The Influence of B-cell Tolerance on Humoral Immunity to HIV-1

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2010
ABSTRACT

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Abstract

Several HIV-1 neutralizing antibodies (e.g. 2F5, 4E10) have been shown to react with self-antigens, suggesting that effective humoral responses to HIV-1 may be constrained by the tolerization of HIV-reactive B cells that also recognize self-antigens. I have tracked the development of 2F5-like HIV-1 gp41 membrane proximal external region (MPER)-reactive B cells throughout ontogeny using B-cell tetramer reagents. In BL/6 mice, MPER-binding populations are lost during normal B-cell development and immunization with HIV-1 MPER antigen does not elicit robust humoral responses. I have identified Kynureninase as a self-antigen that is recognized by 2F5 antibody and, therefore, is a molecule that could mediate the developmental loss of B cells reactive to an epitope shared by HIV gp41 and Kynureninase. To recover these MPER-reactive cells, I describe and characterize a stromal-cell independent culture system that efficiently supports pro-B cell to IgM+ B-cell development with near normal levels of IgH and Igκ diversity. B-cell development in vitro closely follows the patterns of development in vivo with culture derived (CD) B cells demonstrating characteristic patterns of surface antigen expression and gene activation. Immature and transitional B-cell compartments are reduced, due to the induction of tolerance, in the bone marrow of 3H9 IgH knockin mice; however, cultures of 3H9 IgH knockin pro-B cells yields high frequencies of “forbidden”, autoreactive IgM+ B cells. Furthermore, serum IgG autoantibody exceeded
that present in autoimmune, C4\textsuperscript{−\textsubscript{b}} animals following the reconstitution of RAG-1\textsuperscript{−\textsubscript{c}} mice with IgM\textsuperscript{+} CD cells derived from BL/6 mice. I show that HIV-1 MPER-reactive B cells are recovered from both BL/6 and 2F5 IgH knockin bone marrow using this \textit{in vitro} culture system. RAG-1\textsuperscript{−\textsubscript{c}} mice reconstituted with these culture-derived B and T cells generate strong germinal center and antibody responses to HIV-1 MPER antigens. These data demonstrate that the humoral immune response to this HIV-1 gp41 MPER antigen can be restored in mice when the constraints of B-cell tolerance have been relaxed.
Dedication

To my Family & Friends that drove me to completion…

To my Classmates that began this journey with me…

To the People of the world living with HIV/AIDS…
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1. Introduction

1.1 B-cell Tolerance and Immunity to HIV-1 Infection

Subsequent to establishing cellular reservoirs of virus and chronic infection, HIV-1 elicits generalized immune dysregulation, including B-cell activation regardless of antigen specificity. This polyclonal B-cell activation does not result from direct viral infection as HIV-infected B cells are rarely observed in patients (Sodora and Silvestri 2008). Instead, chronic HIV-1 infection drives B-cell activation via systemic inflammatory signals (Sodora and Silvestri 2008), including but not limited to interferons, TNF-family members and interleukins (reviewed in (Moir and Fauci 2009)). This sustained B-cell dysregulation during HIV-1 infection is permissive for the production of serum autoantibody that can result in autoimmune disease (Ng 1996). Thus, the milieu of inflammatory cytokines (e.g. BAFF, TNF (Groom, Kalled et al. 2002; Cain, Kondo et al. 2009)) present during chronic infection appears to relax B-cell tolerance mechanisms (e.g. clonal deletion, anergy and receptor editing (Goodnow 1992)) that normally suppress the capacity of B cells to respond to self-antigens (Fig. 1).

HIV-1 infection commonly induces neutralizing antibodies (Abs), many of which are directed against strain-specific and highly variable regions of the viral envelope (Haynes and Montefiori 2006). Therefore, these Abs exhibit a restricted capacity to control the rapidly mutating HIV-1 (Montefiori 2005). Interestingly, recent evidence
demonstrates that a subset of broadly neutralizing Abs to HIV-1 cross-react with host cellular antigens, raising the possibility that immune tolerance mechanisms limit the development of some HIV-reactive B cells (Haynes, Fleming et al. 2005). In addition, vaccines designed to induce protective B-cell responses have failed to elicit broadly neutralizing Abs, suggesting that the B-cell repertoire is limited in inducing some HIV-neutralizing specificities. This “tolerance hypothesis” proposes that mechanisms of B-cell tolerance that block autoimmune pathology can limit humoral immune responses to some pathogens (e.g. HIV-1); however, reduced immune recognition of these pathogens can also result in chronic inflammation and the relaxation of immune tolerance. While the inflammatory rescue of autoreactive B cells is dangerous with regards to autoimmunity, the benefit of retaining B cells that express Ab specific to self-antigens - that also cross-react with microbial antigens - may be an evolutionary compromise to reduce the threat posed by uncontrolled microbial infections.

In this introduction, I describe the molecular mechanisms that promote a diverse repertoire of Ab specificities formed by V(D)J recombination during mouse and human B-cell development. Furthermore, I discuss mechanisms of tolerance that purge the B-cell repertoire of self-reactive clones and, as a consequence, may limit the recognition of pathogens which express “host-like” antigens. I discuss this “tolerance hypothesis” of Haynes et al. (Haynes, Fleming et al. 2005) as a potential mode of immune evasion.
utilized by HIV-1 to subvert neutralizing B-cell responses. In addition, I explore other mechanisms that can be used by pathogens to subvert immune recognition, including the elicitation of autoantibody. The identification of a novel pool of pathogen-reactive lymphocytes may provide a previously unidentified target(s) for vaccination and provide new insights into host-pathogen interaction.

Figure 1: Inflammation can reduce the effectiveness of tolerance by increasing the production of B-cell survival factors.

During their development in the bone marrow, newly formed B cells may express membrane receptor Abs that are specific for self- (red circle) or foreign (white circle) antigens. The several mechanisms of central tolerance (boxed area) efficiently remove self-reactive B cells, decreasing their frequency in the mature, peripheral B-cell
compartments. In part, B-lymphopoiesis and the maintenance of peripheral B-cell compartments are controlled by the availability of B-cell survival factors, such as BAFF (light blue) (Schneider, MacKay et al. 1999). During inflammation, increased production of these survival factors (darker blue) may promote the survival and/or retention of self-reactive B cells and thereby increases their frequency in peripheral B-cell compartments (Groom, Kalled et al. 2002).

1.2 B-cell Development

B-lymphocytes arise from common lymphoid progenitors (CLP) present in the bone marrow (BM) of adult mice (Kondo, Weissman et al. 1997). Commitment to the B lineage requires expression of specific transcription factors, such as E2a, EBF and Pax5 (Busslinger 2004) that are required for B cell-associated gene transcription and maintenance of B-cell identity (Kallies, Hasbold et al. 2007). E2a is required for initiating EBF and Pax5 expression (Ikawa, Kawamoto et al. 2004) and E2a−/− mice exhibit developmental defects in the BM resulting in a lack of mature B cells, while ectopic expression of EBF in E2a−/− cells restores B-cell development potential (Seet, Brumbaugh et al. 2004). The loss of Pax5 expression in mature B cells, either due to plasma cell differentiation or experimental manipulation, results in i) the decreased expression of surface markers associated with B lymphocytes (Kallies, Hasbold et al. 2007), and ii) the re-expression of genes that are repressed by Pax5 upon B cell commitment, but reactivated during ASC differentiation (e.g. Flt3 and PD-1) (Delogu, Schebesta et al. 2006). These data suggest that B-cell development is controlled by the activation of a
specific genetic profile coupled to the repression of alternative genetic profiles
associated with the development of distinct hematopoietic lineages.

In mice, discrete BM niches are occupied by different B-cell progenitors
depending upon their stage of B-cell maturation (Nagasawa 2006; Pereira, An et al.
2009); however, our understanding of B-cell development is confounded by
heterogeneous BM stromal cell populations (Dorshkind 1990) and the incomplete
knowledge of what factors define these niches. In part, the expression of cytokines that
are essential for B-cell development and survival define these distinct BM niches (Baird,
Gerstein et al. 1999). IL-7 is a key cytokine required during mouse B-cell development in
the BM (Ray, Stoddart et al. 1998). IL-7−/−, IL-7R−/− mice or mice deficient in IL-7 signal
transduction components (e.g. common γ-chain (Kondo, Takeshita et al. 1993)) are
unable to efficiently produce lymphocytes, including B cells (Kondo, Takeshita et al.

While many aspects of human B-cell development mirror that of mouse B
lymphopoiesis, some fundamental differences do exist in B-cell development between
each species. In contrast to B-cell development in mice, human B cells are generated in
primary immunodeficiency patients who lack IL-7, IL-7R or IL-7R signal transduction
components including the common γ-chain (Sugamura, Asao et al. 1996).
While many cell-intrinsic and -extrinsic cues drive the production of B cells, I will focus on the assembly of Ab molecules to describe early stages of B-cell development as re-organization of immunoglobulin loci of the B cell is the strongest evidence for commitment to the B lineage. It is important to note that events leading to Ab production is controlled, in part, by i) signals derived from the cytokine milieu and ii) the expression of specific transcription factors (ten Boekel, Melchers et al. 1995; Strobl, Hofelmayr et al. 2000); however, a detailed description of transcription factors that control B-cell development is beyond the scope of this research and will not be reviewed in detail.

1.2.1 Immunoglobulin and B-cell diversity

The immunoglobulin (Ig) heavy chain (HC) and light chain (LC) gene loci (Igh and Igκ/λ, respectively) encode the B-cell antigen receptor (BCR) (Fig. 2), which is necessary for B-lymphocyte survival and specific recognition of antigens (Carrasco and Batista 2006; Srinivasan, Sasaki et al. 2009). During Ig HC formation, variable- (V), diversity- (D) and joining- (J) region gene segments of the Igh loci are assembled to form a complete VH region-coding DNA segment (Tonegawa 1983). The mouse Igh locus is comprised of ~180 V-segments, ~20 D-segments, 4 J-segments (Lefranc, Clement et al. 2005) arranged in discrete clusters (Fig. 2). After the completion of Igh rearrangement, V- and J-gene segments from the Igκ or Igλ loci are combined to form the Ig LC (Fig. 2). The
HC and LC each contain complementarity determining regions (CDRs) that, combined, form a specific site which determines the specificity of antigen-binding (Carrasco and Batista 2006). Because BCRs are created in the absence of their cognate antigens, B lymphopoiesis produces an un-selected pool of B cells capable of recognizing both foreign and self antigens (reviewed in (Hardy and Hayakawa 2001)) and requires the removal of self-reactive clones to limit autoimmunity.

**Mouse Ig Loci (Igh, Igκ and Igλ)**

The Ig HC is encoded by the *Igh* locus that is comprised of many V-, D-, J- region gene segments that must undergo rearrangement to form one, complete VDJ gene segment that is completed after association with a C-region gene segment. The utilization of different C-region gene segments is dependent on the stage of B-cell maturation and differentiation. The Ig LC is encoded by either the *Igκ* or *Igλ* loci; each locus contains V-
and J- region gene segments that must undergo rearrangement to form one, complete VJ gene segment that is completed after association with a C-region gene segment.

**Figure 3: The stages of B lymphopoiesis**

B cells arise in the adult BM from CLP that progress through a series of developmental stages (Hardy and Hayakawa 2001). Early stages of B-cell development are determined by the rearrangement status of the $Igh$ and $Ig\kappa/\lambda$ loci (Osmond, Rolink et al. 1998). After the formation of the BCR, expression of IgM, IgD and other surface markers (not shown) is used to identify immature, transitional (T1 and T2) and mature B-cell compartments (LeBien and Tedder 2008).

The combinatorial association of V(D)J gene segments that comprise the variable portion of Ig HCs and LCs and the independent pairing of Ig HC + LC generates a diverse array of Abs available for antigen recognition (Tonegawa 1983). This diversity within the HC Ab repertoire is enhanced beyond the potential diversity encoded within
Igh DNA sequence by terminal deoxynucleotidyl transferase (TdT) (Dudley, Chaudhuri et al. 2005). TdT is an enzyme responsible for the template-independent addition of N-nucleotides at Igh gene segment junctions, thereby increasing the diversity and length of CDR3 regions beyond that encoded within Igh loci (Alt and Baltimore 1982).

V(D)J recombination utilizes many enzymes and cellular processes to promote DNA rearrangement while ensuring genomic stability. The process of V(D)J rearrangement requires RAG1/-2 and DNA-dependent protein kinases (PKcs) as they permit double-stranded (ds) DNA cleavage and resolution of dsDNA breaks, respectively (reviewed in (Bosma, Schuler et al. 1988; Dudley, Chaudhuri et al. 2005)). Other proteins, including Artemis and DNA ligase IV, in the nonhomologous end-joining (NHEJ) DNA repair pathway, work in concert with DNA PKcs for the completion of V(D)J recombination (Market and Papavasiliou 2003). During resolution of these dsDNA breaks, imprecise joining of V(D)J gene segments permit unique patterns of template-encoded P-nucleotide additions, yet another mechanism that contributes to BCR diversity (Diaz and Klinman 2000).

Combined, the molecular mechanisms of Ig recombination can generate frame-shift mutations, resulting in truncated or mis-folded non-functional Ig proteins (Baumann, Potash et al. 1985). In-frame rearrangement of the HC and LC loci occur when V(D)J rearrangement maintains the proper reading frame through the constant (C-
regions of Ig HC and LC polypeptides (reviewed in Schlissel 2004). The usage of different C-regions (C\(\mu\), C\(\delta\), C\(\alpha\), C\(\gamma\), and C\(\epsilon\)) of Igh determines the isotype of Ab, each imparting unique biological properties (reviewed in Stavnezer, Guikema et al. 2008). However, developing B cell of the BM only express IgM and IgD Ab isotypes that are created by differential mRNA splicing of the HC VDJ-C\(\mu\)-C\(\delta\) transcript (Stavnezer, Guikema et al. 2008).

During Ig LC rearrangement, V-region and J-region gene segments of either Ig\(\kappa\) or Ig\(\lambda\) are joined to form a complete Vi region-coding DNA segment (Tonegawa 1983) (Fig. 2). Early studies in human fetal liver cells demonstrated that Ig LC rearrangement occurred only after the formation and expression of Ig HCs (Gathings, Lawton et al. 1977). Interestingly, the genomic organization of V- and J-gene segments is different between the Ig\(\kappa\) and Ig\(\lambda\) loci (Fig. 2). The mouse Ig\(\kappa\) locus contains \(~120\) V\(\kappa\) gene segments that covers a \(~3\) megabasepair (Mb) region of DNA followed set of 5 J\(\kappa\) gene segments, one of which is a non-functional pseudogene (Kirschbaum, Jaenichen et al. 1996; Kirschbaum, Pourrajabi et al. 1998), and a single \(\kappa\)-C-region exon that resides 3′ to the J\(\kappa\) gene segment cluster (Kirschbaum, Pourrajabi et al. 1998). The mouse Ig\(\lambda\) locus contains only 3 V\(\lambda\)-segments (V\(\lambda\)l, V\(\lambda\)2 and V\(\lambda\)X) and four J\(\lambda\)-C\(\lambda\) clusters (J\(\lambda\)C\(\lambda\)1 to J\(\lambda\)4C\(\lambda\)4) spread over a \(~0.2\) Mb region (Fig. 2) (reviewed in Sanchez, Rueff-Juy et al. 1996). While the human Igh and Ig\(\kappa\)loci is relatively similar to the mouse Igh and Ig\(\kappa\)
loci, the human Ig\(\lambda\) locus is more diverse than its mouse counterpart (52 V\(\lambda\) genes from 10 V\(\lambda\) families and 7 J\(\lambda\)-C\(\lambda\) gene clusters) (Frippiat, Williams et al. 1995). In part, the size and genomic organization of the Ig\(\kappa/\lambda\) loci may explain why the frequency of Abs that utilize Ig\(\kappa\) or Ig\(\lambda\) LCs varies between different species; in mice the \(\kappa:\lambda\) ratio is 95:5, compared with 60:40 in humans (McGuire and Vitetta 1981).

1.2.2 Pro- and pre-B-cell Development

Early stages of B-cell development are classified based on rearrangement status of Igh and Ig\(\kappa/\lambda\) loci (Chen and Alt 1993) (Fig. 3). In mice, prepro-B cells initiate the rearrangement of D- to J gene segments of the Igh loci (reviewed in (Oltz 2001)) (Fig. 2). Pro-B cells continue the rearrangement process by adding an Igh V-gene segment to the DJ rearrangement previously created (reviewed in (Oltz 2001)) (Fig. 2). Successful in-frame rearrangement of the Igh locus is required for pairing of HC with surrogate LC (\(\lambda5\) and V-preB) to form the pre-BCR (Melchers, Karasuyama et al. 1993) which also contains Ig\(\alpha\) and Ig\(\beta\) signal transduction components (Karasuyama, Rolink et al. 1994; ten Boekel, Melchers et al. 1998). The pre-BCR is necessary for B-cell survival into the pre-B cell stage (Shimizu, Mundt et al. 2002) by delivering tonic signals that increase expression of Bcl-2 family proteins, which inhibit the intrinsic mitochondrial death pathway (Hardy, Li et al. 2000).
Pre-B cells are divided into cycling large pre-B and non-cycling small pre-B compartments (Rolink, Winkler et al. 2000) (Fig. 3). Proliferation at the large pre-B stage expands the numbers of pre-B cells with functional HC rearrangements which

(i) mitigates the apoptotic loss of pro-B cells with non-functional HC rearrangement and 

(ii) gives rise to many small pre-B cells that independently rearrange their Igκ or -λ LC loci, thereby increasing the diversity that each functional Ig HC can be paired with a different Ig LC. Developmental arrest at the pre-B cell stage has been observed in mice with mutations of pre-BCR components, including HC, surrogate LC and Igα (Kitamura, Roes et al. 1991; Kitamura, Kudo et al. 1992; Pelanda, Braun et al. 2002)

The earliest stage of human B-cell development is defined by expression of RAG1/-2 and D- to- J- rearrangements in the Igh loci (reviewed in (Ghia, ten Boekel et al. 1998)). As in mice, functional VDJ rearrangements in human pro-B cells result in the generation of Ig HC polypeptides that associate with the surrogate LC to generate the pre-BCR. The human pre-BCR provides survival and proliferation signals necessary for continued B-cell development through the large and small pre-B stages (reviewed in (Bertrand, Eckfeldt et al. 2000)). Analogous to the deficiencies observed in mouse B-cell development, arrest at the pre-B cell stage has been observed in humans with mutations of pre-BCR components, including HC, surrogate LC and Igα (Conley, Rohrer et al. 2000).
In mice and humans, VJ rearrangement in the $\text{Ig}\kappa$ LC loci tends to occur prior to the $\text{Ig}\lambda$ LC loci (Lewis, Rosenberg et al. 1982; Muller and Reth 1988; Zou, Takeda et al. 1993) but the in-frame rearrangement of V- and J gene segments of either locus allows the expression of IgM Ab (HC+LC) (Wang, Stephan et al. 2002). IgM Ab is first observed in the cytoplasm of small pre-B cells (Wang, Stephan et al. 2002) and in the cytoplasm and surface of more mature B cells subsets (Fig. 3) (Wardemann, Yurasov et al. 2003).

In addition, these early B-cell subsets (pro- and pre-B) are identified by flow cytometry using classification systems based on differential expression of surface antigens (Hardy, Carmack et al. 1991; Osmond, Rolink et al. 1998). In mice, Hardy et al. (Hardy, Carmack et al. 1991) compared the V(D)J rearrangement status of $Igh$ and $Ig\kappa/\lambda$ loci with subpopulations identified by a collection of surface antigens. The surface phenotype of prepro-B (fraction A-CD43$^{hi}$B220$^{lo}$HSA$^{lo}$BP-1$^{neg}$IgM$^{neg}$), early pro-B (fraction B-CD43$^{hi}$B220$^{lo}$HSA$^{int}$BP-1$^{neg}$IgM$^{neg}$), late pro-B and large pre-B (fraction C-C$^{\prime}$-CD43$^{hi}$B220$^{lo}$HSA$^{int}$BP-1$^{hi}$IgM$^{neg}$) and small pre-B (fraction D-CD43$^{lo/neg}$B220$^{lo}$HSA$^{lo}$BP-1$^{lo}$IgM$^{neg}$) cells were identified and characterized. This flow cytometry-based classification scheme permitted the faithful recovery and analysis of mouse B cells at distinct stages of $Igh$ and $Ig\kappa/\lambda$ rearrangement.

The earliest stage of human B-cell development can be defined by their expression pattern of surface antigens (e.g. CD34, CD38, and CD10 (reviewed in (LeBien...
Subsequently, pro-B cells may be identified by the acquisition of CD19 expression (reviewed in (LeBien 2000)) and pre-B cells by the acquisition of CD20 (reviewed in (Welner, Pelayo et al. 2008)). In addition to characterization by surface molecules and Ig gene rearrangements, human B-cell progenitors, pro-B and pre-B cells can be defined by distinctive patterns of gene expression (e.g. E2A, EBF, PAX-5 and BCL-2) that offer an independent gauge of developmental maturity (Hystad, Myklebust et al. 2007).

1.2.3 Immature and transitional B-cell development

BCR+ B-cell compartments are identified by unique combinations of surface antigen expression, Ab isotype and by distinct patterns of localization within secondary lymphoid tissues. In mice, immature and transitional B-cell populations are distinguished based on differential expression of fetal stem-cell antigen (CD93), IgM, IgD, complement- (CD21) and Fc-receptors (CD23) (Allman, Ferguson et al. 1992; Allman, Ferguson et al. 1993; Chung, Sater et al. 2002; Casola 2007).

