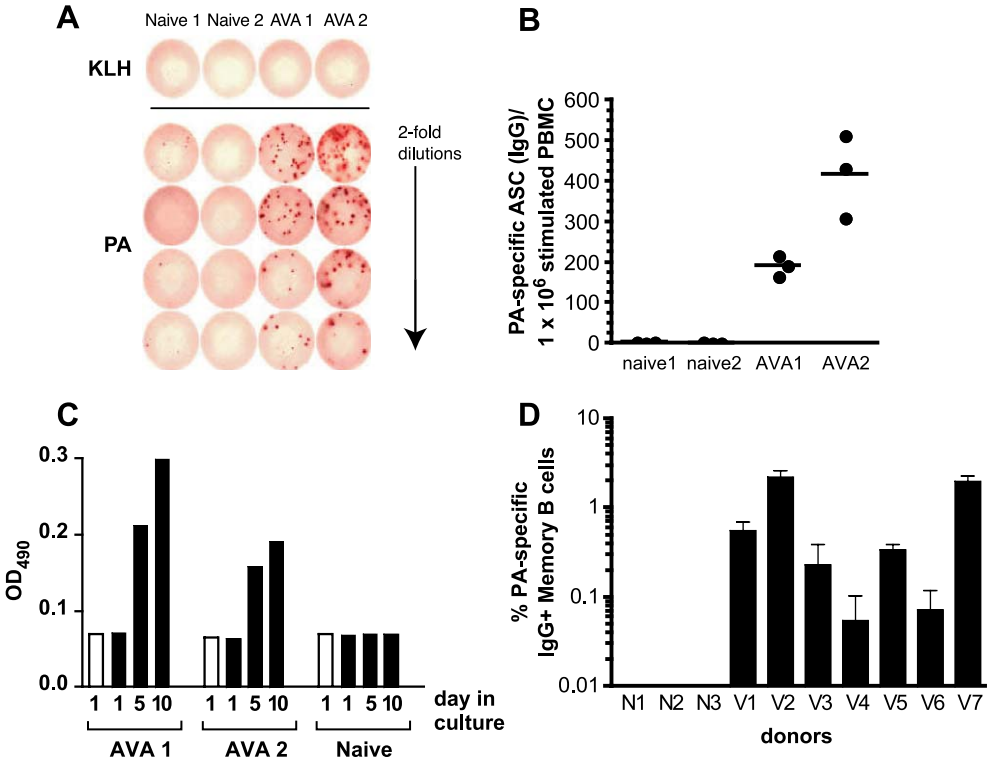

Fig. 2. Anthrax-specific memory B cells in vaccinated individuals. AVA-vaccinated volunteers were tested for the presence of PA-specific memory B cells. PBMCs from two vaccinated (AVA1, AVA2) and unvaccinated (naive1, naive2) volunteers were isolated and stimulated in vitro for 5 days with PWM and SAC. After 5 days in culture, these cells were plated onto ELISPOT plates coated with PA. Individual antibody secreting cells were detected through the use of α-IgGγ antibodies. Wells coated with KLH were used as a specificity control. (A) ELISPOT. Half of a culture well (seeded with 5×10^5 PBMC) was put into the first well of an ELISPOT plate coated with PA or KLH. Twofold serial dilutions of cells were done down the PA-coated wells. (B) Antigen-specific B cells in PBMC. PA-specific ASC detected per million cultured PBMC. Each data point represents results from a single culture well, and three replicates for each donor are shown. The bar represents the arithmetic mean of the three samples. (C) Antibody production in memory B cell cultures. To determine the level of antigen-specific antibody generated by memory B cells in vitro, culture supernatant was collected on days 1, 5, and 10 after start of culture stimulation and PA-specific ELISA was done. Black bars indicate supernatants from mitogen-stimulated cultures, white bars indicate supernatants from medium alone negative control cultures. Two representative AVA-vaccinated individuals are shown along with one unvaccinated individual. (D) Percentage of antigen-specific IgG^+ memory B cells. Data are represented as the percentage PA-specific B cells of the total IgG^+ memory B cells in PBMC. Three unvaccinated (N1–N3) and seven fully vaccinated (V1–V7) individuals are shown. Each vaccinated donor had received a full AVA vaccination schedule and each had received a booster immunization within 2 years prior to the time of experiment. PA-specific IgG^+ memory B cells range from 0.05% to 2.0% of the total $IgG\gamma^+$ memory B cells detected. The limit of detection of these samples ranges from 0.004 to 0.025, with the majority being <0.01. Error bars result from the S.E.M. of six replicate ELISPOT wells per donor.

(Fig. 2A). Supernatants from the memory B cell cultures were tested for anti-anthrax PA antibodies by ELISA. Supernatants of culture wells from AVA-vaccinated individuals tested positive for anti-PA antibodies (Fig. 2C).