Mouse immature B cells (B220intCD93hiIgMloIgDnegCD21negCD23neg) are present in the BM and spleen and express low amounts of surface antigens (e.g. BCR, CD21 and CD23) utilized by mature B cells to promote activation (reviewed in (King and Monroe 2000)). Low expression of BCR and these co-activating receptors limit B-cell activation upon antigen encounter (reviewed in (Fujimoto, Poe et al. 2000)) that can result in
cellular apoptosis or anergy of immature B cells upon cognate antigen exposure (King and Monroe 2000).

The characterization of surface antigens on human immature (CD19+CD10+IgMlo) B cells (Wardemann, Yurasov et al. 2003) has not been as extensive as that for mouse immature B-cell compartment. In some instances, researchers combine newly formed Ig+ B-cell populations into a single compartment for phenotypic comparison to mature B cells (Mei, Yoshida et al. 2007), indicating that researchers have not formed a consensus regarding the surface phenotype of human immature B cells. The development of in vitro culture systems that support B lymphopoiesis from hematopoietic stem cells (Fluckiger, Sanz et al. 1998; Luo, Maarschalk et al. 2009) may provide a novel tool to perform a detailed phenotypic analysis of human immature B cells. However, the phenotype of in vitro-derived B cells must be compared to B cells isolated from human samples to validate any novel insights into the unique surface antigen expression profile of immature B cells.

Mouse immature B cells become mature B lymphocytes after passing through transitional stages of development (Allman, Ferguson et al. 1992; Allman, Ferguson et al. 1993). Mouse transitional B cells are present in BM, blood and spleen, increase expression of BCR and initiate expression of surface antigens (CD21 and CD23) that define mature B-cells (Allman, Lindsley et al. 2001). Transitional-1 (T1) B cells
(B220\textsuperscript{int}CD93\textsuperscript{hi}IgM\textsuperscript{hi}IgD\textsuperscript{neg}CD21\textsuperscript{neg}CD23\textsuperscript{lo/neg}) do not express IgD, CD21 or CD23 antigens that are present on Transitional-2 (T2) B cells (B220\textsuperscript{int}CD93\textsuperscript{int}IgM\textsuperscript{hi}IgD\textsuperscript{lo/hi}CD21\textsuperscript{int}CD23\textsuperscript{hi}). In part, the maturation of T1 B cells into the T2 B-cell compartment is controlled by cell-extrinsic cytokine stimulation that increases the expression of CD23 and CD21 surface antigens (Gorelik, Cutler et al. 2004). Transitional-3 (T3) B cells (B220\textsuperscript{int}CD93\textsuperscript{int}IgM\textsuperscript{lo}IgD\textsuperscript{lo}CD21\textsuperscript{int}CD23\textsuperscript{hi}) were identified as an anergic, “post-selection” compartment of cells that do not give rise to cells of the mature B-cell repertoire (Allman, Lindsley et al. 2001; Teague, Pan et al. 2007).

Human transitional B cells have been isolated from blood and BM (Carsetti, Rosado et al. 2004). These human transitional B cells are identified by expression of the same surface antigens (e.g. IgM, IgD, CD23 and CD21) that are used to identify mouse transitional B cells coupled with the expression of CD10 and CD38 (Carsetti, Rosado et al. 2004; Sims, Ettinger et al. 2005). However, expression of human CD93 is not used as a B-cell developmental marker as in mice (Rolink, Andersson et al. 1998).

Survival of pro- and pre-B cells requires signaling via cell-intrinsic mechanisms (e.g. pre-BCR) (Kitamura, Roes et al. 1991). Progression through the immature and transitional compartments of B lymphopoiesis is controlled by cell-intrinsic and extrinsic stimulation that promotes B-cell survival (Schneider, MacKay et al. 1999; Claudio, Saret et al. 2009). B-cell activating factor belonging to the TNF family (BAFF) is
a cytokine that promotes transitional B-cell survival (Schneider, MacKay et al. 1999) via up-regulation of Bcl-2 family members (Bcl-xL or Bcl-2) as well as down-regulation of the pro-apoptotic molecule Bim (Kayagaki, Yan et al. 2002; Craxton, Draves et al. 2005). Moreover, studies of mice with impaired BAFF signaling but ectopic expression of pro-survival molecules (Bcl-2 and Bcl-xL) have shown that peripheral B-cell development can be restored in the absence of this cell-extrinsic factor (Amanna, Dingwall et al. 2003; Tardivel, Tinel et al. 2004). These observations suggest that cell-extrinsic cues, such as elevated levels of BAFF (Groom, Kalled et al. 2002), promote B-cell survival of immature and transitional B-cell compartments.

Mouse immature and transitional B-cell compartments are the targets of central tolerance mechanisms that purge self-reactive B cells from the mature B-cell repertoire (reviewed in (Goodnow 1992)). Similar to their murine counterparts, Wardemann et al. have demonstrated that human transitional B-cell compartments contain self-reactive cells that are purged by tolerance mechanisms (Wardemann, Yurasov et al. 2003). Our understanding of B-cell tolerance is confounded by some observations that transitional B cells are capable of survival and proliferation after immunization (Ueda, Liao et al. 2007). Other studies demonstrate that mouse T2 B cells respond to BCR ligation by increasing expression of survival signals, proliferating and differentiating; whereas T1 B cells do not (Petro, Gerstein et al. 2002). Transitional B cells of mice can participate in an
immune response if their BCR has affinity for the immunogen and if these cells receive
the same co-stimulation (CD154) and survival (BAFF) cues that are required for mature
B-cell activation (Ueda, Liao et al. 2007). This rescue of antigen-stimulated transitional B-
cell compartments suggests that cell-extrinsic cues that promote B-cell survival can
contribute to autoimmunity by mitigating the induction of B-cell tolerance in activated
transitional B cells (Groom, Kalled et al. 2002).

In humans, transitional B cells isolated from blood do not proliferate in response
to BCR ligation alone (Malaspina, Moir et al. 2006). However, human transitional B cells
will proliferate in response to BCR activation coupled with IL-4 or CD154 stimulation
(Malaspina, Moir et al. 2006). Unlike their mouse homologues, BAFF stimulation cannot
rescue human transitional B cells after BCR ligation (Malaspina, Moir et al. 2006), further
demonstrating that mouse and human B lymphopoiesis are analogous but with some
differences. Similar to mouse transitional compartments, these data also suggest that
human transitional B cells could survive after BCR cross-linking if the proper cell-
extrinsic cues were available to promote cell survival.

1.2.4 Mature B-cell compartments

Mature B-cell compartments are found throughout the body and are enriched in
secondary lymphoid tissues (e.g. spleen and lymph nodes). The spleen is composed of
branched arterial blood vessels that terminate in venous sinuses, designated as red pulp
(Veerman and van Ewijk 1975). These branching central arterioles are surrounded by lymphoid cells, designated as white pulp. White pulp consists of T cell areas and B cell follicles and has similar cellular organization to structures found in lymph nodes (LN) (Mebius and Kraal 2005). The interface between the splenic white pulp and red pulp is referred to as the marginal zone (MZ) (Mebius and Kraal 2005).

In mice and humans, mature splenic B cells are located in follicles with closely associated T-cell zones that surround the central arterioles (reviewed in (Mebius, Streeter et al. 1996)). The MZ of mice is comprised of cells, including metallophilic macrophage (MOMA-1⁺), dendritic cells (DC) and B cells, that surround mature follicular (MF) B cells (reviewed in (Lopes-Carvalho and Kearney 2004)); whereas in humans, MF B cells are encompassed by two areas of MZ cells (inner and outer) that are themselves surrounded by a perifollicular zone containing blood vessels wrapped with macrophages (reviewed in (Dono, Zupo et al. 2003)).

In mice, the expression of IgM, IgD, CD23 and CD21 are used to identify MF and splenic MZ B cells (Ueda, Liao et al. 2007). MF B cells express high levels of IgD and CD23, but low levels of IgM and CD21 (B220⁺IgMloIgDhiCD23hiCD21lo). Cells with this phenotype are also found in the BM, blood, LNs and mucosal areas (reviewed in (Pillai and Cariappa 2009)). Alternatively, MZ B cells express high levels of IgM and CD21, but
low levels of IgD and CD23 (B220^IgM^hiIgD^loCD23^loCD21^hi) and are only found in the spleen (Lopes-Carvalho and Kearney 2004; Pillai, Cariappa et al. 2005).

Toll-like receptors (TLRs) play a key role in pathogen recognition and activation of the immune system (Medzhitov, Preston-Hurlburt et al. 1997). TLRs are a type of pattern recognition receptor that recognize molecules collectively referred to as pathogen-associated molecular patterns (PAMPs) (reviewed in (West, Koblansky et al. 2006)). The differential responses to BCR cross-linking, TLR stimulation (Snapper, Yamada et al. 1993; Oliver, Martin et al. 1997) and regulation of molecules that promote T-cell activation (CD80 and CD86) have been used to distinguish MF and MZ B-cell subsets (Oliver, Martin et al. 1999). These data indicate that each mature B-cell compartment may be specialized to perform activities (e.g. rapid Ab production by MZ B cells in response to TLR ligation) during an immune response that otherwise may result in delayed activation of immune cells (e.g. T cells) and pathogen clearance.

As in the mouse, human naïve mature B cells are present in the BM, blood, spleen, LN and mucosal areas and are identified by their unique pattern of surface antigen expression (CD19^IgM^loIgD^hiCD27^negCD38^negCD10^neg) (reviewed in (Sanz, Wei et al. 2008)). The initial identification of human MZ B cells was defined by their anatomical location and morphology in the spleen (van Krieken, von Schilling et al. 1989; Spencer, Perry et al. 1998). Currently, there is no clear consensus on a unique phenotype for
human MZ B cells due to high variability between individuals (reviewed in (Weill, Weller et al. 2009)). Histological analysis has been used to demonstrate that most human MZ B cells are IgM$h^+$IgD$^-$CD27$^+$ and can be further characterized by differential expression of CD1c, CD21 and CD23 (reviewed in (Weill, Weller et al. 2009)). However, it is clear that the MZ can contain many B-cell populations beyond the MZ phenotype described above, including IgA$^+$ B cells (Steiniger, Timphus et al. 2005). In addition, Weller et al. suggest that IgM$^+$IgD$^+$CD27$^+$ cells in human blood correspond to circulating “splenic” MZ B cells (Weller, Braun et al. 2004); further illustrating that mouse and human MZ B-cell biology is analogous but contains differences.

Lastly, B1 B cells are a third type of mature B cell that are identified by a unique pattern of surface antigen expression (Hayakawa, Hardy et al. 1983). Mouse B1 B cells (B220$^+$IgM$^+$IgD$^-$CD23$^-$CD21$^+$CD11b$^+$) are further subdivided into B1a (CD5$^+$) and B1b (CD5$^-$) subsets (Stall, Adams et al. 1992) while human B1 B cells are defined by their expression of CD5 (Hardy, Hayakawa et al. 1987). In mice, these B1 cells utilize a restricted set of V gene-segments to form their Ab (Pennell, Arnold et al. 1988). Mouse B1 B cells rarely contain N-nucleotide additions, presumably due to the lack of TdT expression (Li, Hayakawa et al. 1993), resulting in a less diverse pool of $Ig\mu$ VDJ gene rearrangements compared to that recovered from B2 B cells.
In mice and humans, B-1 cells are produced primarily during fetal and perinatal development (Hardy and Hayakawa 1991; Bhat, Kantor et al. 1992). The frequency of B1 B cells is low in the spleen and secondary lymphoid tissues; however, the pleural and peritoneal cavities are enriched for B1 B cells (Hayakawa, Hardy et al. 1985; Lalor, Stall et al. 1989).

Currently, there is debate as to whether B1 B cells arise from a precursor that is distinct from the progenitors that give rise to other mature (B2) B-cell compartments (reviewed in (Hardy 2006)). This controversy arose from transplantation experiments demonstrating that hematopoietic tissues of fetal and adult mice were distinct in their ability to reconstitute the B1 B-cell compartment of recipient mice (Hardy and Hayakawa 1991; Kantor, Stall et al. 1992). Donor cells from fetal liver were able to repopulate both B1 and B2 B-cell compartments. In contrast, adult BM cells efficiently reconstituted B2 B cells but were poor at B1 B-cell reconstitution. Recently, a progenitor cell (CD19^+CD45R^-/lo) that does not express lineage-specific surface antigens (Lin^-neg) has been identified in the fetal liver and adult BM that specifically reconstitutes B1 B-cell development in mice (Montecino-Rodriguez, Leathers et al. 2006). However, other research suggests that commitment to either the B1 or B2 lineage is determined by BCR specificity and density (Lam and Rajewsky 1999) and that distinct niche(s) are present during fetal B-cell ontogeny (Witt, Won et al. 2003) that promote B1 B-cell development.
Both mouse and human B1 B cells express low-affinity BCR against conserved bacterial components (Nakamura, Burastero et al. 1988; Boes, Prodeus et al. 1998) and can express “polyreactive” receptors that have low affinities for many different antigens including other immunoglobulins and self antigens (Nakamura, Burastero et al. 1988; Zhou and Notkins 2004). Mouse and human B1 B-cell populations produce most of the circulating natural Ab present in the serum (Nakamura, Burastero et al. 1988; Nakamura, Burastero et al. 1988; Martin and Kearney 2001); however, this natural Ab requires complement fixation for effective clearance of many pathogens (reviewed in Holers and Kulik 2007). Mouse B1 B cells promote host survival by the production of natural Ab that limits the initial dissemination of microbial pathogens and enhances antigen delivery to secondary lymphoid tissues (Ochsenbein, Fehr et al. 1999).

1.2.5 BAFF and mature B-cell numbers

The tumor necrosis factor (TNF) cytokine family is comprised of many related proteins (e.g. TNF, BAFF, LT-α, LT-β, CD178 and CD154) (Ware, VanArsdale et al. 1996) that exhibit homology in their C-terminal, receptor binding domains. TNF-family proteins function in an autocrine, paracrine, or endocrine manner either as integral membrane proteins or as soluble molecules after proteolytically cleavage from the cell surface (reviewed in (Ware, VanArsdale et al. 1996)). Binding of TNF-family cytokines to their respective TNF receptors leads to the activation of several signal transduction
pathways, depending on the family member involved, that can result in either enhanced survival (Schneider, MacKay et al. 1999) or the induction of apoptosis (Pitti, Marsters et al. 1996).

The number of mature peripheral B-cells is regulated, in part, by the systemic availability of at least one TNF-family member, BAFF (reviewed in (Mackay and Browning 2002)). Studies in BAFF transgenic (BAFFTg) animals have demonstrated an increase in numbers of MF and MZ B cells (Mackay, Woodcock et al. 1999); while BAFF-/- and BAFF-R-/- mice exhibit an almost complete loss of T2, MF and MZ B cell compartments (Schiemann, Gommerman et al. 2001; Thompson, Bixler et al. 2001).

A proliferation-inducing ligand (APRIL) is another TNF-family member with partially overlapping functions as BAFF; however, much less is known about its unique role as a B-cell cytokine in either mice or humans (Mackay and Browning 2002). Importantly, APRIL is unable to restore wildtype levels of mature B cells in BAFF-/- mice.

Both BAFF and APRIL can be membrane-bound or cleaved and released as soluble ligands (reviewed in (Bossen and Schneider 2006)). While many cell types express BAFF, including neutrophils, monocytes, macrophages and dendritic cells (Mackay and Browning 2002), less is known about the regulation and expression of APRIL. The differential ability of BAFF and APRIL to bind receptors may account for their divergent biological functions. BAFF binds to three TNF receptor family members:
BAFF-receptor (BAFF-R), transmembrane activator and calcium-modulator and
cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA). APRIL,
however, binds to only two of these receptors - TACI and BCMA (Ng, Mackay et al.
2005; Schneider 2005). Genetic studies have demonstrated that BAFF-R stimulation
activates the alternative NF-kB pathway (Claudio, Brown et al. 2002; Kayagaki, Yan et al.
2002), resulting in the up-regulation of anti-apoptotic factors such as Bcl-2 and Bcl-xl. In
contrast to BAFF-R, both BCMA and TACI activate the classical NF-kB pathway;
however the effects of activating these pathways downstream of TACI and BCMA have
not been tested in vivo. Clearly, activation of the intracellular domain of TACI can induce
apoptosis in transfected cells (Seshasayee, Valdez et al. 2003), indicating that signaling
through BAFF-R and TACI have opposing outcomes. Further investigation is necessary
to elucidate the distinct roles of BAFF and APRIL during mouse and human B-cell
development and differentiation.

Human B cells express BAFF-R and are dependent upon BAFF for their
development and survival. One common variable immunodeficiency (CVID) patient has
been identified with mutation in BAFF-R that results in hypogammaglobulinemia;
however, B-cell development appears intact but mature B-cell compartments are
reduced in number when compared to healthy individuals (Warnatz, Bossaller et al.
2006). These observations are similar to the B lymphocyte developmental defect
observed in A/WySnJ mice (Miller and Hayes 1991). A/WySnJ mice contain a natural mutation in the BAFF-R gene (reviewed in (Mackay and Browning 2002)) that results in a mature B-cell deficiency that is apparent in the spleen, lymph nodes, peritoneum and peripheral blood (Miller and Hayes 1991). This defective BAFF-R allele blocked the production of B220hi IgMlo B cells (Miller and Hayes 1991) and BM chimaeras experiments demonstrated that A/WySnJ B cells were poor competitors for peripheral survival factors, presumably BAFF (Harless, Lentz et al. 2001). These data indicate that signaling through BAFF-R mediates the survival of mature B-cell compartments and defines the carrying capacity for mature B cells in peripheral lymphoid tissues.

Clearly, factors that promote B-cell survival alter B-cell selection and result in autoimmunity (Nisitani, Tsubata et al. 1993; Lang, Arnold et al. 1997). As such, BAFFts mice contain high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies and immunoglobulin deposition in the kidneys (Mackay, Woodcock et al. 1999). These observations predict that B-cell autoimmunity could be modulated by controlling BAFF availability and, in fact, BAFF is a current target for therapeutic intervention in some human autoimmune disorders (Pelletier, Thompson et al. 2003; Pranzatelli, Tate et al. 2008).
1.3 B-cell Activation

Normally, the B-cell repertoire comprises a diverse array of BCR molecules, perhaps $>10^7$ distinct specificities, that allow antigen recognition and promote host survival (reviewed in (Market and Papavasiliou 2003)). The initial B-cell response to antigen is initiated by genetically diverse populations of B cells that recognize this cognate antigen, even with very low affinities (Dal Porto, Haberman et al. 1998; Dal Porto, Haberman et al. 2002). These antigen-activated B cells migrate to nearby T-cell areas to interact with antigen-activated helper T cells and receive survival, proliferation, and differentiation signals in a process termed “linked-recognition” (reviewed in (Kelsoe 1995; Kelsoe 1996; Garside, Ingulli et al. 1998)). Generally, this interaction between antigen-specific T and B lymphocytes initiates two distinct pathways of B-cell differentiation: i) production of short-lived plasmacytes, or Ab-secreting cells, that provide early, low affinity Ab responses and; ii) the germinal center (GC) reaction (Kelsoe 1995; Kelsoe 1996). Combined, these data indicate that humoral immune responses are constrained by the qualities, frequencies, and locations of antigen-specific, naive B cells.

1.3.1 The GC reaction

The GC reaction is the foundation of T<sub>α</sub> humoral responses by promoting the development of higher affinity Ab and memory B-cell compartments (reviewed in
Antigen-activated B cells enter GCs and initiate a complex cellular program that promotes proliferation, somatic hypermutation (SHM), isotype switching and differentiation into antibody forming cells (AFC) and memory cells (McHeyzer-Williams, Driver et al. 2001). The interruption of T-B collaboration, by disruption of CD40-CD154 interaction, blocks the formation of GCs (Han, Hathcock et al. 1995). Similarly, genetic mutation in the mouse and human CD154 gene inhibits the production of GC reactions and results in type-1 hyper-IgM syndrome; a disease characterized by elevated serum IgM, the absence of class-switched Abs and reductions in affinity maturation and B-cell memory (Aruffo, Farrington et al. 1993; Renshaw, Fanslow et al. 1994).

Early during the initiation of GCs, a network of follicular dendritic cells (FDC) is filled with proliferating B cells (Fig. 4). FDCs capture Ab/antigen complexes on their surface, via CD21, and secrete cytokines that support B-cell activation and proliferation (Kosco-Vilbois and Scheidegger 1995). GCs contain two distinct zones of B-cell morphology, the dark and the light zone (Fig. 4). The dark zone is comprised of rapidly dividing B cells called centroblasts; while the light zone consists of B cells, or centrocytes, that express surface Ab and compete for antigens present on FDCs (Liu, Zhang et al. 1991; Rogerson, Hackett et al. 1991).
Figure 4: Diagram of the germinal center reaction

The GC reaction occurs in the follicle (gray area) after B cells (red nucleus) encounter the cognate ligand of their BCR and receive co-stimulation from activated antigen-specific T cells (gray nucleus). The dark zone (blue area) is defined by intense B-cell proliferation, while the light zone (white area) is defined by re-expression of BCR and competition for antigen (black dots) that is retained on the surface of follicular dendritic cells (pink nucleus). The GC reaction alters the repertoire of antigen-specific B cells so that high affinity clones are preferentially expanded, while low affinity clones are eliminated by apoptosis (yellow cells).

Previous studies have suggested that proliferation, mutation and clonal selection of B cells require rapid movement between the light and dark zones of the GC (MacLennan 1994; MacLennan 1994; MacLennan, Casamayor-Palleja et al. 1997; Allen,
Ansel et al. 2004). Recently, these observations have challenged as clonal selection was
demonstrated to occur in the dark zone (Hauser, Junt et al. 2007), indicating that clonal
selection is not dependent upon interzonal migration. Furthermore, these studies
demonstrate that GC B cells are morphologically similar in both the dark and light zones
(Allen, Okada et al. 2007; Hauser, Junt et al. 2007) and indicate that the movement of B
cells within the GC occurs as intrazonal circulation; not as interzonal trafficking
suggested by earlier studies. These conflicting data indicate that our understanding of
the GC reaction is incomplete and requires future studies to elucidate the mechanisms
that control the GC response.

However, it is clear that the GC reaction alters the repertoire of antigen-specific B
cells so that high affinity clones are preferentially expanded, a process termed affinity
maturation (AM) (Jacob, Kassir et al. 1991; Jacob, Kelsoe et al. 1991); while low affinity
clones are eliminated by apoptosis (Rajewsky 1996). AM is driven by activation-induced
cytidine deaminase (AID) (Muramatsu, Sankaranand et al. 1999; Muramatsu, Kinoshita
et al. 2000), an enzyme that is highly expressed in GC B cells (Kuraoka, Liao et al. 2009).
Mutation is largely confined to regions that encode the variable, antigen-binding
portions of the Ig HC and Ig LC loci (~10^6 fold higher than mutation in most other
genomic regions of B cells), the process of somatic hypermutation (SHM) (reviewed in
(Teng and Papavasiliou 2007)). The accumulation of mutations in the Ig loci alters the
binding specificity and affinities of the resultant Abs (Jacob, Miller et al. 1992). B cells that have undergone SHM must compete for antigen as a limiting resources for survival (Jacob, Kassir et al. 1991) and this competition provides a mechanism for the selective survival of higher-affinity B-cell clones.

It has been suggested that clonal expansion and SHM occur in a cyclical process, each round increasing the affinity of Ab for the cognate antigen (Jacob, Kelsoe et al. 1991). However, this mechanism for the induction of AM has been challenged by results indicating that somatic mutation and selection occur normally in mice that cannot retain Ab/antigen complexes on FDC (Hannum, Haberman et al. 2000). Regardless, the GC reaction forms high-affinity memory and AFC that provide long-lived protection against secondary pathogen challenge (Smith, Light et al. 1997; Takahashi, Dutta et al. 1998). These data reveal that the GC reaction must balance migration, proliferation, activation and cell death to enhance humoral immunity to foreign antigen and that our understanding of this complex series of events is incomplete.

1.3.2 Class switch recombination

CSR is an AID-mediated DNA recombination event which diversifies Ab effector functions by replacing the Ig HC Cμ/Cδ gene segments (IgM/IgD Ab) with the Cα, Cγ, or Cε genes segments (IgA, IgG or IgE Abs) (Stavnezer 2000; Manis, Tian et al. 2002) (Fig. 2). Early studies into the process of CSR were performed using mouse B cells stimulated
with LPS in the presence or absence of different cytokines (Lutzker, Rothman et al. 1988) that resulted in differential expression of particular Ig HC genes. CSR also occurs in GCs and appears to be highly dependent on the interaction of CD40 on B cells with CD154 on T cells. This view is supported by observations that IgA, -G, and -E Ab production is severely reduced in patients with a mutation in the CD154 gene (Bhushan and Covey 2001; Durandy 2002).

These observations have recently been extended as CSR is shown to occur in vivo in CD40−/− mice, in T-independent splenic MZ and in intestinal lamina propria immune responses (Renshaw, Fanslow et al. 1994; Fagarasan and Honjo 2000). These findings suggest that molecules, in addition to CD40, are involved in initiating CSR. In addition to B-cell maturation and survival, BAFF has recently been implicated in Ig HC CSR in the absence of CD40 co-stimulation (Schiemann, Gommerman et al. 2001; Avery, Kalled et al. 2003; O’Connor, Raman et al. 2004). In vitro, BAFF stimulation increases AID expression and is involved in promoting α-germline transcript (αGT) and Iα-Cμ transcript (αCT) production, all of which are hallmarks of CSR (Fagarasan, Kinoshita et al. 2001; Litinskiy, Nardelli et al. 2002; Suzuki, Meek et al. 2005).

Some antigens can activate B cells in the absence of helper T cells (Th); a process termed T-independent (Ti) B-cell responses (Fagarasan and Honjo 2000). Ti antigens are divided into Type-1 and Type-2 antigens as their mechanism for B-cell activation differs.
Naïve mature B cells (MF, MZ and B1) express many types of TLRs (Gururajan, Jacob et al. 2007), and these B cells will proliferate and secrete Ab upon TLR stimulation in vitro (Gururajan, Jacob et al. 2007) even in the absence of BCR cross-linking. Most T\textsubscript{i} Type-1 antigens drive polyclonal B cell activation via TLR ligation regardless of BCR specificity, while T\textsubscript{i} Type-2 antigens activate B cells by extensive cross-linking of the BCR which alleviates the requirement of T\textsubscript{H} cytokines necessary to induce B-cell proliferation (Birkeland, Simpson et al. 1987).

In both mice and humans, MZ B cells are constantly exposed to blood-borne pathogens/antigens and contribute to T\textsubscript{i} B cell responses that result in \textit{i}) rapid production of low-affinity Ab or \textit{ii}) migration to T-cell zones as antigen-presenting cells (APC) (reviewed in (Spencer, Perry et al. 1998; Pillai, Cariappa et al. 2005)). It is widely agreed that B-cell memory is the result of T\textsubscript{d} B-cell responses (McHeyzer-Williams and McHeyzer-Williams 2005); however, recent work suggests that some T\textsubscript{i} type-2 antigen immunizations can form antigen-specific memory B cells (Obukhanych and Nussenzweig 2006), indicating that multiple pathways exist that promote humoral immunity to microbial pathogens. These redundant pathways of cellular activation, differentiation and memory cell formation promote host immunity to microbial pathogens that actively evade immune recognition.
1.4 B-cell Tolerance

Ig gene segments that form the BCR are rearranged and combined in the absence of their cognate antigen (Oltz 2001). This process generates a population of newly-formed B lymphocytes that express receptors reactive to self antigens. Early in the 1950s, Billingham, Brent and Medawar demonstrated that allogeneic skin transplant rejection could be reduced by the pre-emptive exposure of foreign, allogeneic cells early in life, a term they referred to as “actively acquired tolerance” (Billingham, Brent et al. 1953; Billingham and Medawar 1953). This work demonstrated that the immune system could be instructed as to the difference between foreign and self-antigens. Later, it was determined that the mature B-cells repertoire is purged of this pool of self-reactive clones through at least three mechanisms: deletion, anergy and receptor editing (reviewed in (Nemazee, Russell et al. 1991; Goodnow, Cyster et al. 1995; Nemazee 2006)).

Clonal deletion, anergy and receptor editing were further characterized using transgenic mouse lines that express i) BCR for authentic self-antigens (Nemazee and Burki 1989; Erikson, Radic et al. 1991) and ii) BCR reactive to lysozyme (Hartley, Crosbie et al. 1991) in mice engineered to express soluble or membrane-bound lysozyme. These experimental models demonstrated that immature and T1 B-cells receive “tolerizing”
apoptotic signals (Hartley, Cooke et al. 1993) and identified anergy (Adams, Basten et al. 1990; Allman, Lindsley et al. 2001) and receptor editing (Gay, Saunders et al. 1993; Tiegs, Russell et al. 1993) by characterization of B-cell populations that escape apoptotic deletion.

The escape of self-reactive B cells from central tolerance does not guarantee survival since autoreactive B cells can be eliminated from the mature repertoire by peripheral tolerance mechanisms (Hartley, Crosbie et al. 1991; Silveira, Dombrowsky et al. 2004). Indeed, there is an inherent danger of acquiring self-reactivity as a result of DNA mutation within the Ig HC and LC loci during the GC reaction and AM. However, mechanisms (e.g. receptor editing, anergy and follicular exclusion (Cook, Basten et al. 1997; Paul, Lutz et al. 2004)) are present within peripheral lymphoid tissue to limit the emergence of self-reactivity that can arise during B-cell activation (Han, Zheng et al. 1995; Pulendran, Kannourakis et al. 1995; Shokat and Goodnow 1995).

The strength of signal received by B lymphocytes through the BCR is determined by Ab/antigen affinity and by co-receptors that can influence (enhance or inhibit) BCR signaling (reviewed in (Tedder, Haas et al. 2002)). The BCR co-receptor complex enhances BCR signaling (reviewed in (Fearon and Carroll 2000)) by lowering the threshold of IgM cross-linking required for B-cell activation; whereas, most receptors on the B-cell surface that bind the C-region of IgHC, denoted Fc receptors, negatively
regulate signaling through the BCR (reviewed in (Ravetch and Kinet 1991)). Mis-
regulation of these BCR co-receptors can influence B-cell tolerance by lowering the
threshold of stimulation that is required for the activation of anergized autoreactive B
cells (reviewed in (Tuscano, Harris et al. 2003; Nitschke 2005)).

Recently, Wardemann and her colleagues demonstrated the effects of tolerizing
processes in humans by expressing Ig HC and LC rearrangements from single
immature, transitional, and mature B cells and determining the frequencies of Abs that
reacted with self-antigens (Fig. 5) (Wardemann, Yurasov et al. 2003; Wardemann,
Hammersen et al. 2004). Wardemann and her colleagues determined that 55-75% of Ab
expressed by immature B cells is autoreactive (Wardemann, Yurasov et al. 2003), a
pattern that holds not only in the BM but for immature cells in peripheral sites as well
(Meffre, Schaefer et al. 2004; Tsuiji, Yurasov et al. 2006). This B-cell repertoire analysis
demonstrated that the frequency of autoreactive B cells declined as cells were recovered
from increasingly mature B-cell compartments (Fig. 5) (Wardemann, Yurasov et al.
2003).
V(D)J recombination is a stochastic process that occurs in the absence of its cognate ligand. Surface expression of IgM (green), IgD (yellow) and other antigens (not shown) are used to identify distinct stages of B-cell development. B cells that express self-reactive BCR must be purged from the mature B-cell repertoire to avoid autoimmunity (red line). Immature and transitional B cells are the central targets of tolerance as the frequency of self-reactive B cells in each compartment decreases as maturation progresses (light blue).

However, the processes of tolerance appear to be incomplete as some (~20%) naïve MF B cells express autoreactive receptors (Wardemann, Yurasov et al. 2003) and the MZ and B1 B-cell pools are further enriched for self-reactivity. Thus, immunological tolerance purges 30-50% of the potential Ab repertoire of humans. Undoubtedly, the spectrum of host antigenic determinants must, to some degree, overlap with the antigens of microbial pathogens. Therefore, some portion of the potential Ab repertoire for exogenous antigens must be lost to the processes of self-tolerance. Whether this loss significantly impacts the host’s ability to establish microbial immunity will depend on the frequency and extent to which microbial and host antigens cross react.
1.5 Autoimmunity

Failure of tolerance mechanisms result in organ-specific or systemic autoimmunity depending on the nature of the defect (Zouali 2001). In many cases, B-cell mediated autoimmune diseases are characterized by long-lived plasma cells that secrete autoantibody (Ahmed and Gray 1996). Furthermore, many autoantibodies are IgG and contain V-gene mutations consistent with SHM and, consequently, have very high affinity for their cognate autoantigen (Jury, Loeffler et al. 1996; Syren, Lindsay et al. 1996). Autoantibody is a key component in the pathogenesis of several autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) (Braun, Sis et al. 2007) and multiple sclerosis (MS) (Archelos, Storch et al. 2000).

In addition to autoantibody production, B cells serve as efficient antigen presenting cells (APC) to autoreactive T cells in RA (Roosnek and Lanzavecchia 1991), MS and type-1 diabetes (T1D) (Serreze, Chapman et al. 1996) as expression of surface autoantibody allows B cells to internalize and present autoantigen far more efficiently than any other professional APC.

RA is a chronic inflammatory disease affecting many tissues, but is primarily targeted to the synovial spaces of joints resulting in the destruction of the cartilage and surrounding soft tissue. Rheumatoid factor (RF), a common autoantibody directed
against the Fc portion of IgG, contributes to the pathogenesis of RA. During the initiation of RA, immune complexes of RF and IgG are deposited in the synovial joints and this deposition results in inflammation and tissue destruction (reviewed in (Levesque 2009)). In mice and humans, CD5+ B cells produce most RF present in serum (Hayakawa, Hardy et al. 1983; Hardy, Hayakawa et al. 1987), demonstrating that human and mouse B1 B cells are a substantial source of autoantibodies. It is interesting that these same mouse and human B1 B-cell populations produce most of the circulating natural Ab present in the serum (Nakamura, Burastero et al. 1988; Nakamura, Burastero et al. 1988; Martin and Kearney 2001) that promotes clearance of many pathogens (reviewed in (Holers and Kulik 2007)). The question remains whether autoantibody and natural Ab produced by B1 B cells are, in fact, from the same pool of Ab that recognizes antigens conserved between mammalian cells and pathogenic microbes.

SLE is a chronic inflammatory disease that targets connective tissue(s) present in multiple organs, including skin, lungs, heart and kidneys. Many factors that contribute to SLE susceptibility including gender and major histocompatibility complex (MHC) haplotype (reviewed in (Rahman and Isenberg 2008)). The classification of SLE severity is described using the American College of Rheumatology criteria (Hochberg 1997) which measures many parameters including joint swelling, neurologic and hematologic disorders rash and autoantibody tiers. As one of many disorders resulting from SLE,
immune complexes are deposited in the kidney resulting in glomerulonephritis and kidney failure (Phadke, Trachtman et al. 1984). If a portion of exogenous antigen recognition is lost while establishing self-tolerance; individuals with active SLE may contain a more diverse repertoire of Abs (compared to normal individuals) that could provide some protection against infection by pathogenic organisms.

As evidenced by the pathogenesis associated with autoimmune diseases, the immune system must be tightly regulated throughout the progression of an immune response (Izcue, Coombes et al. 2006). Microbial infection drives innate cell activation and antigen-specific lymphocyte proliferation. Additionally, inflammation disrupts BM B-cell development to accommodate for expanded granulocyte production, termed “emergency granulopoiesis” (Ueda, Yang et al. 2004; Ueda, Kondo et al. 2005). This process releases developing B cells to peripheral lymphoid tissue where their fate is undetermined (Ueda, Liao et al. 2007); however, these B cells may provide a source of self-reactive clones that eventually arise during chronic inflammation. What is the biological advantage imparted to an organism by releasing potentially harmful B-cell progenitors into the periphery? While the inflammatory rescue of developing autoreactive B cells is dangerous with regards to autoimmunity, the benefit of retaining B cells that express Ab specific to self-antigens - that also cross-react with microbial antigens - may counteract the threat posed by an uncontrolled microbial infection.
For some autoimmune patients, the health benefit imparted by humoral immunity is mitigated by the damage done by aberrant autoimmune B-cell responses. To that end, Rituximab is a successful B-cell depleting Ab therapy used to treat autoimmune diseases (SLE, RA and MS) by reducing clinical symptoms and minimizing tissue damage (Rastetter, Molina et al. 2004). In addition, other therapeutic interventions that alleviate clinical symptoms of autoimmune diseases target pro-inflammatory cytokines (Adorini 2003; Bombardieri, McInnes et al. 2007); however, it is not clear whether these anti-cytokine therapies act directly on autoreactive B cells.

1.6 Immune Evasion

Many pathogenic organisms including protozoan parasites, bacteria, and viruses actively avoid immune surveillance which results in increased microbial proliferation and dissemination. The strategies for this subversion are diverse; for example, many viruses have evolved mechanisms to suppress MHC expression, antigen processing, endocytosis, expression of immune co-stimulator molecules, apoptosis, cytokine production and signaling, and/or complement dependent cytotoxicity (reviewed in (Tortorella, Gewurz et al. 2000)). Often the mechanism responsible for these suppressive pathways is a viral protein(s) that mimics and subverts the function of the host analogue (Tortorella, Gewurz et al. 2000).
Antigenic mimicry has also been suggested as a component – or even the cause – of certain autoimmune diseases by the (inflammatory) rescue of self-reactive B cells normally lost by apoptosis or anergy (von Herrath and Oldstone 1995). The activation of anergized self-reactive B cells by virus decorated with host antigen has been demonstrated in transgenic mice (Kouskoff, Lacaud et al. 2000) and suggested by the strong linkage of autoimmune disease to certain infections. *Trypanosoma cruzi* expresses at least three antigens that are cross-reactive with cardiac muscle (Girones, Cuervo et al. 2005), and immune responses directed at these protozoan antigens results in destruction of cardiac tissue and Chagas’ disease. Additional examples of autoimmune diseases that appear to be induced by microbial antigens include streptococcus-induced carditis, herpes stromal keratitis (Rose and Mackay 2000), and lung damage mediated by Ab to corona virus (Lin, Lin et al. 2005).

In contrast to immune cell activation, antigenic mimicry often acts to suppress immune responses to microbial pathogens. Some microbial pathogens subvert protective immunity by mimicking antigens of their hosts, thereby suppressing immune control. Most mice immunized with *Campylobacter jejuni* LPS contained i) low titers of anti-monosialotetrahexosylganglioside (GM-1) serum IgM Ab titers, ii) undetectable levels of anti-GM-1 serum IgG Ab and iii) did not form humoral memory to this antigen as indicated by a lack of response upon subsequent immunizations (Bowes, Wagner et al.
2002). However, in mice deficient for GalNAc transferase activity (and, consequently, lack complex ganglioside self-antigens) C. jejuni LPS immunization leads uniformly to high titers of anti-GM-1 IgG and robust memory responses (Bowes, Wagner et al. 2002). These data suggest that at least one surface antigen of C. jejuni is invisible to humoral immunity due to the tolerization of B-cell populations that react to GM-1 antigen.

Immune responses to dengue virus (DENV) are especially complex, as humoral immunity is not only protective but also plays a major role in the pathogenesis of the most severe forms of the disease. Humoral protection is largely conferred by neutralizing Abs specific for virus envelope glycoprotein epitopes (Kaufman, Summers et al. 1987) although Abs to precursor membrane and non-structural proteins are also elicited during infection (Kaufman, Summers et al. 1989). The most severe forms of DENV infection are linked to secondary infections that may result in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) characterized by high fever, thrombocytopenia, bleeding disorders, increased vascular permeability, and leakage of intravascular fluid (reviewed in Whitehead, Blaney et al. 2007). DHF and DSS are associated with extraordinary viremias resulting from antibody-dependent enhancement (ADE) of heterotypic infections, a process whereby complexes of DENV and non-neutralizing Ab bind to FcγR+ cells, increasing viral up-take and replication (Goncalvez, Engle et al. 2007). ADE of infections can be mediated in vitro and in vivo by
low doses of neutralizing mAb; ADE of infections in vitro is FcγR-dependent and in vivo ADE is abolished by a CH2γ1 mutation that disables FcγR binding (Goncalvez, Engle et al. 2007).

This model for ADE viremias as the causative agent of DHF and DSS has been disputed by a recent prospective study showing that enhancing Ab activity present in DenV immune plasma does not correlate with the severity of subsequent infections, indicating the presence of additional factors important in DenV immunopathogenesis (Laoprasopwattana, Libraty et al. 2005). At least one of these factors may be the induction of autoantibody. Several mouse mAb generated to purified NS1 protein cross react with fibrinogen and platelets, bind to human endothelial cells, and induce moderate to severe intraperitoneal hemorrhage (Falconar 1997).

These examples of immune evasion illustrate the extent of competition that exists between pathogens and the host immune system. It appears that pathogens have identified and; therefore utilize, distinct structures that provide some degree of protection or escape from the host immune response. A fine line exists between promoting a diverse array of BCR specificities that increases host defense against these microorganisms and generating equally harmful autoreactive B cells that mediate their own pathologies.
1.6.1 HIV-mediated disruption of B-cell homeostasis

Overwhelming viral replication can result in systemic immune dysregulation and pathogen dissemination. During initial HIV-1 infection, viral load can reach as high as $10^5$ to $10^7$ copies/ml in peripheral blood (Holodniy 1999). The primary immune response to HIV-1 usually reduces serum viral load within 3 months; however, viral titers can rebound (by 6-12 months) to levels greater than that observed during initial infection in untreated individuals (Katzenstein, Pedersen et al. 1996).

Early in the HIV-1 epidemic and prior to the advent of effective anti-retroviral therapy (ART), disruption of B-cell homeostasis was commonly observed and initially characterized by hyper-activation and faulty immunoregulation (Lane, Masur et al. 1983; Ammann, Schiffman et al. 1984; Kekow, Kern et al. 1986). B-cell dysregulation in the presence of HIV-1 infection include: hypergammaglobulinaemia, polyclonal B-cell activation, aberrant maturation, elevated plasmacytosis and enhanced B-cell malignancy formation (reviewed in (Moir and Fauci 2009)). Due to chronic antigen stimulation \textit{in vivo} and the lack of additional AFC formation after treatment with B-cell mitogens (Lane, Masur et al. 1983; Yarchoan, Redfield et al. 1986), it is suggested that B cells in HIV-infected patients are “hyper-activated” and spontaneously differentiate to plasma cells without cognate T-cell help. In fact, polyclonal activation of human memory B cells by mitogens and “bystander” T cells can drive short-lived plasma cell differentiation.
(Bernasconi, Traggiai et al. 2002), indicating that inflammation circumvents mechanisms that limit cellular activation.