The number of spots observed in the ELISPOT assay is not a direct reflection of the number of antigen-specific memory B cells seeded in a culture well, as proliferation occurs during the 6-day culture. Therefore, all PBMC samples are assayed both for total IgG memory B cells (spots observed in anti-IgG-coated ELISPOT wells) and antigen-specific memory B cells (spots observed in antigen-coated ELISPOT wells), thereby allowing quantitation of the antigen-specific memory B cells as a percentage of total IgG^+ memory B cells. Seven AVA-vaccinated individuals were tested and all were positive for PA-specific memory B cells (Fig. 2D). Up to 2% of circulating

IgG^+ B cells were PA-specific in these individuals (median = 0.3% of IgG^+ B cells). Donors were at varying time points post-vaccination, and received different numbers of booster immunizations and therefore responses were expected to vary in magnitude.

Since this human memory B cell ELISPOT assay is novel, we performed a series of experiments designed to demonstrate the specificity of this assay for memory B cells. Three of these controls were shown in Fig. 2: (1) antigen-specific memory B cells are not detected in naive individuals, only vaccinated individuals; (2) memory B cells are not detected against an irrelevant protein; and (3) anti-PA antibody was only detected (by ELISA) in cultures wells where PA-specific ASC were detected by ELISPOT. Additional sets of control experiments demonstrated that memory B cell differentiation into ASC in vitro was only detected in the presence of mitogenic stimulation, and no antigen-specific ASC were detectable in peripheral blood directly ex vivo, as expected (data not shown).

4. Vaccine-elicited memory B cells are $CD27^+$

We next wanted to demonstrate that the cells identified as memory B cells in this functional assay possessed canonical characteristics of human memory