Several reports indicate that HIV-1 infection results in the rapid loss of memory B cells (De Milito, Morch et al. 2001; De Milito, Nilsson et al. 2004), including cells specific for non-HIV-1 antigens (Titanji, De Milito et al. 2006). This loss of memory B cells impacts serologic memory to some HIV-1 antigens (e.g., p24) (Binley, Klasse et al. 1997) and to pre-existing memory induced by routine childhood vaccination (e.g., measles virus and tetanus toxoid) (Titanji, De Milito et al. 2006). This phenomenon has important implications for vertically-infected children, as control of HIV-1 by ART must occur within the first year of life to ensure the establishment of humoral memory to childhood vaccination (Pensieroso, Cagigi et al. 2009). These data suggest that HIV-1 affects humoral immune responses by suppressing the induction of novel memory cells and reducing existing memory B-cell compartments.

While direct interaction of HIV-1 with B cells has been observed (De Milito, Morch et al. 2001), sometimes via complement-coated viral particles and CD21 (De Milito, Nilsson et al. 2004), HIV-infected B cells are rarely observed in the peripheral blood of patients. These and other observations suggest that persistent HIV-1 infection drives B-cell activation via systemic inflammatory signals (Sodora and Silvestri 2008), not through direct infection of B cells by virus. These inflammatory mediators include
but are not limited to interferons, TNF-family members and interleukins (reviewed in (Moir and Fauci 2009)). Analogous to the mobilization of developing B cells in mice by pro-inflammatory cytokines (Ueda, Yang et al. 2004), the frequency of transitional B cells are expanded in the periphery of HIV-infected individuals (Malaspina, Moir et al. 2006) and may contribute to another prevalent disorder, the presence of serum autoantibodies (Ng 1996).

The frequency and distribution of lymphocytes (T- and B-cells) within gut-associated lymphoid tissue (GALT) is severely disrupted early after HIV-1 infection (Mehandru, Poles et al. 2004; Levesque, Moody et al. 2009), contributing to systemic inflammation by disrupting homeostatic control of commensal flora (reviewed in (Izcue, Coombes et al. 2009)). The disruption of GALT results in enhanced cell-extrinsic cues that promote B-cell survival and contribute to autoimmunity by mitigating the induction of B-cell tolerance (Groom, Kalled et al. 2002). Combined, these data suggest that persistent HIV infection and the subsequent dysregulation of intestinal immunity to commensal flora contribute to chronic inflammatory conditions that are permissive for the relaxation of B-cell tolerance.

**1.6.2 Autoantibody and HIV-1 infection**

Pathogens that are successful in establishing long-term infections promote chronic inflammation, sometimes resulting in the induction of tertiary lymphoid tissues
The milieu of cytokines present during inflammation can enhance cell survival and contribute to the breaking of B-cell tolerance (Groom, Kalled et al. 2002). Low levels of non-pathogenic serum autoantibody are routinely observed during HIV-1 infection. Reports of HIV-1 infection and coincident rheumatologic disorders began to appear in the mid-1980s (Johnson, Duvic et al. 1985; Winchester, Bernstein et al. 1987).

Depending upon the cohort examined, RF has been observed in 5-60% of HIV-1+ patients (Kopelman and Zolla-Pazner 1988; Procaccia, Blasio et al. 1991; Medina-Rodriguez, Guzman et al. 1993; Massabki, Accetturi et al. 1997). Reactive arthritis, previously named Reiter’s syndrome, is initiated by infection of a distal anatomical location (intestines, genitals or urinary tract) that results in arthritic inflammation of large joints, eyes and skin (references within (Panush, Wallace et al. 2007)). Not surprisingly, HIV-1 infection can induce reactive arthritis (Brancato, Itescu et al. 1989) similar to many other pathogens (reviewed in (Rohekar and Pope 2009)). Whether direct recognition of HIV-1 or the destruction of GALT and microbial dissemination drives the induction of reactive arthritis remains unclear. Other rheumatic disorders have been described in HIV-1+ patients including: septic arthritis, myalgias, Sjogren’s syndrome, avascular bone necrosis and dermatomyositis.
Anti-nuclear antibodies (ANA) are autoantibodies reactive with various nuclear antigens including DNA, RNA, nuclear pore complex, histones and combinations of these molecular components. ANA are readily detected in systemic autoimmune diseases including SLE, Sjogren's syndrome, autoimmune hepatitis, scleroderma, T1D, polymyositis, and mixed connective tissue disease (described in (Kavanaugh, Tomar et al. 2000)). In some HIV-1+ patients (>25%), ANA are present at low, but detectable concentrations (>1:40 titer) in serum (Berman, Espinoza et al. 1988; Kopelman and Zolla-Pazner 1988; Savige, Chang et al. 1994). The frequency of HIV-1+ patients with ANA is much lower than individuals with active SLE disease (~75-85%) but very similar to patients with inactive SLE disease (~10-25%) (Souza, da Silva et al. 2009). Furthermore, the serum titer of ANA in patients with active SLE is far greater (1:100 – 1:10,000) than observed during HIV-1 infection (Kallenberg, van der Meulen et al. 1983). At present, it is unknown whether human B1 B cells contribute to ANA production; however, it is reasonable to speculate that HIV-mediated dysregulation of B1 B cells, a known reservoir of autoantibody, could contribute to the presence of ANA.

Elicitation of autoantibody against hematopoietic cells and their cellular components has been previously characterized as a part of HIV-1 pathogenesis. Neutropenia, or an insufficiency of neutrophils, has been associated with HIV-1 infection and is present in ~40% of patients with AIDS (Murphy, Metcalfe et al. 1987;
Zon, Arkin et al. 1987). Neutrophils are a target of autoantibody which, in association with production defects, contributes to neutropenia (Klaassen, Goldschmeding et al. 1991; Klaassen, Vlekke et al. 1991). Furthermore, autoantibody has been observed against granule- (elastase, lactoferrin and myeloperoxidase), cytoplasmic- and membrane-associated components of neutrophils (Klaassen, Goldschmeding et al. 1992; Savige, Chang et al. 1994). Whether anti-neutrophil Abs are sufficient to mediate the neutropenia associated with HIV-1 infection remains unclear. However, recent studies concluded that there was no correlation between presence of anti-granulocyte Abs and the degree of neutropenia observed in HIV-infected children (Weinberg, GA 1996 Sep 15-18; 200 (abstract no. I77) NLM Gateway).

Red blood cell (RBC) and platelet autoantibodies are readily detected during HIV-1 infection (Stricker, Abrams et al. 1985; Toy, Reid et al. 1985; van der Lelie, Lange et al. 1987; Klaassen, Mulder et al. 1990). The frequency of platelets tends to inversely correlate with the amount of platelet autoantibody in HIV-1+ patients, suggesting a pathologic effect. Compared to HIV-1 uninfected controls, platelet numbers are already significantly reduced in HIV-1+ subjects, indicating that the presence of anti-platelet Ab is only one of several factors contributing to platelet deficiency.

Anemia, or insufficiency of RBC, is a common hematologic abnormality after HIV-1 exposure and becomes progressively worse in late-stage disease, affecting most
patients (60 to 80%) (Toy, Reid et al. 1985; Zon, Arkin et al. 1987). Currently, it is unclear whether autoantibodies contribute to anemia via hemolysis, enhanced phagocytosis and/or antibody-dependant cell-mediated cytotoxicity (ADCC) of RBCs. Other cellular targets of autoantibody during HIV-1 infection include: natural killer (NK) cells (Klaassen, Goldschmeding et al. 1992), CD4+ and CD8+ T cells (Williams, Masur et al. 1984; Dorsett, Cronin et al. 1985; Keay, Wecksler et al. 1995).

Cellular infection by HIV-1 can alter the lipid content of cell membranes (Apostolov, Barker et al. 1987; Klein, Bruser et al. 1992). Anti-lipid autoantibodies arise in many forms and may, in part, represent binding to self-antigens or newly exposed autoantigens (Amara, Chaugier et al. 1994). Anti-cardiolipin Abs are i) present in diseases such as SLE and primary anti-phospholipid syndrome (APS) (autoimmune-derived) (Hunt, McNeil et al. 1992) and ii) result from polyclonal B-cell activation induced by Epstein-Barr virus, leishmaniasis and HIV-1 (infection-derived) (Matsuura, Igarashi et al. 1992). HIV-infected patients (~40%) make anti-phospholipid Abs likely as a result of polyclonal B-cell activation (Petrovas, Vlachyiannopoulos et al. 1999). In this regard, Moody et al. recently demonstrated that some anti-lipid Abs from autoimmune patients inhibit binding of CCR5-utilizing HIV-1 to peripheral blood CD4 T cells (Moody, Liao et al.). This inhibition is mediated by the Ab binding to blood monocytes and inducing their production of CCR5-blocking chemokines. In this example, a
neutralizing Ab acts without binding the virion (Moody, Liao et al.). It remains unknown whether autoimmune patients with defective tolerance can make broadly neutralizing, envelope Ab after infection with HIV-1.

Research suggests that protective, HIV-neutralizing Abs inhibit viral entry into target cells through interaction of both the lipid membrane and proteins of virus (Ofek, Tang et al. 2004; Cardoso, Zwick et al. 2005). Furthermore, there is evidence that the normal formation of membrane microdomains, such as lipid rafts, is required for efficient HIV-1 infection (Manes, del Real et al. 2000; Nisole, Krust et al. 2002). As such, novel anti-cholesterol Abs have been used to limit viral replication by preventing “the proper spatio-temporal juxtaposition of HIV-1 glycoproteins with CD4 and chemokine receptors, thus negatively interfering with virus attachment/entry” (Beck, Balogh et al.). Therefore, it is reasonable to study these lipid-reactive Abs identified in HIV-1 infected patients as precursors to efficacious anti-HIV-1 envelope Ab responses. It is important to recognize that not all anti-lipid Abs are pathogenic as anti-lipid Abs are thought to serve as critical mediators of cholesterol homeostasis (Alving and Wassef 1999).

1.7 The Tolerance Hypothesis

HIV-1 may have evolved to reduce MPER Ab responses by having conserved neutralizing epitopes that mimic autoantibody epitopes. The “tolerance hypothesis” states that Abs targeting critical, neutralizing epitopes of pathogens are derived from
autoreactive B cell clones that are normally deleted or made tolerant upon antigenic stimulation. Exploitation of immunological tolerance by viruses and other human pathogens in order to mitigate humoral immunity can be detected by comparing the Ab repertoires of B-cell populations before and after their tolerization. If the Ab repertoires for self- and microbial antigens do not substantially overlap, then the extensive losses of autoreactive cells typical of B-cell maturation (Wardemann, Yurasov et al. 2003; Meffre, Schaefer et al. 2004; Wardemann and Nussenzweig 2007) will not diminish the numbers of B cells specific for viral, bacterial, etc. antigens (in fact, it will increase their relative frequencies). On the other hand, if pathogens commonly express antigens that cross-react with their host, self-tolerance will also reduce the size and diversity of the Ab repertoire to these foreign determinants (Fig. 6). Interestingly, the frequency of neutralizing Abs reactive to broadly conserved portions of Influenza hemagglutinin (HA) is extraordinarily low (~10^-9) (Ekiert, Bhabha et al. 2009). Future experiments should test if this rarity results from the induction of B-cell tolerance in the progenitor pool that is reactive to these conserved regions of HA.
Figure 6: Mechanisms of tolerance block the development of B cells that bear receptors specific for cross-reactive antigens present on microbes and host tissues.

The primary B-cell repertoire is composed of cellular clones specific for self-antigens (red circles), foreign or non-self antigens (white), and antigen subsets that are present on both foreign-, e.g. microbial, and self structures (green). During development, tolerance mechanisms (boxed area) limit the maturation of B-cell clones that react with “self-“ and “shared” antigens, whereas B cells specific for foreign determinants are not affected. The fates of these two classes of self-reactive B cells include i) apoptotic deletion (broken lines), ii) induction of anergy (single receptor) or iii) receptor editing (blue receptors). Therefore, the frequencies of B cells specific for “self-“ and “shared-antigens” are minimal in the mature, peripheral B-cell compartments compared to those B lymphocytes specific for foreign antigens.
HIV-1 may offer another example of antigenic mimicry as a protective adaptation to avoid humoral immunity (Haynes, Fleming et al. 2005), indicating an immunological advantage for retaining a limited pool of mildly self-reactive lymphocytes. Protective Abs that neutralize multiple HIV-1 clades have been identified but are rare (Muster, Steindl et al. 1993; Zwick, Labrijn et al. 2001; Nelson, Brunel et al. 2007). HIV-1 infection commonly induces primarily Abs that are non-neutralizing or are against highly variable envelope regions that result in restricted type- or isolate-specific neutralization (Haynes and Montefiori 2006). Many theories have been offered for the scarcity of HIV-1 neutralizing Ab, including the complex nature and genetic plasticity of HIV-1 epitopes, the shielding of crucial antigen sites by glycosylation, competitive suppression by highly immunogenic but non-neutralizing surface antigens, and insufficient diversity in the primary repertoire of Ab specificities (reviewed in (Burton, Desrosiers et al. 2004)).

Although each of these arguments is plausible, the recent finding that most HIV-1 neutralizing Ab react with host antigens offers an alternative explanation for their rarity (Haynes, Fleming et al. 2005; Alam, McAdams et al. 2007). These recent reports indicate that the broadly neutralizing anti-HIV-1 Ab repertoire may be lost due to cross-reactivity to self-antigens, via the induction of B-cell tolerance. Two Abs, 2F5 and 4E10, neutralize HIV-1’s ability to infect cells and show considerable binding to HEp-2 cells, a
measurement commonly used to show ANA reactivity. These Abs also bind other self-antigens such as cardiolipin and phosphotyrdlsrme (PS).

Recent demonstrations that most HIV-1 neutralizing Ab exhibit weak but crucial binding to host membrane antigens (Ofek, McKee et al.; Scherer, Leaman et al.; Alam, Morelli et al. 2009) add additional support to the hypothesis that the HIV-1 neutralizing Ab repertoire might be lost as a consequence of B-cell tolerance mechanisms. Indeed, a 2F5 Ig heavy chain “knock-in” (2F5 HC-KI) mouse line was recently generated to determine whether 2F5 HC encoded receptors are sufficiently cross-reactive to activate B-cell tolerance mechanisms (Verkoczy, Diaz et al.). Verkoczy et al. clearly demonstrate that expression the 2F5 HC elicits a developmental blockade that prevents the maturation of small pre-B cells to immature B cells (Verkoczy, Diaz et al.), a defect first observed for B cells expressing receptors reactive with DNA- or MHC I (Tiegs, Russell et al. 1993; Chen, Nagy et al. 1995). This demonstration is unequivocal proof that at least one broadly neutralizing, human Ab for HIV-1 is sufficiently cross-reactive to self-antigen(s) to elicit immunological tolerance.

Therefore, Haynes et al. (Haynes, Fleming et al. 2005) predicts that most B cells specific for HIV-1 epitopes that also react to self-antigens will be subject to tolerization during development. Given that many HIV-1 infected patients and vaccinees generate non-neutralizing Abs (Haynes and Montefiori 2006), the tolerance hypothesis predicts
that non-neutralizing Abs - unlike the rare, broadly neutralizing Abs - may not exhibit substantial autoreactivity and do not invoke B-cell tolerance.

The capacity for viral neutralization is commonly measured in the serum of patients using strains of HIV-1 isolated from the infected individual (autologous) and from other HIV-infected persons (heterologous). Recent longitudinal studies have monitored the emergence of these neutralizing autologous and heterologous Ab responses in recently HIV-infected individuals (Gray, Moore et al. 2007; Gray, Taylor et al. 2009; Shen, Parks et al. 2009). Early after infection (~3-6 mo), strain-specific neutralizing Abs can be formed to reduce viral burden; however, these Abs rarely contain heterologous neutralizing capacity. After brief interruption of ART, viremia rapidly ensues with a concomitant autologous Ab response that eventually limits viral replication (Montefiori, Hill et al. 2001). Unfortunately, HIV-1 usually evolves just as rapidly to circumvent neutralization, indicating that neutralizing Ab exerts selective pressure(s) that the virus is well equipped to avoid. This is precisely why novel immunogens must elicit neutralizing Ab responses to conserved regions of HIV-1 that cannot easily be modified without severe repercussions to replication “fitness”.

In the infrequent instance that broadly neutralizing Ab is formed, it is only after months (10-20) of chronic antigen exposure (Shen, Parks et al. 2009) and autoimmunity. Unfortunately, patients that make neutralizing Ab after viral reservoirs have been
established do not control viral load (Shen, Parks et al. 2009), since humoral immunity is
not effective for clearing intracellular pathogens.

The MZ and B1 B cell compartments are candidates to target for efficacious HIV-1 vaccine
design as both types of B cells are enriched for self-reactive clones compared to MF B cells (Pillai, Cariappa et al. 2004), the normal targets of B-cell vaccines. The retention of some autoreactive B cells and the conservation of the MZ and B1 B-cell compartments may represent an “evolutionary compromise” for the capacity to respond to microbial antigens that are structurally similar to those of the host. Expansion of these B-cell pools or the utilization of autoreactive strains of mice may provide insight into HIV-1 humoral immunity upon immunization. However, the recent demonstration that expression the 2F5 HC elicits a developmental blockade at the transition from mouse small pre-B cells to immature B cells (Verkoczy, Diaz et al.) indicates that these rare, broadly neutralizing Ab will be absent from the mature B-cell repertoire. Alternatively, whether the inflammatory release of developing, immature B cells to the periphery increases the “precursor pool” for rare broadly neutralizing B cells should be explored.

1.7.1 SLE and HIV-1 infection

As noted, a number of rheumatologic disorders can arise after the establishment of a chronic HIV-1 infection. Presumably, these autoimmune syndromes are the result of T- and B-cell dysregulation and it may be that these late events are important in
understanding the complex biology of HIV-1 infection. We note that the occasional production of broadly reactive neutralizing Ab comes only after months (10-20) of chronic HIV-1 infection, antigen exposure and induction of autoantibody (Shen, Parks et al. 2009). Could it be that the immune dysregulation that follows HIV-1 infection creates conditions that are permissive for both autoreactivity and neutralizing Ab production?

The tolerance hypothesis predicts that individuals with active humoral autoimmunity might have a lower incidence of HIV-1 infection. SLE is understood to be a disease of impaired early B-cell tolerance (Yurasov, Wardemann et al. 2005) and if developing neutralizing Ab+ B cells are lost to immunological tolerance (Verkoczy, Diaz et al.), then neutralizing Ab+ B cells will be more frequent in SLE patients. It follows that patients with active SLE may contain Abs that provide a degree of protection from HIV-1 infection.

Several investigators have described a lower-than-expected frequency of coincident SLE and HIV-1 infection and suggest this is related to the spectrum of Abs that SLE patients can make (Kaye 1989). As of 2004, only 32 cases of combined SLE and HIV-1 disease have been reported (Palacios, Santos et al. 2002; Palacios and Santos 2004). Of the 32 SLE/HIV-1+ individuals, only 21 cases met full diagnostic criteria for SLE, suggesting that coincidence of SLE and HIV-1 infection is even more rare. Based on the prevalence of each disease, this observed frequency of SLE/HIV-1+ patients is much
lower (<10%) than the expected frequency of coincidence that was predicted (~400 cases) in the early 1990’s.

Production of broadly neutralizing Ab by HIV-1+ autoimmune disease patients would provide strong evidence for the suppression of neutralizing Ab by B-cell tolerance mechanisms. Prospective studies in autoimmune patients (SLE and APS) have been initiated to i) determine the incidence of HIV-1 infection and ii) characterize anti-HIV-1 neutralizing Abs that are elicited after HIV-1 infection (Moody, MA, Haynes, BF, unpublished). However, if either HIV-1 infected autoimmune patients or autoimmune animal models do not make broadly neutralizing Ab, then other mechanisms that control these Ab species will have to be explored.

It should be noted that the elevated level of IL-16 in SLE patients has been suggested as the mechanism behind reduced HIV-1 infection (Sekigawa, Lee et al. 2000), since IL-16 has previously been reported to limit viral infection (Zhou, Goldstein et al. 1997) and viral promoter activity (Maciaszek, Parada et al. 1997). Future studies should examine the incidence of HIV-1 within multiple cohorts of patients with SLE and other autoimmune diseases (RA, MS and/or Sjogren’s) to determine whether autoimmunity truly limits infection with HIV-1.

In part, the tolerance hypothesis can be studied in mouse models since many autoantigens are conserved throughout phylogeny. It has been determined that HIV-1
MPER-specific B cells are differentially regulated in both normal (BL/6 and BALB/c) and autoimmune (MRL/lpr−) mice (Gray, Moore et al. 2007) and unpublished results Verkoczy and Haynes). For example, Abs that target the 2F5 gp41 epitope, but not the 4E10 peptide epitope, are constitutively produced in MRL/lpr− mice. After cloning, sequencing and re-expression of 2F5-epitope reactive Abs, many questions can be asked about the capacity for viral neutralization, bias in V(D)J usage in IgH and IgK/λ and whether these Abs are cross-reactive with host cellular antigens.

The studies performed in this thesis test whether B-cell populations that are potential progenitors of broadly neutralizing Ab to HIV-1 are removed from the mature repertoire by mechanisms of tolerance. In BL/6 mice, I demonstrate that MPER-binding populations are lost during normal B-cell development and immunization with HIV-1 MPER antigen does not elicit robust humoral responses. I describe and characterize a stromal-cell independent culture system that efficiently supports pro-B cell to IgM+ B-cell development that contain frequent self-reactive B-cells, including HIV-1 MPER-reactive B cell recovered from both BL/6 and 2F5 Igh “knock-in” BM. Furthermore, I demonstrate that Rag1null, lymphopenic mice reconstituted with these culture-derived B and T cells i) contain serum IgG autoantibody which exceeds that present in autoimmune, C4− animals, and ii) generate strong germinal center and Ab responses to HIV-1 MPER antigens upon immunization. Furthermore, co-workers and I have
identified a potential self-antigen that contains the nominal 2F5 epitope, binds 2F5 Ab and is conserved throughout mammalian phylogeny. Combined, these data demonstrate that the humoral immune response to this HIV-1 gp41 MPER antigen can be restored in mice when the constraints of B-cell tolerance have been relaxed.
2. Materials and Methods

2.1 Mice

C57BL/6, RAG1 deficient (B6.129S7-Rag1tm1Mom/J) mice (both, The Jackson Laboratory, Bar Harbor ME), and congenic C4+/+ (Fischer, Ma et al. 1996) mice were maintained in the Duke University vivarium. Congenic 3H9R HC-KI (Chen, Nagy et al. 1995) mice were kindly provided by Dr. R. Eisenberg (University of Pennsylvania). 2F5 HC-KI (Verkoczy, Diaz et al.) mice were provided by Dr. Verkoczy (Duke University). RAG1 deficient mice were reconstituted with $2 \times 10^7$ CD cells by i.v. injection; these CD-RAG mice were held for 3-5 weeks before their use in experiments. RAG1 deficient mice were reconstituted with $2 \times 10^7$ LN cells by i.v. injection; these LN-RAG mice were held for 3-5 weeks before their use in experiments. Mice were housed in specific pathogen-free conditions at the Duke University Animal Care Facility and given sterile bedding, water and food; animals were entered into experiment protocols at 6-8 wk of age. All experiments were approved by the Duke University Animal Care and Use Committee.

2.2 Tissue Preparation

Mice were sacrificed by cervical dislocation. BM was collected from long bones of the hind legs by flushing with 1 ml cold, Iscove’s modified Dulbecco’s Medium (IMDM) containing 10% defined fetal bovine serum (HyClone, Logan Utah), $5.5 \times 10^{-5}$ M 2-ME, penicillin (10 Units/ml), and streptomycin (10μg/ml). BM was disaggregated and dispersed into single-cell suspensions by repeated pipetting. The preparation of cells
from spleen, lymph node and thymus has been described (Chen, Koralov et al. 2000). Viable cells from dissociated tissues were enumerated in hemocytometers by Trypan Blue exclusion.

### 2.3 Flow Cytometry

To identify, characterize, and isolate lymphocytes, mAb included: B220-PacificBlue (RA3-6B2), CD23-biotin (B3B4), CD93-APC (AA4.1), BP-1-PE (6C3), CD24-biotin (M1/69), CD24-FITC (M1/69), GL7-FITC (GL7), CD43-APC (S7) and APC-Alexa750-conjugated streptavidin were purchased from BD Pharmingen (San Diego, CA); and anti-mouse IgM-PEcy7 (eB121-15F9), anti-mouse IgD-FITC (11-26), CD21-PE (eBio8D9), CD5-PE (53-7.3), CD1d-PE (1B1), CD80-FITC (16-10A1), CD86-PE (GL1), CD4-PEcy7 (L3T4), CD8-PEcy5 (Ly-2), CD44-FITC (IM7), CD62L-PE (MEL-14), Gr-1-PEcy5 (Ly6G), CD11b-PEcy5 (M1/70) and TCRβ-APC (H57-597) were purchased from eBioscience (San Diego, CA). 10^6 cells were suspended in FACS Buffer and labeled with mAb described above. FACS buffer contained 1xPBS (pH7.2) with 3% FBS (Sigma) and 0.01% Sodium Azide. Propidium iodide (PI) was used to exclude dead cells from our samples. All FACS analysis was performed using a BD LSRII or Canto cytometer and presented with FlowJo software. Cell sorting was performed on a BD FACS Vantage cytometer.
2.4 B-cell Culture System

BM contents from single mice were dispersed in 5 ml of IMDM by repeated pipetting; cell suspensions from multiple mice were routinely pooled in 10 cm² culture dishes. BM cells were incubated (15 min; 37°C) to permit cell attachment (Fig. 7). Non-adherent cells were recovered by centrifugation (4°C; 400 x G; 5 min), resuspended in 1 ml ACK buffer (1 min; 0°C) to remove erythrocytes, and immediately washed in 10 ml IMDM (Chen, Koralov et al. 2000). Washed live cells from BM were enumerated in Trypan Blue and transferred into T-75 culture flasks (7.5 x 10⁵ cells/ml; 25 ml resulting in 1.875 x 10⁷ cells/T-75 flask) (Fig. 7) for 4 d in IMDM containing 10% defined - embryonic stem cell tested- fetal bovine serum (HyClone, Logan Utah), 5.5 x 10⁻⁵ M 2-ME, penicillin (10 Units/ml), and streptomycin (10 μg/ml) supplemented with recombinant mouse IL-7 (10 ng/ml) (R&D Systems, Minneapolis, MN). Typically, non-adherent BM cells were twice cultured in IL-7 containing IMDM (4 days, each) followed by a single round of culture in IMDM containing BAFF (20-100 ng/ml; 3-4 days) (Fig. 7). Between sequential cultures, cells were washed and re-plated at the initial concentration.

Figure 7: In vitro B-cell culture system diagram
2.5 ELISA

2.5.1 Characterization of serum immunoglobulin reconstitution

ELISA plates (BD Falcon) were coated (overnight, 4°C) with 2 μg/ml (50 μl/well) of goat anti-mouse Ig(H+L) (Southern Biotechnology Associates, Birmingham, AL) in carbonate buffer (0.1M; pH9.5). Coated plates were washed with 1xPBS (pH7.4) containing 0.1% Tween-20 and 0.5% BSA (USB Corporation). Wells were incubated (2hrs; 25°C) with blocking buffer (PBS (pH7.4), 0.5% BSA). Serum samples were initially diluted 1:1000; followed by serial 3-fold dilutions. Purified mouse IgM (B1-8) and IgG (H33Lγ1) mAbs were used to generate a standard curve (30 μg/ml to 0.5 ng/ml) to determine serum Ab concentrations. HRP-conjugated goat anti-mouse IgM and goat anti-mouse IgG were used to detect bound Ab (Southern Biotechnology Associates, Birmingham, AL).

2.5.2 Characterization of serum Ab responses

ELISA plates (BD Falcon) were coated (overnight, 4°C) with 2-5 μg/ml (50 μl/well) of capture reagent (NIP-BSA or DP178-Q16L) in carbonate buffer (0.1M; pH9.5). Coated plates were washed with 1xPBS (pH7.4) containing 0.1% Tween-20 and 0.5% BSA (USB Corporation). Wells were incubated (2hrs; 25°C) with blocking buffer (PBS (pH7.4), 0.5% BSA, 0.1% Tween-20). Serum samples were initially diluted from 1:5 to 1:50; followed by serial 3-fold dilutions. Purified mouse IgG (H33Lγ1 and 13H11) mAbs were used to generate a standard curve (10-30 μg/ml to 1.5-5 ng/ml) to determine serum Ab
concentrations. HRP-conjugated goat anti-mouse IgG was used to detect bound Ab (Southern Biotechnology Associates, Birmingham, AL). Only samples that fell within the linear portion of our standard curve were used for analysis.

2.5.3 NIH-3T3 cell ELISA

1-2x10^4 NIH-3T3 cells were plated in 96-well tissue culture plates. After 24 hrs, NIH-3T3 cells were fixed with methanol:acetone (1:1) (10 min; -20°C). Fixed NIH-3T3 cells were rehydrated and blocked (PBS with 0.5% BSA, 0.1% Tween-20 and 1.0% normal goat serum; overnight; 4°C). The mAbs were incubated in wells (2hrs; rt) as indicated. After extensive washing (4x, 200μl, 5min each), bound Ab was detected using goat anti-mouse IgM-HRP and IgG-HRP Abs. Purified recombinant 2F5 mAb was used as a positive control for detection and developed with goat anti-human IgG-HRP Ab.

2.6 ELISpot

ELISpot plates (Millipore) were coated (overnight; 4°C) with 2 μg/ml (50 μl/well) of goat anti-mouse Ig(H+L) in carbonate buffer (0.1M; pH9.5). Coated plates were washed with PBS (pH7.4) containing 0.1% Tween-20 and 0.5% BSA. Wells were incubated (2hrs; 25°C) with blocking buffer (PBS (pH7.4), 0.5% BSA). B cells were incubated (3x10^4 cells/well; 200μl IMDM) with LPS (5μg/ml; 1-3 d). Antigen-specific AFC: LPS-activated B cells were washed (3x; 5ml IMDM) and were incubated (0.5-1x10^3 cells/well) (37°C; 4hrs) in IMDM. Plates were washed with dH20 (2x; 200μl/well) and blocking buffer (1x; 200μl/well). Plated were incubated with blocking buffer (1-2 d; 4°C).
Membranes were probed with 20 μM biotin-DP178-Q16L or biotin-R4A peptide (2 h; 25°C). Streptavidin-AP (Southern Biotech) and SIGMA FAST BCIP/NBT (Sigma) were used to develop spots. Total AFC: LPS-activated B cells were washed and plated (2.5-5x10^2 cells/well; triplicate). Plates were washed and re-blocked as described above. Membranes were probed with goat-anti-mouse IgM-AP and/or IgG-AP detection Ab.

### 2.7 Immunofluorescence Assays on NIH-3T3 and C. luciliae

NIH-3T3 cells (1-2x10^4 cells/ml; 10mls) were plated onto 10cm tissue culture plates (24hrs; 37°C) containing sterile glass coverslips. Coverslips were removed and immersed (10 min; -20°C) in methanol:acetone (1:1) for cell fixation. Slides containing C. luciliae (Scimedx Corporation, Denville, NJ) or coverslips containing NIH-3T3 cells were rehydrated (PBS (pH7.4); 30 min; 25°C). Samples were blocked (2 hr; 25°C) using PBS (pH7.2) containing rat anti-mouse CD16/CD32 (1%), purified rat IgG (5%), FBS (10%) and Tween-20 (0.1%). Samples were washed (1 min) in PBS (pH7.2) containing BSA (1%) and Tween-20 (0.1%). Samples were labeled with 2F5 mAb, serum or 2-ME treated hybridoma Ab (2hrs; 25°C) followed by extensive washing (2x 150mls; 10min each; 1x 150mls; overnight). Ab was detected using goat anti-human IgG-FITC, goat anti-mouse Igκ-FITC or goat anti-mouse Igλ-FITC Ab (2hrs; 25°C) followed by extensive washing (3x 150mls; 10min each). Coverslips were mounted to slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope.
**2.8 Sequence Analysis of V(D)J Rearrangements**

Genomic DNA was isolated from sorted B-cell subsets from cultures or BL/6 mice by phenol-chloroform extraction (Invitrogen). V(D)J rearrangements were amplified by semi-nested PCR using *Pfu* polymerase (Stratagene, La Jolla, CA) (Han, Dillon et al. 1997) with 5` primers specific for VH1, V\(\kappa\)4 or V\(\kappa\)5 genes, and a reverse primer specific for JH2 or J\(\kappa\)2 (Table 2). This approach allows the amplification of VH- or V\(\kappa\)- to JH1 or JH2 and J\(\kappa\)1 or J\(\kappa\)2, respectively. Amplified V(D)J products were gel purified and ligated into pCR2.1 plasmid (Invitrogen) and cloned by bacterial transformation (Jacob, Kelsoe et al. 1991). Cloned V(D)J inserts were sequenced in an Applied Biosystems automated DNA sequencer and analyzed by IMGT/V-QUEST (http://imgt.cines.fr) and NCBI blast search (http://www.ncbi.nlm.nih.gov/BLAST) software. LC rearrangements of 3H9 CD hybridomas were amplified by PCR as previously described (Rohatgi, Ganju et al. 2008) and sequenced as described above.

**2.9 Quantitative Real-Time PCR**

A. Genomic DNA was isolated from sorted B-cell subsets of cultures or BL/6 mice by phenol-chloroform extraction (Invitrogen). VDJ rearrangements were amplified from ~20ng of genomic DNA by q-rtPCR method using SYBR Green PCR reagent (Applied Bioscience) with forward primers specific for VH1, VH2 or VH9 genes and a reverse primer specific for JH1 (Table 2). The relative abundance of rearranged DNA
was normalized to CD14 signal and was calculated by the comparative threshold cycle method (Ueda, Liao et al. 2007).

B. Expression of B-cell mRNA was measured by q-rtPCR. Briefly, total RNA was extracted from sorted cell populations (~10^6 cells) using Trizol/chloroform extraction (Invitrogen). cDNA was prepared by standard methods (Ueda, Kondo et al. 2005; Ueda, Liao et al. 2007) using oligo(dT)12-18 primers and Superscript III reverse transcriptase (both, Invitrogen, Carlsbad CA). PCR primers used in this study are listed (Table 2) or previously described (Lazorchak, Schlissel et al. 2006). The relative expression levels of each message were normalized to Igβ message and were calculated by the comparative threshold cycle method (Ueda, Liao et al. 2007). All measurements were performed using a BioRad iCycler equipped with a MyiQ optical module (Bio-Rad).

2.9 Peptides and Tetramers

All peptides were synthesized by SynPep Corporation (Dublin, CA). All tetramers were created as previously described (Moody, MA submitted manuscript). Peptides used for immunization, ELISA, tetramer and ELISPOT analysis include: DP178-(biotin)-YTSLIHSLIESQNQ(QorL)EKNEQELLELDKWASLWNWF, SP62-(biotin)-GGGQQEKNEQELLELDKWASLWN, R4A-(biotin)-GGGGDWEYSVWLSN. All tetramer reagents used for FACS were labeled with APC to track antigen-specific B-cell populations. Empty-tetramer reagent was biotin-saturated streptavidin-APC that did not contain peptide. All tetramer reagents were used at ~10 nM concentration (~125 ng)
to label cells for 30 mins on ice. Cells were then washed and labeled with mAb (described above) to determine specific cell populations.

2.10 Immunizations

4-Hydroxy-3-nitrophenylacetyl (NP) hapten is conjugated to chicken gamma globulin (CGG) and used for immunizations: 6-8 wk old BL/6 mice were immunized (ip) with NP13-CGG (5 μg) precipitated in alum and suspended in 200 μl PBS. CD-RAG mice were immunized with equivalent amounts of antigen 3.5 wk after CD B cell transfer. Mice were bled before and 12d after immunizations to determine (4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP) -specific serum Ab levels. DP178-Q16L immunizations: 6-8 wk old BL/6 mice were immunized (ip) 1-2 times with DP178-Q16L peptide (10 μg) precipitated in alum and suspended in 200μl PBS. CD-RAG mice were immunized (ip) 1-2 times with DP178-Q16L peptide (10 μg) precipitated in alum and suspended in 200μl PBS 3.5-4 wk after CD B cell transfer. Mice were bled as indicated to determine antigen-specific serum Ab levels. Spleen and MLN were harvested 16 d post-immunization and analyzed via FACS and immunofluorescent labeling of tissue sections.

2.10 Histology

A portion of the spleen and individual MLN from naïve and immunized mice were embedded in OCT compound and snap frozen using N2-chilled 2-methylbutane and stored at -80°C. 5 μm sections were prepared using a cryostat and poly-lysine coated slides. Sections were fixed with 1:1 Acetone:Methanol for 10 min at -20°C and labeled
with B220-biotin, TCRβ-PE (red) and GL-7-FITC (green) mAb. FITC signal was amplified using anti-FITC-AF488 mAb. Streptavidin-AlexaFluor350 was used to amplify B220-biotin signal (blue). Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope.

2.11 Cell Lines

H33Lγ1 (NP-reactive; IgG isotype) and B1-8 (NP-reactive; IgM isotype) cell lines were grown in RPMI media containing 10% FCS, 10⁻⁴M 2-ME and penicillin/streptomycin (P/S) antibiotics as previously described (Kuraoka, Liao et al. 2009). Purified H33Lγ1 and B1-8 mAbs were used to generate standard curves to determine NIP-specific serum Ab concentrations elicited upon NP-CGG immunization. Additionally, these mAbs were used to generate standard curves to determine serum Ig reconstitution after adoptive transfer of B-cell populations into RAG-deficient mice. P3 and 13H11 (MPER-reactive; IgG isotype) cell lines were grown and maintained in DMEM media (Gibco) containing 10% FCS, 10⁻⁴M 2-ME and penicillin (10 Units/ml), and streptomycin (10μg/ml) antibiotics. R4A (DNA-reactive, IgG isotype) cell line was grown and maintained in DMEM media (Gibco) containing 20% FCS, 1% MEM non-essential amino acids, 10⁻⁴M 2-ME and penicillin (10 Units/ml), and streptomycin (10μg/ml) as previously described (Shefner, Kleiner et al. 1991). The P3, 13H11 and R4A cell lines were used to demonstrate that labeling by MPER tetramer was peptide-specific.
Additionally, purified 13H11 mAb was used to generate standard curves to determine MPER-specific serum IgG concentrations elicited upon MPER immunization.

### 2.12 Hybridomas

Activated cells (LPS; 5μg/ml, BAFF; 20ng/ml; 3 days) were electrofused with either NS1 or NS0-bcl2 fusion partners to generate hybridomas as previously described (Yu, McGraw et al. 2008). Cells were screened for reactivity to *Crithidia luciliae*, HIV-1 MPER antigen and/or NIH-3T3 cells. Antigen-reactive wells were expanded and sub-cloned (0.3 cells/well) to generate mAbs. Abs were recovered from culture supernant by Protein G column purification and re-screened on their original antigens.

The utility of *in vitro* culture methods to identify factors necessary for B-lymphopoiesis is well known (Ray, Stoddart et al. 1998; Cho, Webber et al. 1999; Hess, Werner et al. 2001). Most culture systems used to study lymphopoiesis and the development of hematopoietic progenitor cells (HPC) employ stromal cell lines, e.g., OP9 (Nakano, Kodama et al. 1994) and S17 (Collins and Dorshkind 1987), to provide necessary growth and differentiation factors (Billips, Petitte et al. 1992). These stromal cells provide essential cytokines that support hematopoiesis (Baird, Gerstein et al. 1999) and express adhesion molecules that *in vivo*, define specialized BM niches that promote B-cell development (Tokoyoda, Egawa et al. 2004). The capacity of stromal cell culture systems to support the development of B-lineage cells from HPC has been well characterized (Rolink, Kudo et al. 1991; Nakano, Kodama et al. 1994) and contributed to the discovery of IL-7 as a key cytokine for the stromal-dependent phase of mouse B-cell development (Cumano, Dorshkind et al. 1990; Billips, Petitte et al. 1992). However, stromal cell cultures are incapable of efficient *de novo* production of IgM+ B cells without the addition of exogenous cytokines (Flt-3L) or B-cell mitogens (LPS) (Ray, Stoddart et al. 1998; Cho, Webber et al. 1999).

In part, the presence of IL-7 in stromal cell cultures limits IgM+ B-cell development. B-cell precursors maintained in the presence of IL-7 continue to proliferate, retain their Igκ and Igλ gene loci in germ-line configuration and, therefore,
do not express surface IgM (ten Boekel, Melchers et al. 1995). Withdrawal of IL-7 from B-cell cultures decreases proliferation and is associated with increased expression of genes (RAG1/2) that are required for light chain (LC) gene rearrangements (Rolink, Kudo et al. 1991; Rolink, Streb et al. 1991). IL-7-mediated regulation of B-cell maturation is suggested to occur either by direct control of Ig recombinase gene activity (Billips, Nunez et al. 1995) or by controlling cell cycle (Li, Dordai et al. 1996). B-cell receptor (BCR) transgenic (Tg) pre-B cells require IL-7 for proliferation but are able to bypass IL-7-mediated developmental blockade as LC rearrangement is no longer required to continue differentiation (Melamed, Kench et al. 1997; Tze, Baness et al. 2000). Several groups have demonstrated that B lymphopoiesis can occur in the absence of BM stromal cells if the proper cytokines are provided (Tze, Baness et al. 2000; Luo, Maarschalk et al. 2009). Indeed, Claudio et al. reported that the removal of IL-7 and addition of BAFF to their B-cell culture system yields surface IgM+ B cells (Claudio, Brown et al. 2002).

BAFF is a cytokine that promotes B-cell survival (Schneider, MacKay et al. 1999) and B-cell maturation (Batten, Groom et al. 2000; Rolink, Tschopp et al. 2002; Gorelik, Cutler et al. 2004). BAFF−/− and BAFF-R−/− mice show an increase T1 B-cell compartment and have substantially reduced amounts of more mature B-cell subsets (Schiemann, Gommerman et al. 2001; Thompson, Bixler et al. 2001). Conversely, BAFF Tg mice have elevated numbers of mature B cells and develop autoimmune-like manifestations (Mackay, Woodcock et al. 1999). BAFF signaling is mediated through three
independently regulated receptors on B cells: B cell activating factor receptor (BAFF-R), transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA) (reviewed in (Mackay, Schneider et al. 2003)).

One clear limitation of stromal-independent cultures is that newly-formed B-cell populations have not been systematically characterized. I describe and detail a stromal-independent culture system that supports the survival, proliferation and differentiation of virtually all BM B-cell developmental stages. These culture-derived (CD) B-lineage cells are phenotypically and genotypically similar to their in vivo counterparts. Furthermore, I show that this culture system permits the development of autoreactive B cells which are normally purged during their development in the BM. CD cells were used to reconstitute peripheral lymphoid tissues of RAG\(^{-}\) mice and restored both serum IgM and IgG to control levels. CD B cells maintained their bias toward autoreactive specificities even after transfer to RAG\(^{-}\) hosts.