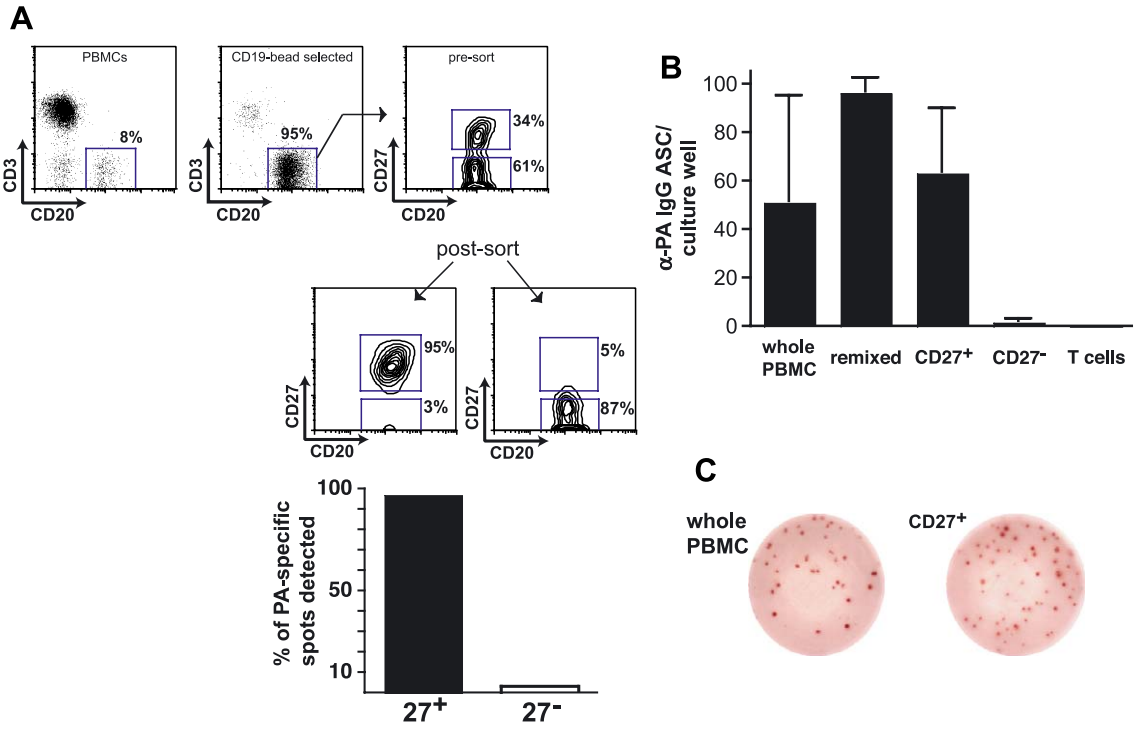

Fig. 3. Identification of anthrax-specific memory B cells as $CD27^+$ B cells. (A) Purification of $CD27^+$ and $CD27^-$ B cells. PBMCs isolated from an AVA-vaccinated individual were enriched for B cells by anti-CD19 antibody-linked paramagnetic bead selection. The B cell enriched fraction was FACS-sorted based on CD27 expression to give 90–95% pure populations of $CD27^+$ and $CD27^-$ B cells. The sorted $CD27^+$ and $CD27^-$ fractions were cultured with B cell-depleted PBMC (< 0.5% $CD20^+$). Cells were cultured for 6 days with PWM and SAC and total numbers of IgG secreting and PA-specific IgG secreting cells were then measured by ELISPOT. Graph shows the percentage of PA-specific IgG ASC that were detected in each fraction; 95% of the ASC were observed in the $CD27^+$ fraction. (B) Antigen-specific B cells are $CD27^+$. The number of PA-specific B cells detected per culture well is shown for each combination of B and T cells. "T cells": wells containing only B cell-depleted PBMC. "Remixed": wells containing cells that were fractionated into non-B cells, $CD19^+CD27^+$, and $CD19^+CD27^-$ populations and then reconstituted in appropriate ratios. The sorted $CD27^+$ and $CD27^-$ fractions were each separately cultured with B cell-depleted PBMC (< 0.5% $CD20^+$) ("$CD27^+$" and "$CD27^-$"). Data shown are from one experiment, and are representative of the two experiments done. (C) ELISPOTs. There is no qualitative difference in ASC spots produced from sorted and unsorted B cell populations.

B cells. CD27 has recently been identified as a good surface marker for human memory B cells, as determined by identification of somatic hypermutations of heavy- and light-chain loci in $CD27^+$ B cells and not in $CD27^-$ B cells (Agematsu et al., 1998, 2000; Klein et al., 1998; Tangye et al., 1998). Therefore, we performed cell sorting analysis to determine if the anthrax-specific memory B cells we describe were $CD27^+$ memory B cells.

B cells comprise ~5–20% of human peripheral blood lymphocytes. Approximately 30–50% of the B cells are $CD27^+$ (Fig. 3A). Human peripheral blood from an AVA-vaccinated individual was isolated and then separated into B cell and non-B cell populations using anti-CD19 magnetic bead purification. The $CD19^+CD20^+$ cells were then further purified by flow cytometric cell sorting to separate $CD27^-$ (naive) and $CD27^+$ (memory) B cells (Fig. 3A). Purity of the populations was checked by flow cytometry. $CD27^-$ naive B cells and $CD27^+$ memory B cells were then separately cultured with autologous non-B cells for 6 days in vitro using our standard memory B cell culture conditions. On day 6, the cultures were assessed for both total memory B cells and anthrax-specific memory B cells by ELISPOT. The majority of the total IgG^+ B cells (data not shown) and, importantly, the anthrax-specific memory B cells were found in the $CD27^+$ B cell population (Fig. 3A–C). This demonstrates that our assay accurately detects antigen-specific memory B cells elicited by a human vaccine and that these cells reside in the $CD27^+$ B cell compartment.

5. Detecting other responses: smallpox vaccine- or anthrax infection-specific memory B cells

To demonstrate that this assay is useful for the detection of other antigen-specific B cell responses, we explored antigen-specific memory B cell responses in smallpox vaccine (vaccinia virus; DryVax) recipients. Immune memory after smallpox vaccination (DryVax) is a valuable benchmark for understanding the kinetics and longevity of B cell memory in the absence of re-exposure to antigen, since immunization of the US population was stopped in 1972 and smallpox disease was declared eradicated worldwide in 1980 (Fenner et al., 1988). In addition, there is currently great public health interest in smallpox immunity due to the possible threat of bioterrorism (Fauci, 2002). We used vaccinia virus-infected