3.1 Results

3.1.1 Efficient generation of IgM\(^{+}\)IgD\(^{+}\) B cells in vitro

Non-adherent BM cells were collected and prepared for culture in tissue culture flasks (see 2.4). At the initiation of culture, 25-30% of BM cells were B220\(^{+}\); however, after 4 days with IL-7, \(\geq\)80% of 1\(^{°}\) CD cells expressed B220 and cultures increased 2-3 fold in B220\(^{+}\) cell number (Table 1). To identify the B cells present in BM and recovered in 1\(^{°}\) cultures, each cohort was labeled with mAb to B220, IgM, IgD, CD23 and CD21 to
identify B-cell subsets (Fig. 8) (Ueda, Liao et al. 2007). The frequency of B220+ pro-/pre-
(IgMnegIgDnegCD93hiCD23negCD21neg), immature (IgMloIgDnegCD93hiCD23negCD21neg), T1
(IgMhiIgDhiCD93intCD23negCD21neg), T2 (IgMhiIgDhiCD93intCD23hiCD21int) and mature
(IgMloIgDhiCD93negCD23hiCD21int) B cells was analyzed (Fig. 8). Each CD B-cell
population expressed CD93, CD23 and CD21 at similar levels compared to BM B
lymphocytes (data not shown). Pro-/pre-B cells were the most abundant population of 1°
CD cells (~80% of B220+ cells) (Fig. 8 and Table 1). Immature and T1 B cells were each
present at 5-10% in the cultures; whereas, T2 and mature B cells were each present at
≤5% (Fig. 8 and Table 1). In addition to these characterizations, BM and 1° CD cells were
analyzed using Hardy’s method (Hardy, Carmack et al. 1991) to define stages of B-cell
ontogeny. Fraction “B” (early pro-B) (B220loCD43hiBP-1loHSAint) and fraction “C-C” (late
pro- and preB-I) (B220loCD43hiBP-1hiHSAint) cells constituted ~70% and ~25%,
respectively, of the B220loCD43hi CD cells (Fig. 8). Compared to BM, our cultures
produced a substantial enrichment for pro- and preB-I compartments.

Table 1: Generation of CD B cells

<table>
<thead>
<tr>
<th>a. Generation of CD B cells (x10^6)</th>
<th>B220+ (x10^6)</th>
<th>Pro/pre (x10^6)</th>
<th>Immature (x10^6)</th>
<th>T1 (x10^6)</th>
<th>T2 (x10^6)</th>
<th>Mature (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>18.8</td>
<td>5.3±0.5</td>
<td>1.9±0.2</td>
<td>4.9±1.4</td>
<td>2.4±0.8</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>1° CD IL-7</td>
<td>18.8</td>
<td>15.5±5.2</td>
<td>12.6±4.2</td>
<td>14.1±4.8</td>
<td>4.8±7.3</td>
<td>3.3±0.6</td>
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<tr>
<td>2° CD IL-7</td>
<td>18.8</td>
<td>52.3±12.3</td>
<td>51.8±11.8</td>
<td>5.6±1.9</td>
<td>2.5±0.5</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>3° CD IAFF</td>
<td>18.8</td>
<td>8.2±1.3</td>
<td>5.5±0.4</td>
<td>7.1±1.7</td>
<td>0.3±0.7</td>
<td>0.3±0.5</td>
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</table>

<table>
<thead>
<tr>
<th>b. Generation of CD B cells (%)</th>
<th>B220+ (%)</th>
<th>Pro/pre (%)</th>
<th>Immature (%)</th>
<th>T1 (%)</th>
<th>T2 (%)</th>
<th>Mature (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>18.8</td>
<td>25.8±4.2</td>
<td>58.2±0.8</td>
<td>11.4±3.0</td>
<td>3.8±1.0</td>
<td>4.2±2.9</td>
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<td>1° CD IL-7</td>
<td>18.8</td>
<td>84.8±2.0</td>
<td>81.7±1.8</td>
<td>62.1±1.2</td>
<td>5.6±6.6</td>
<td>21.0±0.3</td>
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<tr>
<td>2° CD IL-7</td>
<td>18.8</td>
<td>96.6±1.0</td>
<td>97.2±0.5</td>
<td>10.0±1.1</td>
<td>0.5±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>3° CD IAFF</td>
<td>18.8</td>
<td>96.7±0.9</td>
<td>67.8±5.5</td>
<td>86.0±0.5</td>
<td>7.7±0.6</td>
<td>65.3±0.5</td>
</tr>
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</table>

At the initiation of each culture, cells (18.8x10^6/T-75 flask) were cultured in media with
the indicated cytokines (see Section 2.4). Samples of each culture were analysed by flow
cytometry to determine which cell population were present. The numbers (a) and percent (b) of B220+ cells were determined. Distinct B-cell subsets were identified by flow cytometry (Fig. 8) and the number and percent of each compartment (pro/pre, immature, T1, T2 and mature) was determined. Data for each B-cell compartment is determined from the B220+ gate.

CD B cells were re-cultured in new IL-7+ media (2° CD); then cultured cells were labeled with mAb to identify B-cell subsets (Fig. 8). The number of cells recovered from 2° CD cultures was 2-4 times greater than input values and 95% of cells expressed B220 (Table 1). The most abundant population (95%) of 2° CD cells were pro-/pre-B cells whereas IgM+ cells were minor populations (<5%) of cells (Fig. 8 and Table 1). 2° CD cells were analyzed using Hardy’s method, and we observed that Fraction “A” (pro-B) (B220loCD43hiBP-1loHSAlo), “B” and “C-C’’ cells comprised <1%, 10-15% and 80-85% of the cultures, respectively (Fig. 8).
Figure 8: Efficient generation of IgM⁺IgD⁺ B cells in vitro.

Non-adherent BL/6 BM cells (left) were cultured with 10 ng/ml IL-7 for 4 d (1° CD) or 8 d (2° CD). Cells were labeled with mAbs to B220, IgM, IgD, CD23, CD21 and CD93 (top panels) to identify pro-/pre-B, immature B, T1 B, T2 B and mature B cells. Cells were also labeled with mAb to HSA, BP-1, CD43, B220 and IgM to identify early B-cell compartments using Hardy’s method (bottom panels). Additionally, 2° CD cells were washed to remove IL-7 and then cultured with 20ng/ml BAFF for 3-4 d (3° CD). Cells were labeled with mAb to B220, IgM, IgD, CD23, CD21 and CD93 (right panels). Histograms of CD93 expression on each population are shown (bottom right). Results are representative of multiple (n≥5) independent experiments.
B-cell progenitors occupy independent BM niches depending upon their relative maturity (Nagasawa 2006; Pereira, An et al. 2009). While repeated IL-7+ cultures increased B220+ cell numbers (2- to 4-fold over each input value), this technique only expanded the pro-/pre-B cell compartments (Table 1). Earlier work showed that the removal of IL-7 and addition of BAFF resulted in the emergence of Ig+ B cells *in vitro* (Claudio, Brown et al. 2002), and I tested whether BAFF would enrich B220+IgM+IgD+ B cells in these cultures. 2° CD cells were cultured with BAFF for 3-4 days and then labeled with mAb to B220, IgM, IgD, CD23, CD21 and CD93 to identify B-cell subsets (Ueda, Liao et al. 2007). BAFF+ cultures (3° CD) yielded fewer numbers of cells as input values, but were enriched (>20%) for IgM+IgD+ B cells. 3° CD cells contained pro-/pre-B cells (65%), immature B cells (9%), T1 B cells (8%), T2 B cells (7%) and few (<1%) mature B cells (Table 1 and Fig. 8, right). CD93 was highly expressed on pro-/pre-B cell compartments and its expression decreased as maturation increased through the T2 B-cell compartment (Fig. 8, right). CD23 and CD21 expression increased at the transitional B-cell compartments similar to BM (Fig. 10). These results indicated that pro-/pre-B cells that expanded during the IL-7+ cultures retained their potential to differentiate. The sequential culture system of 1° (IL-7), 2° (IL-7) and 3° (BAFF) generated a large number of Ig+ B cells grown independently of the BM microenvironment.
3.1.1.1 T cells in B-cell culture system

Since mature T cells are present at low frequencies within the BM (Fig. 9A, top), I examined whether BM T cells survived in the cultures by FACS using mAbs for CD4, CD8, CD62L and CD44 (Kaech, Wherry et al. 2002). In BM, I observed that ~5% of B220neg cells expressed CD4 or CD8 and the majority (>50-60%) of these cells were CD62LhiCD44lo, a phenotype consistent with a mature, naïve T cells (Fig. 9A). In 1° and 2° CD cultures, 5-20% of cells were B220neg (Fig. 8) and 25-50% of these CD cells expressed CD4 or CD8 (Fig. 9A). Again, the majority (>80%) of these T cells exhibited a phenotype (CD62LhiCD44lo) consistent with naïve T cells. I determined the absolute number of cells with each T-cell phenotype that were recovered from 1° and 2° cultures (Fig. 9B). Both CD4+CD62LhiCD44lo and CD8+CD62LhiCD44lo cells decreased ~2-fold between 1° and 2° cultures. The number of CD4+CD62LhiCD44hi and CD8+CD62LhiCD44hi cells showed little or no change between 1° and 2° cultures. These data indicated that mature T cells survived through each IL-7+ phase of the culture system.
Figure 9: Mature T cells are maintained in BM culture system.  

(A) Non-adherent BL/6 BM cells (top) were cultured with 10 ng/ml IL-7 for 4 d (1° CD, middle) or 8 d (2° CD, bottom). Each population of cells was labeled with mAbs to B220, CD4, CD8, CD62L and CD44 to determine T-cell phenotypes present using flow cytometry.  

(B) The number of live cells was determined using a hemacytometer with Trypan Blue exclusion. Data are presented as the mean ± S.D. of the number of each T-cell phenotype in multiple cultures (n=6-8 independent cultures). These data demonstrate that mature T cells survive in vitro.
3.1.2 CD B cells have phenotypic and gene expression profile similar to BM B cells

I characterized CD B cells for surface antigens that are differentially expressed by distinct B-cell subsets (Carsetti, Rosado et al. 2004; Lopes-Carvalho and Kearney 2004; Pillai, Cariappa et al. 2005; Hardy 2006) from BM, spleen and peritoneal cavity (PC). Cells from each tissue were labeled with mAb to B220, IgM, IgD and CD23, CD21, CD5, CD1d or CD11b. BM and CD B cells were characterized by their B220, IgM and IgD expression (Gorelik, Cutler et al. 2004). Splenic B cells (B220⁺) were divided into mature follicular (mature) (IgM⁻IgD⁻CD23⁺CD21⁻), T2 (IgM⁺IgD⁻CD23⁺CD21⁻), T1 (IgM⁺IgD⁻CD23⁺CD21⁻) and MZ (IgM⁺IgD⁻CD23⁺CD21⁺) B cells (Lopes-Carvalho and Kearney 2004). Resident PC cells were divided into B1 B cells (B220⁻IgM⁺IgD⁻CD5⁻CD23⁻CD21⁻) and mature B cells as described (Martin and Kearney 2001).

CD B cells most closely resembled BM B-cell compartments (Fig. 10). Early CD and BM B-cell fractions (pro-/pre- to T1) were indistinguishable for all markers tested (Fig. 10A-B). The T2 and mature B cells generated in vitro displayed increased expression of CD23 and CD21 when compared to their respective BM counterparts (Fig. 10A-B), consistent with previous reports that BAFF signaling induces increased CD23 and CD21 expression (Gorelik, Cutler et al. 2004). All CD B cells, regardless of their developmental status, express low levels of CD5 (Fig. 10A). As determined by MFI, CD5 expression by
pro-/pre-B and immature B cells is ≤15% of peritoneal B1 cells whereas transitional and mature phenotype CD B cells express CD5 at levels <33% of freshly isolated B1 cells (Figs. 10A and 10D). CD1d is used for lipid-antigen presentation by B cells and is highly expressed on MZ B cells (Amano, Baumgarth et al. 1998; Lee, Abeyratne et al. 1998). Expression of CD1d increases during maturation from pro-/pre-B cells to T1 B cells, followed by decreased expression as B cells continue to mature (Fig. 10B). CD B cells express CD1d similar to BM B cells; however, T2 B cells sustain CD1d expression (Fig. 10A). Taken together, the surface phenotype of CD B cells suggests that we are generating B cells in vitro that are similar to BM-derived B cells.
Figure 10: CD B cells have phenotypic and gene expression profile similar to BM B cells

Non-adherent BL/6 BM cells were cultured using standard conditions (1°-IL-7, 2°-IL-7, 3°-BAFF) to generate (A) CD B cells. (B) BM (C) spleen and (D) peritoneal cavity cells were harvested for comparison of surface antigen expression. Each group of cells were
labeled with mAbs to B220, IgM, IgD and CD23, CD21, CD5, CD11b or CD1d. Flow diagrams were gated on live, single B220+ cells. Pro-/pre-, immature, T1, T2 and mature, MZ B and B1 B cells were identified as previously described. (E) CD B cells and ex vivo BM cells were labeled with mAbs to B220, IgM, IgD, CD23 and CD21 to identify pro-/pre-, immature/T1 and T2/mature B cells. RNA was isolated from FACS sorted ex vivo BM (black) and CD B-cell (gray) compartments. cDNA was created using oligo(dT)12-18 and Superscript III reverse transcriptase. q-rtPCR was performed on cDNA samples (n=2-3) from the B-cell compartments described above. Values for each reaction were normalized to Igβ expression. Significant differences (*; p < 0.05) between groups were determined by (two-tailed) Student’s t-test.

To directly compare gene expression, I had control and CD B cells sorted (pro/pre, imm/T1 and T2/mature) to harvest mRNA for quantitative real-time PCR (q-rtPCR). I measured the expression of genes crucial for B-cell development, survival and V(D)J recombination of immunoglobulin (Ig) loci. RAG1/2 and TdT are critical enzymes in Ig gene rearrangement and diversification (Dudley, Chaudhuri et al. 2005). λ5 is an essential component of the pre-BCR, and necessary for survival beyond the pro-B cell stage (Shimizu, Mundt et al. 2002). Pax-5 is a transcription factor required to induce and maintain B-cell identity (Cobaleda, Schebesta et al. 2007). The expression level and temporal regulation of each transcript was comparable between BM and CD B-cell fractions (Fig. 10E). I compared the expression of BAFF-R and TACI in each sorted B-cell compartment. The expression of each receptor gene was similar between BM and CD B cells for each B-cell population (Fig. 10E) demonstrating that CD B cells share the gene expression profiles of their BM counterparts.
Table 2: Primer list

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
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</tr>
<tr>
<td>α5</td>
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</tr>
<tr>
<td></td>
<td>TTG GTG TGT TGG GAG GGT TGG</td>
</tr>
<tr>
<td>BAFF-R</td>
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<td></td>
<td>GGC GGT GTG TGC ATG TTC TTT GAG</td>
</tr>
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<tr>
<td>β-actin</td>
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</tr>
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<td>RAG1</td>
<td>CCA AGC TGC AGA CAT TCT AGC ACT C</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>TdT</td>
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</tr>
<tr>
<td></td>
<td>CTG GGC TGC TIG AAG TTT TCC</td>
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<td>qPCR-gDNA</td>
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</tr>
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</tr>
<tr>
<td></td>
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<td>IgH-V1</td>
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</tr>
<tr>
<td>IgH-V2</td>
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<tr>
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</tr>
<tr>
<td>JH1</td>
<td>CCC GCT TCA GAA TGG AAT GTG C</td>
</tr>
</tbody>
</table>

**Sequence Analysis**

| IgH-V1 T6 out | CAG GTT CAG CTG/CAG CAG CAG TCT G |
| IgH-V1 T6 in  | TGTAT CTT GCA AGG CTT CTG GCT ACA C |
| IgH-V1 20 out | AGG TCC AGC TGC AGG AGT CTG |
| IgH-V1 20 in  | TCA GTG AAG ATG TCC TGC AA |
| JH5        | CCC TAG TCC TCCATT ACC TGA A |
| Igx-V4 (Xe3) | GAC ATT CAG GTG ACC CAG TCT CCA |
| Igx-V4 (Xe5) | CCC AGT TGG (GGTGT CAG TGG CAG TCG |
|            | continued (AVG)T (AV)G (AV)GAC |
| Igx-V5 (Xe23) | CTC TGG CAT CCC CTC DAG G |
| Igx-V6 (Xe23) | TGG CAG TGG ATG AGG GAC AG |
| Jx2        | TTC CTC GTT AAC ACC TGG TGT GA |
3.1.3 CD B cells express diverse *Igh* and *Igκ* rearrangements

3.1.3.1 Diversity in *Igh* family repertoire

To ensure that CD B cells express a diverse set of heavy chain (HC) and LC genes, I amplified V<sub>H</sub>1-, V<sub>H</sub>2- and V<sub>H</sub>9- rearrangements to J<sub>H</sub>1 by PCR. A non-rearranging gene, CD14, was used to normalize the amount of input DNA (~20ng). Genomic DNA from unsorted BL/6 BM served as a positive control for each PCR reaction; concurrently, sorted pro-/pre-B cells from BL/6 BM and CD B-cell samples were directly compared (Fig. 11). The V<sub>H</sub>1 family (69 members) is located at the 5` distal end of the *Igh* locus (IMGT/V-Quest). Both BM and CD samples showed equal V<sub>H</sub>1 usage (~3% of CD14 signal) (Fig. 11). The V<sub>H</sub>2 family (9 members) is located at the 3` region of the *Igh* locus, proximal to the D cluster (IMGT/V-Quest). BM and CD samples showed similar (~0.8% of CD14 signal) usage of V<sub>H</sub>2 gene segments (Fig. 11). Lastly, we examined V<sub>H</sub>9 (4 members), located in the center of the *Igh* locus (IMGT/V-Quest) and determined that BM and CD samples contained similar (~0.1% of CD14 signal) amounts of these V-gene segments (Fig. 11). These results indicated that the stochastic nature of HC V-gene selection during rearrangement of the BCR is intact for B cells grown in our culture system.
Figure 11: CD B cells exhibit non-biased usage of several VH-family genes

Non-adherent BL/6 BM cells were cultured (1°-IL-7, 2°-IL-7) to generate CD B cells. Cells were harvested and labeled with mAbs to B220, IgM and IgD. Pro-/pre-B cells were sorted from BM (B6) or 2° CD B cells (CD) and genomic DNA was extracted from 10⁶ cells using proteinase K digestion and phenol-chloroform extraction. Whole BM (BM) gDNA sample was used as positive control for detection. ~20 ng of gDNA was amplified by q-rtPCR using V_H1-, V_H2- or V_H9-family specific 5` primer with an intronic J_H1 specific 3` primer. CD14-specific PCR reaction was used to normalize input quantities of gDNA. Values are represented as the percent of CD14 signal. Experiment was performed on sorted samples (n=3 each) of BL/6 BM and 2° CD pro-/pre-B cells.

3.1.3.2 Junctional diversity in CDR3 V(D)J regions

The mature B-cell repertoire contains a diverse array of BCR specificities that promote antigen recognition and host survival (reviewed in (Market and Papavasiliou 2003)). Several mechanisms, such as combinatorial association of variable- (V), diversity- (D) and joining- (J) gene segments, operate in developing B lymphocytes to promote BCR diversity (Chen and Alt 1993; Benedict, Gilfillan et al. 2000). Non-template (N-) nucleotide addition and imprecise joining of recombining VDJ gene segments further contribute to the diversity of the BCR repertoire (Benedict and Kearney 1999). If few pro-
/pre-B cells preferentially expanded during IL-7+ cultures, then IgM+ cells that arise during BAFF+ cultures would contain a restricted subset of IgH rearrangements. Therefore, I sequenced the complementarity determining region-3 (CDR3) regions of IgH and Igκ genes amplified from sorted pro-/pre- and im/T1 CD B cells.

Table 3: Sequence analysis of CD B cells

<table>
<thead>
<tr>
<th></th>
<th>V(_{\mu})1</th>
<th>V(_{\kappa}4/\kappa5)</th>
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<tbody>
<tr>
<td>BL6 BM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pro-/pre-</td>
<td>28</td>
<td>81</td>
</tr>
<tr>
<td>in/T1</td>
<td>N.D.</td>
<td>55</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>V(_{\mu})1</th>
<th>V(_{\kappa}4/\kappa5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pro-/pre-</td>
<td>118</td>
<td>54</td>
</tr>
<tr>
<td>im/T1</td>
<td>N.D.</td>
<td>59</td>
</tr>
</tbody>
</table>

I recovered unique HC CDR3 sequences (~100) and analyzed IgH rearrangements to both J\(_{\mu}1\) and J\(_{\mu}2\) gene segments in CD pro-/pre-B cells (subset shown in Table 3 and Table 4). Over thirty distinct V\(_{\mu}1\) family members were recovered, representing ~50% of V\(_{\mu}1\) gene segments available (Table 3). These V\(_{\mu}1\) gene segments were associated with 11 different D gene segments (Table 3 and Table 4) and contained both P- and N-nucleotide addition at V-D and D-J junctions, resulting in further diversification of the IgH repertoire (Table 4). This analysis was extended to the V\(_{\kappa}4\) and V\(_{\kappa}5\) LC loci in pre- and im/T1 CD B cells. Similar mechanisms of diversity, with the exception of N-
nucleotide addition, were detected in the LC repertoire (Table 3 and Table 4). Taken together, this sequencing data demonstrates that CD B-cell development supports typical, diverse V(D)J rearrangements that are observed in BM counterparts.

**Table 4: Sequence analysis of V(D)J CDR3 rearrangements in CD B cells**

<table>
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<tr>
<th>Seq #</th>
<th>VH-family</th>
<th>D-segment</th>
<th>3’ portion of V-segment</th>
<th>N or P nucleotides</th>
<th>D-region</th>
<th>N or P nucleotides</th>
<th>5’ portion of JH</th>
<th>J-region</th>
<th>Fm 5</th>
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<tr>
<td>1</td>
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<td>P.16.1</td>
<td>TAT TGT GA</td>
<td>A CCG ACC C</td>
<td>TT AT ACT ACG G</td>
<td>G</td>
<td>AC TGG</td>
<td>JH2</td>
<td>nf</td>
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<tr>
<td>2</td>
<td>VH1.24</td>
<td>SP.12</td>
<td>TAC TGT CCA GAG</td>
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<td>GC TTC AAC</td>
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<td>ACT AGC GTA G</td>
<td>TTC GGG GCT ACT</td>
<td>TC TGT GTC TG</td>
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<td>AGC C</td>
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</table>

* F = functional rearrangement, and nf = non-functional rearrangement.

The molecular mechanism of Ig recombination permits the addition and/or removal of nucleotides which can generate frame-shift mutations, resulting in a truncated, non-functional immunoglobulin protein (Baumann, Potash et al. 1985). I determined the reading frame of CDR3 sequences based on two criteria: a conserved cysteine residue of the framework-3 (FW3) region of V-gene segments and the JH to Cμ
or Jκ to Cκ splice site present in each J-gene segment. The Igh and Igκ genes were analyzed for productive (P) and non-productive (nP) rearrangements and both P and nP rearrangements were observed (Table 4). Compared to previously described B-cell culture systems (Rolink, Kudo et al. 1991; Ray, Stoddart et al. 1998; Tze, Baness et al. 2000), I demonstrate that CD B cells utilize each element of V(D)J recombination that contributes to BCR diversity.

3.1.4 CD B cells rapidly differentiate to AFC and upregulate co-stimulatory molecules on TLR stimulation

To determine whether CD B cells were functional, I compared the response of mature B cells, MZ B cells (Snapper, Yamada et al. 1993; Oliver, Martin et al. 1997) and 3° CD B cells to Toll-like receptor (TLR) stimulation. Spleen cells were labeled with mAb to B220, IgM, IgD, CD23 and CD21 to sort mature and MZ B cells; while, CD B cells were sorted for B220+CD43negCD93lo cells. Sorted mature, MZ and CD B cells were stimulated with LPS for up to 3 days and were tested for IgM AFC differentiation. Mature- and MZ-derived AFC were low (~1% of input cells) after 2 days of LPS stimulation, but increased (~10% of input cells) by day 3 (Fig. 12A). CD B cells differentiated into IgM AFC by day 2 (~3% of input cells) and reached levels similar to mature and MZ compartments by day 3 (Fig. 12A). These results demonstrated that CD B cells were capable of rapid differentiation to AFC after LPS stimulation. Regulation of surface CD80 and CD86 expression after LPS stimulation is used to distinguish MF and MZ B cells (Oliver, Martin et al. 1999). LPS-treated mature, MZ and CD B cells were analyzed for CD80 and
CD86 expression. Each group increased CD80 and CD86 expression within 48 hrs of LPS stimulation with MZ B cells expressing the highest levels (Fig. 12B). CD B cells expressed an intermediate level of each co-stimulation molecule compared to mature and MZ B cells (Fig. 12B). Each group reduced CD80/CD86 expression by the third day of LPS stimulation (Fig. 12B). These data demonstrate that CD B cells were able to differentiate in manners similar to splenic B-cell compartments in response to TLR ligation.
Figure 12: CD B cells rapidly differentiate to AFC and increase CD80/CD86 expression upon TLR stimulation.

CD43^CD93^lo CD B cells and mature splenic B-cell populations (MF and MZ) were sorted using FACS. Sorted cells were cultured with 5μg/ml LPS for 24hr, 48hr or 72hr. (A) Number of IgM AFC was determined by ELISpot after LPS stimulation for mature (●), MZ (■) or CD (▲) B cells. ELISPOT plates coated with anti-mouse Ig(H+L) polyclonal Ab and assays were performed in triplicate for each condition tested. Values represent mean and standard deviation of triplicate samples. (B) Expression of CD80 and CD86 was measured on mature, MZ and CD43^CD93^lo CD B cells after LPS stimulation. Cells were labeled with mAbs to B220, CD80 and CD86 after LPS treatment for 48hr (blue) or 72hr (green) and compared to ex vivo splenocytes (red).
3.1.5 Elevated frequencies of autoreactive CD B cells

There are several co-receptors that modulate signaling through the BCR, such as CD19, CD22 and CD21 (Chen, Koralov et al. 2000; Nitschke 2005). Mis-expression of these co-receptors can influence the intensity of down-stream signal transduction, sometimes resulting in autoimmunity (Tuscano, Harris et al. 2003). Since IgM+IgD+ CD B cells had elevated expression of CD23 and CD21 (Fig. 10A) and developed without the BM micro-environment associated with central tolerance (Sandel and Monroe 1999; Sandel, Gendelman et al. 2001), I tested whether this culture system permitted the formation of autoreactive B cells. Previous work has demonstrated that the 3H9 HC encodes dsDNA and ssDNA binding with various LC (Shlomchik, Mascelli et al. 1990). The 3H9 IgH transgenic and “knock-in” (HC-KI) mice have been created to study the processes that promote self-tolerance (Erikson, Radic et al. 1991; Chen, Nagy et al. 1995).

I analyzed B-cell compartments of 3H9 HC-KI mice and confirmed that the frequency of BM immature and transitional B cells was substantially reduced (25-40% of BL/6 controls) (Fig. 13A, left). Furthermore, the number of immature/transitional B cells in BM of 3H9 HC-KI mice was reduced (20-30% of BL/6 controls) (Fig. 14). After IL-7+ expansion of pro-/pre-B cells from 3H9 HC-KI and control BM, I tested whether 3H9 HC-KI immature and transitional B cells would develop in the presence of BAFF. Equal numbers of cells were recovered from BAFF+ cultures of 3H9 HC-KI and control cells (data not shown). The frequency of 3H9+ immature CD B cells was equal to control
immature CD B cells; whereas, the frequency of 3H9+ transitional B cells approached (~65%) that of control cultures (Fig. 13A, right). The frequencies of immature and transitional 3H9+ CD B cells were much greater (~4-fold and ~10-fold, respectively) than the 3H9 HC-KI BM compartments (Fig. 13A, bottom). These data suggested that autoreactive B cells lost during development in BM are recovered using this culture system.

**Figure 13: Autoreactive transitional B cells are generated using BM culture system.**

(A) Non-adherent BL/6 (top) and 3H9 HC-KI (bottom) BM cells (left) were cultured with 10 ng/ml IL-7 (4 d) followed by culture with 20ng/ml BAFF (4 d) (right). Cells were labeled with mAbs to B220, IgM, IgD, CD23, CD21 and CD93 to identify B-cell subsets. Representative flow diagrams were pre-gated on live, single B220+CD93+ cells. FACS plots are representative of multiple (n=3) independent experiments. (B) Summary of 3H9 CD hybridomas for labeling of *C. luciliae* (see Fig. 14B for representative images), expression of 3H9 transcripts and identification of Vκ/Jκ and Vλ/Jλ usage.

To directly assess whether DNA-reactive B cells were generated in 3H9 HC-KI BM cultures, I activated 3H9 CD B cells (Fig. 13A, bottom right) with LPS and BAFF for
fusion with NS0-bcl2 cells to create hybridomas (Yu, McGraw et al. 2008). Cells were sub-cloned at 0.3 cells/well and each mAb was characterized. All of 3H9 CD hybridoma lines were IgM and 11 (91.7%) of these 3H9 CD hybridomas utilized a κLC. One hybridoma (8.3%) utilized a λLC (Fig. 13B). mRNA from each cloned hybridoma was isolated to generate cDNA and to confirm the presence of 3H9 Igh transcripts (Fig. 13B).

C. luciliae are commonly used to detect Abs that react with native DNA (ADA) (Gilkeson, Pippen et al. 1995). Seven of the 3H9 CD hybridomas (6/11 κLC and 1/1 λLC) reacted with native DNA (Fig. 13B and Fig. 14B). Many families of light chains (Vk3, Vk4, Vk8, Vk9, Vk21, Vk23 and Vλ1) combine with the 3H9 HC and impart DNA-reactivity (Shlomchik, Mascelli et al. 1990; Radic, Erikson et al. 1993). I sequenced the LC loci of our 3H9 CD hybridomas and readily detected Vk- and Vλ-family members that are “permissive” for DNA binding (Fig. 13B). These data demonstrate that autoreactive B cells which are lost during development in BM are recovered using this culture system.
Figure 14: CD system recovers autoreactive B-cell development.

(A) BM cells were harvested from BL/6 (n=4) and 3H9 HC-KI mice (n=2). The number of live cells was determined using a hemacytometer with Trypan Blue exclusion. The frequency of specific B-cell compartments were identified as previously described (Fig. 10B) and used to enumerate the size of each compartment. (B) Ab from 3H9 CD hybridomas (50μg/ml) was treated with 2-ME (0.01M, 2hrs) and used to label C. luciliae. Bound Ab was detected using either anti-mouse Igκ- or Igλ-FITC Abs. Representative examples of strong (++), weak (+) and no (-) DNA binding activity are presented. All images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope with an exposure time of 500 ms.
3.1.6 CD cells reconstitute peripheral immune tissue of lymphocyte-deficient hosts

I tested whether CD cells could reconstitute lymphopenic recipients. BL/6 BM cells were cultured in IL-7+ media to generate 2° CD B cells which were then cultured with BAFF to generate IgM+ CD B cells for injection into B6.RAG1-/- recipients (CD-RAG mice) (section 2.1). Recipient mice were sacrificed 4wks post-transfer to analyze reconstitution of B-cell compartments in peripheral lymphoid tissue. Spleen and LN cells from control and CD-RAG mice were labeled with mAb to B220, IgM, IgD, CD23 and CD21 for comparison. BL/6 mice had a normal distribution of both splenic and LN B-cell populations as previously reported (Fig. 15A) (Allman and Pillai 2008). The spleen of CD-RAG mice contained B cells (~10% of splenocytes) that expressed elevated (~2-fold) CD23 and CD21 and were enriched for IgMhiIgDlo cells (Fig. 15B), consistent with a MZ B cell phenotype. The LNs of CD-RAG animals contained B220+ cells that expressed levels of IgM, IgD, CD23 and CD21 similar to control LNs (Fig. 15B). CD B cells represented ~30% of the lymph node cells recovered (data not shown). The recovery of B cells from spleen and LN demonstrate that CD B cells are able to traffic appropriately to peripheral lymphoid organs and survive for extended periods of time. Then, I tested whether the donor cells could reconstitute the serum Ab of recipient animals. Serum from CD-RAG mice was analyzed by IgM- and IgG-specific ELISA over the course of four weeks (Fig. 15C). CD B cells were able to restore serum IgM concentration
progressively over the reconstitution period to near normal levels. Serum IgG was undetectable at day 7, but was comparable to normal controls by 4 wk post-reconstitution.
Figure 15: CD B cells reconstitute peripheral immune tissue of lymphocyte-deficient hosts.
Non-adherent BL/6 BM cells were cultured (1°-IL-7, 2°-IL-7, 3°-BAFF) to generate CD B cells for injection into B6.RAG−/− mice. At 4 wk post-transfer, spleen and LN cells from (A) BL/6 or (B) B6.RAG−/− mice reconstituted with 3° CD B cells were labeled with mAbs to B220, IgM, IgD, CD23 and CD21. Flow diagrams were pre-gated on live, single, B220+ cells and were representative of each mouse analyzed (n>3). (C) Sera from CD-RAG mice were collected via retro-orbital eye bleeding at 1 wk, 2 wk, 3 wk and 4 wk post-transfer. Serum from un-manipulated BL/6 (B6) and RAG-deficient (RAG) mice was collected and used as controls. Concentrations of serum IgM and IgG were determined for each sample using anti-mouse IgM- or IgG-specific ELISAs including standard curves. Each group contained three mice (n=3) except for D21 & 28 time points (n=2).

3.1.6.1 Reconstitution of hematopoiesis in CD-RAG mice

I investigated whether reconstitution of lymphopoiesis occurred in CD-RAG mice after cell transfer. I observed very few (<1%) BM B220+ cells that co-expressed IgM+ (Fig. 16A). The thymus did not contain CD4+CD8+ (DP), CD4+ (CD4-SP) or CD8+ (CD8-SP) T-cell progenitors, indicating that CD cells do not contain T-cell progenitors at the time of transfer to RAG-deficient mice (Fig. 16B). However, mature T-cell populations were retained through our culture system (Fig. 9) leading me to examine mature T-cell reconstitution of CD-RAG mice. I harvested the spleen and LNs of BL/6, RAG1−/− and CD-RAG mice to analyze mature T-cell compartments. The spleen and LNs of BL/6 mice contained both CD4+ and CD8+ cells that were predominately (>80%) CD62LhiCD44lo “naïve” phenotype and a small frequency (~5-10%) of CD44hi “activated” or “memory” cells (Fig. 16C-D, top). RAG-1−/− mice did not contain any CD4+ or CD8+ cells that I could detect (Fig. 16C-D, middle). Mature T cells were present in the spleen and LNs of CD-RAG mice at ~5% and ~35% of the B220neg compartment, respectively, (Fig. 16C-D,
bottom). Both CD4+ and CD8+ cells in CD-RAG mice were predominately (~70-80%) CD44hi, consistent with homeostatic driven proliferation (Kieper and Jameson 1999). These data indicated that mature T cells which survived in vitro were able to partially reconstitute the peripheral lymphoid tissue of RAG1+ mice.
Figure 16: Mature T-cells are present in secondary lymphoid tissues of CD-RAG mice but B- and T-lymphopoiesis is not restored.

Non-adherent BL/6 BM cells were cultured using standard conditions to generate 3° CD B cells (BAFF+) and were injected into B6.RAG<sup>+</sup> mice. At 5 wk post-transfer, (A) BM, (B) thymus, (C) spleen and (D) LN cells from CD.RAG or BL/6, B6.RAG<sup>+</sup> mice were labeled with mAbs to (A) B220, IgM, IgD or (B) CD4, CD8, CD25, CD44 or (C & D) B220, CD4, CD8, CD44, CD62L. Flow diagrams were pre-gated on (A) live, single, B220<sup>+</sup> cells or (B) live, single cells or (C & D) live, single, B220<sup>+</sup> cells.
3.1.7 Serum IgG autoantibody is present in CD-RAG mice

Autoreactive B cells can be eliminated from the mature B-cell repertoire by peripheral tolerance mechanisms (Hartley, Crosbie et al. 1991; Silveira, Dombrowsky et al. 2004). Therefore, I tested whether autoreactive B cells were retained in vivo after reconstitution of RAG-/- mice. I screened serum collected from CD-RAG animals for the presence of IgG autoantibody. I included NIH-3T3 cells as a source of mouse cellular antigens during these immunofluorescence studies. As a positive control, serum from C4+/− mice contained detectable levels of anti-nuclear antibodies (ANA) and ADA as previously reported (Fig. 17, top) (Chen, Koralov et al. 2000). Serum from BL/6 mice contained barely detectable levels of ANA and ADA (Fig. 17, bottom) and Ig-deficient RAG1−/− serum served as a negative control for labeling (data not shown). Conversely, the serum of CD-RAG mice contained ANA and ADA at levels similar to C4+/− mice (Fig. 17, middle). The recovery of serum IgG autoantibody suggested that RAG-/- hosts permitted autoreactive CD B cells survival long enough to contribute to serum Ab restoration.

Combined, the characterization of in vitro B-cell development that occurred in this culture system suggested that I could study i) B-cell populations that were normally forbidden in the mature peripheral B-cell repertoire, and ii) humoral immune responses to antigens that are normally under immuno-regulatory control.
Control serum was obtained from BL/6, B6.RAG−/− and C4−/− mice and from CD-RAG mice at 4wk post-transfer. Slides containing NIH-3T3 (left) and C. luciliae (right) were labeled with serum (1:20dil) from each mouse group. After overnight washing, Ab bound to cells was detected using rat anti-mouse IgG-FITC Ab. All images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope with an exposure time of 60ms (C. luciliae) or 100ms (NIH-3T3). Scale bar equals 10μm for all images.
4. Enhanced Ab Responses to an HIV-1 MPER Antigen in Mice Reconstituted with Cultured Lymphocytes

While serum Ab responses are generated in HIV-infected individuals, humoral responses are weak and emerge only after the initial immune suppression of HIV-1 replication (Parren and Burton 2001). These responses select for HIV-1 mutants resistant to Ab neutralization (Burton, Desrosiers et al. 2004) as the genetic plasticity of the lentivirus reduces the efficacy of both Ab and cellular responses in controlling HIV infection (Burton, Desrosiers et al. 2004), indicating that Ab exerts selective pressure that HIV-1 is well equipped to avoid. The MPER of the HIV-1 gp41 is a conserved structure crucial for viral fusion with target cell membranes (Wyatt and Sodroski 1998) and considered a strong candidate for an effective HIV-1 vaccine (Haynes and Montefiori 2006) (Fig. 18). Rarely, protective humoral responses are directed against the MPER of HIV-1 after infection (Shen, Parks et al. 2009) (Wyatt and Sodroski 1998). A series of protective Abs, 2F5, 4E10, and Z13, that react with linear epitopes of the HIV-1 MPER have been recovered (Muster, Steindl et al. 1993; Zwick, Labrijn et al. 2001; Nelson, Brunel et al. 2007); however, vaccination strategies do not routinely induce robust anti-MPER responses (Eckhart, Raffelsberger et al. 1996; Coeffier, Clement et al. 2000; Derby, Kraft et al. 2006).
The “infection spike” of HIV-1 consists of a heterotrimeric complex of gp120 (red ovals) and gp41 (light blue cylinders) molecules buried in the viral lipid membrane (yellow circles) (Earl, Doms et al. 1990). gp120 mediates viral attachment to target cells via interaction with CD4 and chemokine receptors (Wyatt, Kwong et al. 1998). After attachment of viral particles, gp120 and gp41 undergo conformational changes to bring viral and target cell membranes in close proximity to each other (Pancera, Majeed et al.). The precise mechanism of membrane fusion is not entirely understood.

Several arguments have been proposed to explain why the Ab response against HIV-1 is ineffective at controlling viral spread. These arguments include the high frequency of genomic mutation that alter HIV-1 epitopes, shielding of crucial antigenic determinants by glycosylation, competitive suppression by highly immunogenic but non-neutralizing surface antigens, and insufficient diversity in the primary repertoire of
Ab specificities (Burton, Desrosiers et al. 2004). While each argument is plausible, the demonstration that 2F5 and 4E10 Abs exhibit substantial affinities for self-antigens offers an alternative explanation for the rarity of protective Ab responses (Haynes, Moody et al. 2005). If epitopes of HIV-1 mimic self-antigens of the host, the normal processes of immunological tolerance will purge these epitope-specific B cells from the mature repertoire and consequently impair Ab responses. Therefore, the fate of self-/HIV-reactive B cells should be investigated to determine whether i) these cells are purged from the mature B-cell repertoire during their development or ii) these cells are available for immune activation but held in an anergic state. This information is vital to the logical design of future vaccines that attempt to elicit Ab responses to the MPER of HIV-1.

The influence of humoral tolerance on MPER-reactive B-cell development has recently been investigated (Verkoczy, Diaz et al.). In addition to the HIV-1 MPER, 2F5 mAb recognizes a cellular antigen(s) present in both humans and mice (Verkoczy, Diaz et al.) and B-cell development in 2F5 Igh “knock-in” (2F5 HC-KI) mice is blocked at the transition of small pre-B to immature and transitional B cells in the BM (Verkoczy, Diaz et al.). This developmental blockade is remarkably similar to that observed in mice expressing BCR for MHC (Nemazee and Burki 1989) and double-stranded DNA (Chen, Nagy et al. 1995). Unlike the studies that utilize BCRs with known specificity to self-antigens (MHC and DNA), the self-antigen(s) that mediates the selection of 2F5 HC-KI B cell development has not been identified. Because 2F5 and 4E10 mAbs demonstrate
measurable binding to many biological structures (e.g., lipids and protein), it is not clear what interaction is mediating this induction of tolerance in MPER-reactive B cells (Scherer, Leaman et al.; Alam, Morelli et al. 2009).

The 2F5 mAb requires both lipid reactivity and affinity for a linear peptide epitope of the HIV-1 gp41 MPER to block HIV-1 infection of target cells (Alam, Morelli et al. 2009). The structures of 2F5 and 4E10 mAbs reveal distinct regions for binding MPER peptide and lipid antigens (Scherer, Leaman et al.; Alam, Morelli et al. 2009). The hydrophobic H3 loop mediates the lipid reactivity of 2F5; whereas, the MPER peptide binding maps to the CDR3 region (Alam, Morelli et al. 2009). Mutation in either paratopic region of 2F5 alone marginally interferes with the antigen reactivity of the alternate, non-mutated paratope (Alam, Morelli et al. 2009). However, mutation of either paratopic region will significantly reduce the ability of 2F5 to neutralize HIV-1 infection (Alam, Morelli et al. 2009). These data suggest that removal of B cells that express Ab with either lipid-reactivity or specificity for the appropriate MPER peptide specificity would result in the loss of HIV-1 neutralizing activity. To test this hypothesis, I determined if B cells specific for the 2F5 peptide epitope of MPER, independent of association with lipids, were tolerized during their development in the BM.
Ab molecules (dark blue) bind antigen in its native conformation. Linear peptide epitopes (orange lines) are biotinylated and attached to streptavidin (light blue & green) to form tetramer reagents. These tetramers are labeled with fluorescent molecules and used to identify antigen-specific B-cell compartments.