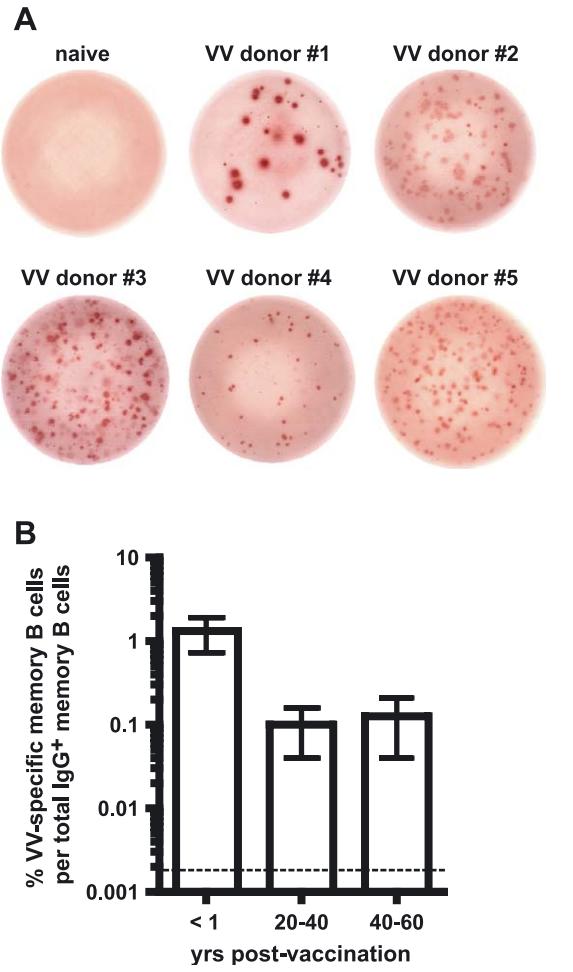

Fig. 4. Anti-vaccinia virus memory B cells in DryVax (smallpox vaccine) immunized individuals. (A) Individuals were tested for vaccinia virus-specific memory B cells using VV-infected cell lysate as the ELISPOT coating antigen. HeLa cell lysate was used as a control antigen. One non-vaccinated ("naïve") individual and several vaccinated ("VV donor") individuals are shown. One of many replicate wells is shown for each. (B) Vaccinees were divided into three group (<1, 20–40, and 40–60 years post-vaccination) and mean VV-specific memory B cell levels were compared. Error bars represent 95% confidence interval. There is a statistically significant difference between <1 vs. 20–40 years ($P<0.001$, two-tailed, one-way ANOVA) and <1 vs. 40–60 years ($P<0.001$). No statistically significant difference is observed between 20 and 40 years vs. 40–60 years ($P \gg 0.05$). Limit of detection is indicated by dashed line. (Data from Crotty et al., 2003a).

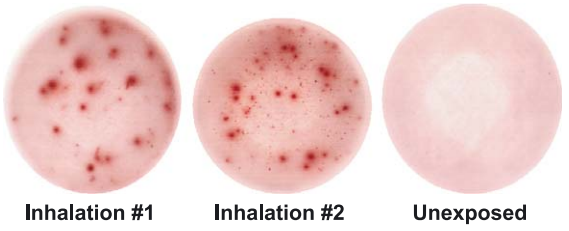

Fig. 5. Anti-anthrax memory B cells in anthrax-exposed individuals. Shown in the left and center wells are PA-specific IgGγ spots from two individuals who developed inhalational anthrax from the Fall 2001 bioterrorist attacks. Samples are approximately 1 year post-exposure. Shown in the right well is an example of an unvaccinated/unexposed donor.

cell lysate as the antigen, and uninfected HeLa cells were used as the appropriate negative control antigen. Vaccinia virus (VV)-specific memory B cells were detected in vaccinated individuals (Fig. 4). In contrast to the anti-anthrax PA responses (which were relatively uniform), a variety of VV-specific spot sizes and spot densities were observed in VV-immunized individuals (Fig. 4A), perhaps reflective of responses directed against a variety of VV protein antigens. Peak anti-VV responses of 1–3% of circulating IgG^+ memory B cells were observed, similar to what we have observed with anthrax (Fig. 4B). Impressively, responses were detectable for up to 60 years post-vaccination (Fig. 4B) (Crotty et al., 2003a).

In addition, PA-specific memory B cells were detected in individuals afflicted by inhalation anthrax after exposure to anthrax in the terrorism incidents of the Fall of 2001 (Fig. 5, and Quinn et al., submitted). These individuals were tested for PA-specific memory B cells at approximately 1 year post-exposure. These results demonstrate that PA-specific memory B cells are elicited by anthrax infection as well as by AVA vaccination.

6. Discussion

Efficient techniques are available for detecting human antigen-specific memory $CD8^+$ T cells, memory $CD4^+$ T cells, and antibodies, but there is a dearth of techniques for detecting human memory B cells. It is unclear whether memory B cell and serum antibody levels correlate well for most antigens, and it is therefore important to be able to track memory B cells as an independent parameter of antigen-specific immune memory.

The strengths of this ELISPOT memory B cell assay are: (1) that it is readily generalizable to virtually any antigen (since the stimulation conditions are nonspecific, and the only antigen-specific step is the coating of the ELISPOT plate); (2) it provides an accessible quantitation of memory B cell numbers (percent of circulating IgG^+ memory B cells that are antigen-specific); and (3) it uses relatively inexpensive reagents (pokeweed mitogen (PWM), SAC, and CpG) and a modest amount of human blood (5 ml). Additionally, we have demonstrated that the vaccine-elicited antigen-specific memory B cells do indeed reside in the $CD27^+$ compartment previously identified as the compartment containing the majority of B cells possessing hypermutated Ig heavy and light chains.

Two previously available techniques for tracking human memory B cells involve either direct detection of antigen-binding cells by flow cytometry (Leyendeckers et al., 2002), or long-term culture (14–28 days) followed by ELISA (Bernasconi et al., 2002). Direct detection of memory B cells by antigen-binding is an appealing technique, and is of great value because it does not involve any stimulation, and allows for further phenotypic analysis of individual cells (Leyendeckers et al., 1999, 2002; Maruyama et al., 2000; Gonzalez et al., 2003; Weitkamp et al., 2003). However, development of antigen-binding flow cytometry assays can be tricky (Bell and Gray, 2003), and the low frequency of most antigen-specific B cells causes detection problems. Detecting memory B cells by bulk culture followed by ELISA on the supernatants is a strategy similar to ours (Bernasconi et al., 2002). We prefer the ELISPOT assay because it gives an estimate of the actual frequency of the antigen-specific memory B cells, and is more rapid (5–6 days instead of 14+).

One to two percent of IgG^+ memory B cells in PBMC are smallpox or anthrax vaccine-specific after recent immunization, as determined using our memory B cell ELISPOT assay. By using best estimates of the relevant parameters (1.25×10^6 PBMC recovered per milliliter of blood collected into CPT; B cells are 10% of PBMC; memory B cells are 30% of B cells; IgG^+ memory B cells are 50% of memory B cells), we can calculate that there are approximately 200–400 anthrax or smallpox vaccine-specific IgG^+ memory B

cells per milliliter of blood (of the ~ 125,000 total B cells/ml blood). This translates to 1–2 million anthrax or smallpox-specific memory B cells circulating in the blood of a recently immunized person.

This assay can be readily adapted for use in detecting virtually any antigen recognized by human B cells (we have also recently validated this memory B cell assay in non-human primates, with minor modifications (unpublished data)). Here we have demonstrated the ability to detect memory B cells specific for two different antigens of interest—anthrax PA protein, and vaccinia virus—and we are in the process of expanding that repertoire. We have recently completed studies determining the magnitude and longevity of memory B cell responses after vaccination against smallpox (Crotty et al., 2003a) and the magnitude of memory B cell responses after bioterrorism-related anthrax (Quinn et al., submitted). This assay is also being implemented as part of a large (1500 volunteers) multi-center vaccine trial (the Anthrax Vaccine Research Program, AVRP) to enable us to track humoral immune responses longitudinally over a 4-year time course. In addition, this assay is being used to determine the development of immunological memory in individuals infected with or exposed to monkeypox in the recent Midwest outbreak (I. Damon et al., unpublished data). As such, this assay makes a variety of questions accessible regarding human memory B cells and the longevity of human immune memory.

Acknowledgements

This work was funded by the Anthrax Vaccine Research Program (AVRP) and grants from the National Institutes of Health. Shane Crotty was funded by a Cancer Research Institute Postdoctoral fellowship.

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Chapter 4, Supplemental Figures 1 and 2:

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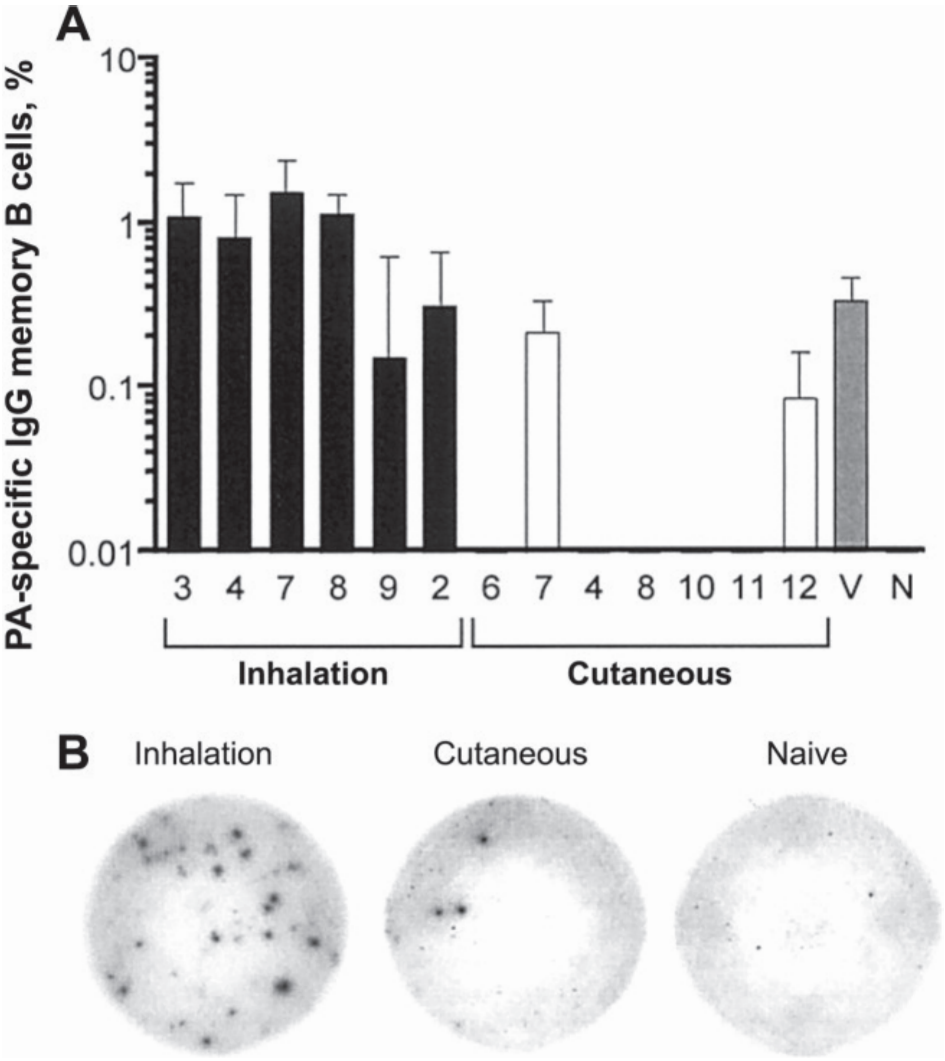
> Quinn, C.P., P.M. Dull, V. Semenova, H. Li, S. Crotty, T.H. Taylor, E. Steward-Clark, K.L. Stamey, D.S. Schmidt, K.W. Stinson, A.E. Freeman, C.M. Elie, S.K. Martin, C. Greene, **R.D. Aubert**, J. Glidewell, B.A. Perkins, R. Ahmed, and D.S. Stephens. 2004. Immune responses to *Bacillus anthracis* protective antigen in patients with bioterrorism-related cutaneous or inhalation anthrax. *J Infect Dis* 190:1228-1236.

Supplemental Figure 4-1: Enzyme-linked immunospot (ELISPOT)) assay analysis of the frequency of protective antigen (PA)-specific IgG memory B cells in patients with inhalation and cutaneous anthrax. Surviving patients with bioterrorism-related anthrax were tested for PA-specific IgG B cell memory 8-14 months after the onset of symptoms, by screening peripheral-blood mononuclear cells (PBMCs) using a novel ELISPOT-based functional memory B cell assay. **A)** PA-specific IgG memory B cells were detected in serum samples in all 6 of the patients with inhalation anthrax who survived and in 2 of the 7 patients with cutaneous anthrax who were tested. Patients are indicated in the abscissae by their cutanious- or inhalation-anthrax case no. Data for a representative vaccinated individual (≥4 doses of anthrax vaccine absorbed) who had received a booster dose within the last year are shown for comparison (V). Data for a naïve individual (N) are shown as a negative control. The lower limit of detection was 0.002-0.01 antigen-specific cells/10^6 PMBCs. **B)** ELISPOT images of PA-specific IgG memory B cells, from CA-7, from a representative patient with inhalation anthrax, and

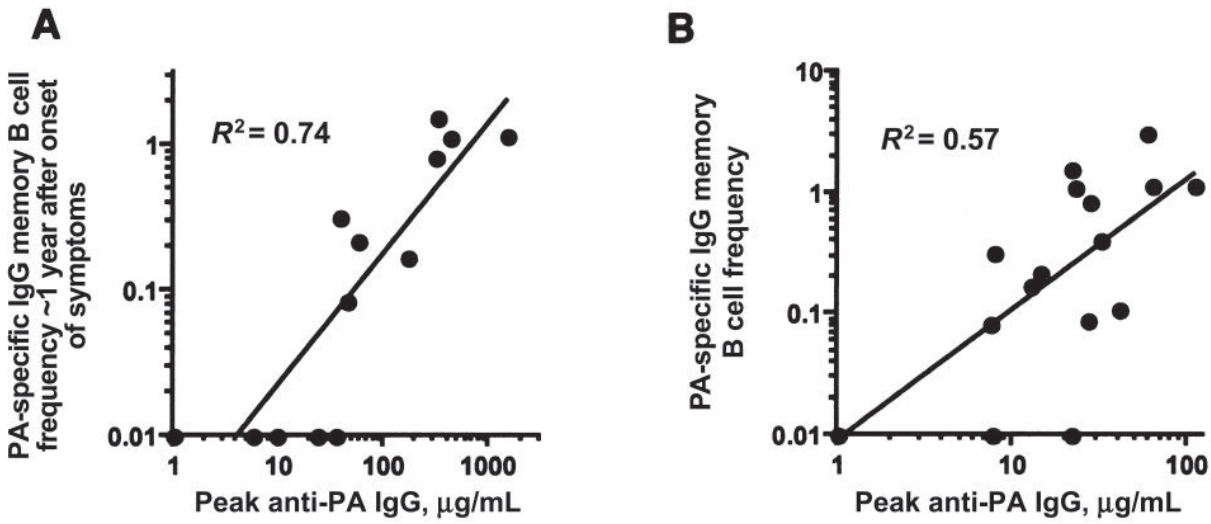
from a naïve individual (shown as a control).

Supplemental Figure 4-2: A) Relationship between peak anti-protective antigen (PA) IgG levels and the subsequent magnitude of PA-specific IgG B cell memory ~1 year after the onset of symptoms (R2=0.74, linear regression). Non-responders are graphed at the origin. **B)** Comparison of anti-PA IgG levels and PA-specific IgG memory B cell frequencies, obtained from the same venipuncture. A positive correlation was observed (R2=0.57, linear regression). Data represented in this graph were from venipuncture taken between 3 and 14 months after exposure. Non-responders are graphed at the origin.

Supplemental Figure 4-1



Supplemental Figure 4-2



Conclusions and Future Directions:

Part I: Cellular and humoral immunity during chronic viral infection

The global burden of people living with HIV, Hepatitis B, and HCV is more than 40, 250, and 170 million people each, respectively(196-198). Although developing vaccines for disease prevention is important, the need for therapeutic interventions in chronically infected patients remains crucial to improving global health. Therapeutic vaccine strategies have had only limited clinical success(199-202), and better understanding the regulation of the immune system during this chronic phase will help us design better therapeutic strategies. CD8 T cells are critical for the clearance of virally infected cells, however, exhaustion and CTL escape remain important barriers to therapeutic success. In these studies we have examined the role the CD4 T cells and B cells in altering viral replication and CD8 T cell exhaustion.

In the first study we were able to show that short alterations in CD4 T cell activation and signaling via CD40:CD40L can lead to long-term impairment of humoral immune responses. These animals had significantly reduced long-term antibody responses and had increased viral burden and CD8 T cell exhaustion. While treatment with hyperimmune serum has been shown to prevent the exhaustion of CD8 T cells in CD40L deficient animals(203), these experiments treated the mice immediately following LCMV infection and this strategy remains clinically less useful as patients are rarely diagnosed during the acute stages of infection. More interestingly, both B cells and CD4 T cells have been shown to help prevent exhaustion of CD8 T cells transferred into chronically infected hosts(183). Additionally, CD8 T cell immunotherapy has shown

promise clinically in the treatment of malignancies(185). In the second study, we took a similar approach by transferring naïve antigen-specific CD4 T cells into LCMV chronically infected mice. Transgenic CD4 T cells transferred into these chronically infected hosts divided, produced cytokine, and survived long-term in vivo. Strikingly, following CD4 T cell transfer there was enhanced proliferation and function by exhausted CD8 T cells. This enhancement of CD8 T cell function was to a wide-range of LCMV epitopes, which may be important in preventing viral escape. This enhancement depended on antigen-specific CD4 T cell transfer, and also resulted in improved antibody responses in chronically infected mice. This enhanced immunity lead to decreases in serum viral loads and overall viral burdens over the long-term post transfer. This data suggests that an immune transfer strategy might be useful in improving CD8 T cell function after the establishment of CD8 T cell exhaustion during chronic disease.

CD4 T cells in CD40L deficient animals have defects in proliferation and cytokine function following LCMV infection. These mice failed to develop a long-lived antibody response to LCMV or VSV(43, 44, 170). However, CD4 T cells from CD40 deficient mice were capable of providing help when transferred into naïve animals and challenged with a T-dependent antigen(172). From this data, researchers concluded that the CD4 T cell defect was not due to a requirement for CD40 on the antigen presenting cell during CD4 T cell priming. In the second chapter we show an important role for the CD40/CD40L pathway during B cell priming and for prevention of CD8 T cell exhaustion following LCMV clone-13 infection. Indeed, CD40L knockout CD4 T cells could be transferred to chronically infected recipients to examine whether this molecule is important (either directly or through an antigen presenting cell). However, another

way to approach this question is to address whether B cells were necessary for the CD8 T cell rescue by transferring transgenic CD4 T cells into the μMT mice, which do not clear LCMV clone-13 infection. The need for CD4 T cell help to activate antigen-presenting cells is thought to be less important for viral infections where the antigen-presenting cell is directly infected and activated through TLR signaling. However, antigen-presenting cell dysfunction has been well documented in both murine models(53, 54) and human chronic infection(204-207). Therefore, the rescue of CD8 T cell function by CD4 T cells may still be occurring through CD4 T cell activation of the antigen-presenting cell. This is a difficult question to address with this model. One way to investigate these types of questions would be to look at differences in priming by therapeutic vaccination with MHC class I versus class II knockout dendritic cells loaded with LCMV peptides.

The activation of naïve CD4 T cells in the chronic environment is altered and further studies are ongoing to better understand how these transferred CD4 T cells differ from those activated during acute infection. Indeed, a study looking at CD8 T cell activation during the acute versus chronic stage of polyoma virus has shown different requirements for co-stimulatory molecules in the latter primed CD8 T cells(208). The availability of antigen and professional antigen presenting cells is altered during the establishment of a chronic infection. It remains unclear where these cells are being activated and by what cell types; for example are there differential fates for cells primed in the lymph nodes versus the peripheral tissues? Although all the transferred cells were recruited into the response and underwent 4-7 rounds of division (Chapter 3, Figure1), these activated CD4 T cells also have altered levels of activation markers such as CD62L, CD69 and CD25 (R. Aubert, unpublished observations). This leads to the question of

whether memory CD4 T cells or in vitro activated and rested CD4 T cells would also persist following transfer and if they could mediate help to exhausted CD8 T cells. These studies have not yet been done and would bring further clinical relevance to this model of CD4 T cell immunotherapy.

This study did not examine the mechanism of CD4 T cell help to exhausted CD8 T cells following in vivo transfer. This could be easily studied by crossing the transgenic LMCV-specific CD4 T cell mice with mice deficient in a number of potential targets. Although previous studies have shown that IL-2 treatment enhances CD8 T cell responses in chronic infection(194), these transferred CD4 T cells make refractory levels of cytokine, with very low amounts of IL-2 compared to cells activated during an acute viral infection. IFNγ, as well as IFNα/β have been suggested to be essential for CD8 T cell proliferation and/or survival and may also be important for CD8 T cell rescue(21, 209). However, inhibition of cytokine production by transferred CD4 T cells likely represents an important checkpoint to prevent fatal immunopathology. These mice lose significant body weight following CD4 T cell transfer and alterations in the proliferation or differentiation of these CD4 T cells may result in poor clinical outcome.

CD8 T cell immunotherapy has been used to treat malignancies(185) and has shown some promise to limit viral infections in bone marrow transplant recipients(186, 210). However, rapidly replicating pathogens such as HIV and HCV have shown a propensity for the evolution of escape mutations(189, 211) and this could limit the usefulness of such therapies(188). Additionally, CD4 T cell responses appear to be critical for the maintenance of CD8 T cells during chronic viral infection(86, 163). Therefore we examined whether direct CD4 T cell immunotherapy would modulate CD8

T cell exhaustion in chronically infected recipients. An alternate strategy would be to develop therapeutic vaccines that target both CD4 T cell and CD8 T cell epitopes. One advantage to the direct immune transfer approach is that frequencies of antigen-specific CD4 T cells may be too low to be boosted during chronic infection. Secondly, these transferred CD4 T cells can be engineered to express molecules that may skew the immune response away from a tolerogenic or Th2 phenotype. IL-2 producing CD4 T cells has been shown to correlate with strong CTL responses and viral control(162). Most importantly, targeting of CD4 T cells lead to improvements in CD8 T cell responses across a wide-range of major and minor viral epitopes, thereby lessening the chance of viral escape. Finally, CD4 T cell responses may also enhance antibody responses and these antibodies may help lower viral titers and prevent re-exhaustion of the recovered CD8 T cells. The studies here begin to examine how CD4 T cell immunotherapy can influence the exhaustion of CD8 T cells and suggest that such a strategy may be a useful approach to overcome chronic infection.

Part II: Measuring humoral immunity following acute vaccination

Humoral memory consists of two components: the long-lived plasma cells which reside primarily in the bone marrow and secrete antibodies, and circulating memory B cells which are primed to rapidly divide and differentiate into plasma cells following antigen re-encounter. Memory B cells have also been suggested to be important in replenishing plasma cell numbers in the absence of antigen exposure(153). However, very little is known about the kinetics or size of the memory B cell population in humans. In this study we have designed an assay to measure circulating memory B cells in the

peripheral blood of humans following vaccination or antigen exposure. This assay is readily adaptable to a wide range of bacterial or viral pathogens. In addition, we have shown that the cells detected by our assay originate from the CD27+CD19+ memory B cell population. Finally, we used this assay to detect memory B cells following two different types of vaccinations, as well as in patients exposed to anthrax in 2001. This assay provides a useful tool to examine the size and longetivity of memory B cells in humans following vaccination or infection.

Modern vaccine development has increasingly relied on development and testing in non-human primate models. We have concurrently developed our assay to measure memory B cells in the peripheral blood of rhesus macaques. Our initial studies of anthrax exposed humans showed a strong correlation between peak IgG titer and level of memory B cells approximately one year post infection. By working in non-human primate models, we can assess more closely these correlations between antibody titer and memory B cell levels post vaccination and examine if there are differential effects on memory B cells and antibody following booster immunizations. Additionally, the ability to collect tissue samples in non-human primates provides us the ability to ask how well assaying the blood captures the level of memory B cells present in the lymph nodes or spleen.

Most importantly, by using the rhesus macaque model we can begin to address the biology of memory B cells during antigen challenge. A critical question is: in the absence of high levels of circulating antibody, can memory B cells divide and differentiate into plasma cells and provide protection? Although many vaccines induce potent long-lived antibody responses, others have shown a drop to low levels within a few years post vaccination. One of these vaccines, the hepatitis B (HBV) vaccine

requires frequent booster immunization for high-risk groups such as healthcare workers. However, it is difficult to maintain complete vaccination coverage of these individuals and understanding if memory B cells provide some protection would help shape post-exposure strategies.

In this study we have developed an assay that provides an alternative approach to measuring B cell memory. Although serum antibody levels are critical for defense against foreign pathogens, circulating memory B cells are primed for differentiation into potent immune effectors. By measuring memory B cells in humans, we have developed a useful tool for measuring vaccine efficacy and helping us better understand the maintenence of serological memory in humans.

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**Note that Chapter 4 references are not listed below; they are located within the chapter text.

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