Even without resorting to transgenic or “knock-in” mice, it is possible to identify specific B cells either by antigen-binding (McHeyzer-Williams, Nossal et al. 1991; Lalor, Nossal et al. 1992; McHeyzer-Williams, McLean et al. 1993) or by clone-specific (anti-idiotope) mAb (Reth, Imanishi-Kari et al. 1979; Takemori, Tesch et al. 1982). The development of B-cell tetramers, analogous to those routinely used to identify antigen-specific T cells (Altman, Moss et al. 1996), has greatly enhanced the ability to identify and isolate antigen-specific B cells despite their low frequencies (Fig. 19) (Newman, Rice et al. 2003). I have used B-cell tetramers to identify MPER peptide-reactive B cells within central and peripheral lymphoid tissues and to follow the fates of tetramer-binding cells.
The “tolerance hypothesis” predicts that HIV-1 gp41 MPER tetramer-binding cells should be enriched in developmentally immature B-cell compartments, but rare or absent in mature B-cell populations.

I have described a BM culture system that supports the survival, proliferation and differentiation of virtually all B2-lineage developmental stages (Holl, Haynes et al. 2010). These CD B-lineage cells are phenotypically and functionally similar to their in vivo counterparts (Holl, Haynes et al. 2010) but develop in the absence of many self antigens and absent the environment of the BM (Sandel and Monroe 1999; Sandel, Gendelman et al. 2001). CD B cells are enriched for autoreactive specificities, and maintain this bias even after transfer to RAG-1 deficient hosts (Holl, Haynes et al. 2010). Importantly, these cultures contain MPER-reactive B cells and mice reconstituted with CD B cells generated both robust GC responses and serum IgG Ab upon immunization with HIV-1 peptide. C57BL/6 control animals responded poorly as I have shown that these MPER-reactive B-cell subsets were lost beyond the BM transitional B cell stages in vivo.

4.1 Results

4.1.1 Labeling of cells with MPER tetramer is inhibited only by homologous peptide reagents

The human 2F5 mAb reacts with self-antigen(s) (Haynes, Fleming et al. 2005) that are expressed in both mice and humans ((Verkoczy, Diaz et al.) and (Fig. 20 and Fig. 21); whereas, non-neutralizing MPER Abs (e.g. 13H11) and a series of irrelevant antigen-
reactive Abs (e.g. B1-8 and H33Lγ1) do not demonstrate measurable binding to NIH-3T3 cells (Fig. 20).

**Figure 20: 2F5 mAb binds NIH-3T3 cells in ELISA**

1-2x10^4 NIH-3T3 cells were plated in 96-well tissue culture plates. After 24 hrs, NIH-3T3 cells were fixed with methanol:acetone (1:1) for 10 min at -20°C. Fixed NIH-3T3 cells were rehydrated and blocked overnight in PBS with 0.5% BSA, 0.1% Tween-20 and 1.0% normal goat serum. The mAbs B1-8 (Δ), H33Lγ1 (∗) and 13H11 (■) were incubated in wells at rt for 2hrs. After extensive washing, bound Ab was detected using goat anti-mouse IgM-HRP and IgG-HRP Abs. Purified recombinant 2F5 mAb (♦) was used as a positive control for detection and developed with goat anti-human IgG-HRP Ab.

MPER peptide and lipid antigens are bound by 2F5 and 4E10 mAbs via distinct paratopic regions. As indicated previously, the hydrophobic H3 loop potentially mediates the lipid reactivity of 2F5; whereas, the MPER peptide binding of 2F5 mAb maps to a distinct region of the CDR3. 2F5 mAb could recognize and label NIH-3T3 cells
via interaction with peptide and/or lipid antigens. I determined if labeling of NIH-3T3 cells by 2F5 mAb could be inhibited by pre-treatment with MPER peptide antigen independent of lipid antigen(s).

![Inhibitor: 2F5 (M) vs. JRFL, DP178, R4A peptides](image)

**Figure 21: Inhibition of 2F5 binding to 3T3 cells by recombinant HIV-1 gp140 (JRFL), and the DP178 and R4A peptides.**

To determine whether 2F5 reactivity to fixed 3T3 cells could be inhibited by proteins/polypeptides containing the 2F5 MPER core epitope (ELDKWA), we reacted 2F5 monoclonal (10µg/ml) antibody with increasing molar concentrations of homologous (JRFL and DP178) or heterologous (R4A) inhibitors (1 hr, 25 °C). These mixtures were subsequently added to hydrated/blocke...
were overlayed with goat anti-human IgG-FITC (1:400 in PBS with 0.1% Tween-20 and 0.5% BSA). After 1 hr., slides were washed, coversliped in Fluoromount-G. Twenty-four hr. later, fluorescence images were acquired using a Zeiss Axiovert 200M confocal microscope at 200x magnification and a fixed 300 msec exposure time.

Homologous inhibitors, the JRFL protein and, to a lesser extent, DP178 polypeptide, inhibited 2F5 binding to NIH-3T3 cells (Fig. 21). An irrelevant polypeptide, R4A, showed no inhibition (Fig. 21). These data demonstrate that a substantial amount of 2F5 reactivity to fixed NIH-3T3 cells is determined by protein-protein interaction rather than un-specific lipid binding. Furthermore, these inhibition data indicated that determining the frequency of MPER peptide-reactive B cells throughout ontogeny would directly test if 2F5-like B cells were influenced by mechanisms of B-cell tolerance.

To identify and enumerate mouse B cells that bind to the 2F5 epitope, B-cell tetramer reagents were generated that carry HIV epitopes, including MPER antigens (2F5, 4E10) or irrelevant peptide motifs (Moody et al. submitted manuscript) (Fig. 22 and 23B-D). The specificity of tetramer binding was determined by competitive inhibition using 13H11 cells that expresses MPER-specific IgG1 receptors reactive with a 2F5-proximal determinant (Alam, Scearce et al. 2008). Some 60% of 13H11 cells were labeled by APC-conjugated MPER tetramer whereas APC-conjugated empty (no peptide) or R4A tetramers labeled ≤20% of 13H11 cells (Fig. 22 and 23B-C). The parental fusion line, P3, was not labeled significantly by any tetramer (Fig. 23B). To ensure the specificity of tetramer binding, 13H11 cells were incubated with either unlabeled tetramer or free peptide (0.6 to 20-fold molar excess) and subsequently labeled with APC-conjugated
MPER-tetramer (representative histograms in Fig. 22). Homologous peptide and unlabeled tetramer reduced both the frequency and intensity (MFI) of 13H11 cells labeled by APC-conjugated MPER-tetramer in a dose-dependent manner to background levels (Fig. 23B-C). In contrast, addition of unlabeled heterologous peptide or tetramer resulted in little (≤10%) to no reduction of MPER-tetramer labeling as determined by reductions in the frequency or MFI of MPER-labeled cells (Figs. 23B-C).

Next, I incubated BM B cells with MPER-tetramer to determine the specificity of any labeling (Fig. 23D). BL/6 BM cells (~2×10⁶) were incubated in medium alone or in medium containing unlabeled MPER peptide (10-fold molar excess), washed and then exposed to APC-conjugated MPER tetramer. After washing, BM cells were reacted with B220 mAb to identify B-lineage cells. BM cell populations contained a small (≤0.2%), but reproducible, population of MPER-tetramer+ B220+ cells; in BM samples pre-incubated with soluble MPER peptide, tetramer labeling frequencies were consistently reduced by ≥80% (Fig. 23D). I conclude that the substantial majority of B cells labeled by MPER-tetramer specifically bound the MPER-peptide, and that the MPER-tetramer identifies antigen-specific B-cell populations (Fig. 23D).
Figure 22: Specific labeling of B-cell lines by MPER tetramer

13H11 cells (1-1.3 x 10^6) were incubated in PBS + 3% FCS alone or buffer containing equivalent molar excess amounts of either i) unlabeled MPER-tetramer, ii) MPER peptide, iii) unlabeled R4A tetramer or iv) R4A peptide for 30 min. at 0°C. Unlabeled peptide and tetramer concentrations were established to represent 0.6, 1.3, 2.5 and 5.0 M excess of tetramer-associated peptide epitope. Subsequently, cells were labeled with 125 ng of APC-conjugated MPER-tetramer for 30 min. on ice. Independent aliquots of 13H11 cells were labeled with APC-conjugated Empty-tetramer as a negative control for peptide-independent binding. 13H11 cells were subsequently analyzed by flow cytometry and the fraction of tetramer binding cells (inset no.) and the M.F.I. of tetramer+ cells were determined compared to unlabeled controls. Each histogram is representative of at least 3 independent measurements (n≥3) compiled over 2 independent experiments. All data was acquired using a BD LSRII cytometer and histograms were created using FlowJo software.
Figure 23: Labeling by MPER-tetramer is blocked by homologous (MPER) but not heterologous (R4A) reagents

(A) NIH-3T3 cells were labeled with 2F5 mAb and bound Ab was detected using anti-human IgG-FITC. Scale bar equals 20 μm for all images. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope at 200x magnification.

13H11 cells (1-1.3 x 10^6) were incubated in PBS + 3% FCS containing equivalent molar excess amounts of either unlabeled MPER peptide (○), unlabeled MPER-tetramer (●) or control unlabeled R4A peptide (△) and unlabeled R4A tetramer (▲) for 30 min at 0°C. Unlabeled peptide and tetramer concentrations were established to represent 0.6, 1.3, 2.5 and 5.0 M excess of labeled tetramer-associated peptide epitope. Subsequently, cells were labeled (L) with 125 ng of APC-conjugated MPER-tetramer for 30 min on ice. Other 13H11 cells were labeled with either APC-conjugated Empty-tetramer (short dashed line) or R4A-tetramer (long dashed line) as negative controls for binding. Also, P3 (◊)
cells were labeled with APC-conjugated MPER-tetramer as a negative control for binding. Cells were analyzed by FACS and (B) fraction of tetramer-binding cells and (C) M.F.I. of tetramer+ cells was determined. Each data point represents the average of at least 3 independent measurements (n≥3) compiled over 2 independent experiments. (D) BL/6 BM cells (10^6) were incubated alone or with 10-fold molar excess of MPER peptide. Then, BM cells were incubated alone or labeled with 125 ng of APC-conjugated MPER-tetramer. All samples were washed and labeled with mAb to B220. FACS plots are pre-gated on live, single, B220+ cells. Data presented are representative of 2 independent experiments.

4.1.2 HIV-1 MPER-reactive B cells are lost during the transitional stage of B-cell development

The developmental impairments of B cells in 2F5 HC-KI mice is consistent with the hypothesis that B cells recognizing some HIV gp41 MPER epitopes are removed by the mechanisms of self-tolerance (Verkoczy, Diaz et al.). To determine whether MPER-reactive B cells expressing endogenous Ig rearrangements might also be lost to tolerance mechanisms, I determined the frequencies of MPER-tetramer binding in specific B-cell compartments in the BM and spleen by labeling BL/6 BM cells and splenocytes with control and MPER tetramers along with mAbs that define specific subsets of B cells (Fig. 24) (Ueda, Liao et al. 2007).
BM and spleen cells were harvested from BL/6 mice. Cells (10⁶) were labeled with mAb to B220, IgM, IgD, CD23, CD21 and CD93. Flow diagrams were gated on live, single B220⁺ cells. Pre/Pro B (B220intIgMnegIgDnegCD23negCD21negCD93hi), Immature B (B220intIgMloIgDnegCD23negCD21negCD93hi), T1 B (B220intIgMhiIgDhiCD23hiCD21negCD93bi), T2 B (B220hiIgMhiIgDhiCD23biCD21intCD93int), MZ B cells (B220biIgMbiIgDbiCD23biCD21biCD93neg) and mature B (B220hiIgMbiIgDhiCD23biCD21biCD93neg) cells were identified in the BM and/or spleen. Data was acquired using a BD LSRII flow cytometer and analyzed using FlowJo software.

B220biIgMneg B cells from BM (pro/preB and plasmablasts/-cytes) do not exhibit significant MPER-tetramer binding, whereas immature (~0.2%) and transitional (T) 1

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**Figure 24: Cell surface markers used to define BM and splenic B-cell populations**

BM and spleen cells were harvested from BL/6 mice. Cells (10⁶) were labeled with mAb to B220, IgM, IgD, CD23, CD21 and CD93. Flow diagrams were gated on live, single B220⁺ cells. Pre/Pro B (B220intIgMnegIgDnegCD23negCD21negCD93hi), Immature B (B220intIgMloIgDnegCD23negCD21negCD93hi), T1 B (B220intIgMhiIgDhiCD23hiCD21negCD93bi), T2 B (B220hiIgMhiIgDhiCD23biCD21intCD93int), MZ B cells (B220biIgMbiIgDbiCD23biCD21biCD93neg) and mature B (B220hiIgMbiIgDhiCD23biCD21biCD93neg) cells were identified in the BM and/or spleen. Data was acquired using a BD LSRII flow cytometer and analyzed using FlowJo software.

B220biIgMneg B cells from BM (pro/preB and plasmablasts/-cytes) do not exhibit significant MPER-tetramer binding, whereas immature (~0.2%) and transitional (T) 1
and -2 (~0.4%) BM B cells were labeled by MPER-tetramer at a low, but significant frequencies (Fig. 25). Significantly, splenic T1 and T2 B cells that were otherwise phenotypically similar to their BM counterparts, exhibited lower frequencies of MPER-tetramer labeling (p<0.05) that were indistinguishable from that of empty-tetramer controls (Fig. 25). Reactivity with the MPER-tetramer was not a general property of BM B cells as mature, recirculating, B cells present in the BM exhibited a low (~0.2%) frequency of MPER-binding cells (Fig. 25). I provide representative examples of tetramer+ B cells compartments (Fig. 26).

In the spleen, the frequencies of mature follicular (MF) and marginal zone (MZ) B cells that bound MPER-tetramer (~0.2%) were not significantly different (p≥0.20) from the frequencies of cells labeled by empty tetramer (Fig. 25), suggesting that mature MPER-reactive B cells are rare.

Taken together, these data are consistent with the generation of HIV-1 MPER-reactive B cells and their subsequent loss during the T1 and T2 stages of B-cell development in the BM. Indeed, this period of development known to be a major checkpoint of central B-cell tolerance (Melchers and Rolink 2006). To my knowledge, these experiments constitute the first demonstration of developmentally regulated reductions in the numbers of antigen-specific B cells in normal mice (Melchers and Rolink 2006).
Figure 25: MPER-tetramer binding B cells are lost in BM transitional compartments

BL/6 BM and spleen cells were harvested for flow cytometry. Cells (10⁶) were labeled with 125 ng of MPER-APC (♦️) or R4A-APC (●️) tetramer. Cells were washed and labeled with mAb to B220, IgM, IgD, CD23 and CD21. Specific B-cell compartments of the BM (closed) and spleen (open) were identified (Ueda, Liao et al. 2007). Unlabeled control samples were acquired to determine background signal. Data presented as the average and S.E.M. of the percent (%) of tetramer⁺ cells within each B-cell compartment. Each group contained multiple mice (♦️; n=9 BM & Spl, ●️; n=4 BM & Spl for R4A) compiled over 2-3 independent experiment. Significant differences (*; p ≤ 0.05) between groups were determined by (two-tailed) Student’s t-test.
Figure 26: Developing BM B cell populations bind MPER peptide-loaded tetramer.

Non-adherent BM and spleen cells were harvested and stained with 125ng of SP62-APC tetramer. Cells were washed and stained with mAb to B220, IgM, IgD, CD23 and CD21. SP62-tetramer binding was assessed on each population using a BD LSRII flow
cytometer. Plots are pre-gated on single, live, B220+ cells. Analysis of specific B cell subsets was performed using surface staining criteria previously described.

### 4.1.3 In vitro B-cell culture system rescues 2F5 HC-KI immature and transitional B-cell development

Earlier, I developed and characterized stomal cell-independent, B-cell cultures that generate substantial numbers of IgM+ B cells, including those normally lost to immunological tolerance in the BM (Holl, Haynes et al. 2010). This method allows the development of “forbidden”, autoreactive B cells (e.g., DNA-specific 3H9 HC-KI) in vitro and their transfer and persistence into RAG1 deficient recipients (Holl, Haynes et al. 2010).

![Figure 27: In vitro cultures permit the development of 2F5 HC-KI transitional B cells that are absent in BM](image)

Non-adherent BL/6 (left) and 2F5 HC-KI (right) BM cells (top) were cultured with 10 ng/ml IL-7 followed by culture with 20ng/ml BAFF (bottom). Cells were labeled with mAbs to B220, IgM, IgD, CD23, CD21 and CD93 to identify B-cell subsets. FACS diagrams were pre-gated on live, single B220+CD93+ cells using FlowJo software. FACS plots are representative of multiple (n=3) independent experiments.
To determine whether this *in vitro* recovery might extend to autoreactive B cells that also recognize MPER epitopes, I cultured non-adherent BM cells from 2F5 HC-KI mice (Verkoczy, Diaz et al.) and characterized their capacity to support B-cell development *in vitro*. As expected (Verkoczy, Diaz et al.), the BM of 2F5 HC-KI mice contains significantly (p<0.01) reduced numbers of immature and T1 B cells (~10% of BL/6 controls (Fig. 27); nonetheless, following the culture of 2F5-HC KI BM in IL-7 and BAFF (Holl, Haynes et al. 2010) I recovered substantial numbers (~35% of BL/6 controls) of immature and T1/T2 2F5 HC-KI B cells (Fig. 27). Frequencies of immature and transitional 2F5 HC-KI CD B cells were much greater (~10-fold) than the corresponding 2F5 HC-KI compartments in BM (Fig. 27, right panels), demonstrating that this culture system is permissive for the development of 2F5 HC-KI B cells that are normally lost during development (Holl, Haynes et al. 2010).

### 4.1.4 CD B cells contain HIV-1 MPER-reactive cells

The specificity of B lymphocytes that can generate Ab specific for the 2F5 MPER epitope has yet to be characterized. As our CD B cells are grown *in vitro*, absent the normal BM micro-environment that promotes tolerization of self-reactive B cells (Sandel and Monroe 1999; Sandel, Gendelman et al. 2001), I determined whether the culture system supports the development of MPER-reactive B cells from BL/6 mouse BM. CD B cells were labeled with control (empty), R4A- or MPER-tetramers (Fig. 23B-C) along
with B220, IgM and IgD mAb (Representative FACS profiles in Fig. 28). By this method, I identified MPER-specific B cells (0.2 – 0.4% of T1 or T2 compartments) as tetramer positive cells (Fig. 29A).

Non-adherent BM cells were cultured using standard conditions to generate Ig⁺ B cells. 10⁶ cells were harvested and stained with 125ng of SP62-APC, R4A-APC or empty-APC tetramer. Cells were washed and stained with mAbs to B220, IgM and IgD. Tetramer binding was assessed on each population using a BD LSRII flow cytometer. Plots are pre-gated on single, live, B220⁺ cells. Analysis of specific B cell subsets was performed using surface staining criteria previously described.

Figure 28: Examples of Tetramer⁺ CD B-cell compartments.
Figure 29: CD B cell populations bind MPER peptide-loaded tetramer

BM cells were cultured using a standard method to generate Ig⁺ B cells (Holl, Haynes et al. 2010). (A) Cells were labeled with 125 ng of MPER-APC (♦), R4A-APC (●) or empty-APC (▲) tetramer. Cells were washed and labeled with mAb to B220, IgM and IgD. Tetramer binding was assessed on each population using a BD LSRII flow cytometer. Analysis of specific B-cell subsets was performed using surface staining criteria previously described (Ueda, Liao et al. 2007). Each data point represents the average and S.E.M. of multiple independent measurements (♦; n=14, ●; n=10, ▲; n=7) for each population compiled over 3-4 independent experiments. Significant differences (*; p < 0.05) between groups were determined by (two-tailed) Student’s t-test. (B) BM cells were cultured to generate Ig⁺ B cells (Holl, Haynes et al. 2010). Cells were labeled with 125 ng /10⁶cells of MPER-APC tetramer and with mAb to B220. Live MPER-tetramer⁺B220⁺ cells were sorted using BD FACSVantage flow cytometer. Unselected and selected
(MPER^B220^) cells were stimulated using 5 μg/ml LPS and 20 ng/ml BAFF for 72h. Cells were harvested, washed and 1.5-2x10^3 cells were incubated for 4h on ELISpot plates coated with goat anti-mouse Ig(H+L) capture reagent. Membranes were probed with either 20 μM biotin-MPER or biotin-R4A peptide to determine the frequency and enrichment of MPER peptide-specific cells. Streptavidin-AP and SIGMA FAST BCIP/NBT were used to develop antigen-specific spots. ELISpot images are representative of 2 independent experiments performed in duplicate.

B220^lo^Ig^neg^ B cells from these cultures do not exhibit significant MPER-tetramer binding, whereas immature (~0.15%) and T1/T2 (~0.35%) CD B cells were labeled by MPER-tetramer at low, but significant frequencies (Fig. 29A). Few immature and T1 CD B cells bound the empty-tetramer (≤0.1%), suggesting that the majority (~75%) of labeling by MPER- and R4A-tetramers was peptide-specific (Fig. 29A). For T1 and T2 CD B cells, the frequencies of MPER-reactive cells are not significantly different (p=0.10 and 0.40, respectively) than that observed in the transitional compartments of BM (Fig. 25).

These data demonstrate that MPER-reactive B cells are efficiently generated from BL/6 BM using this culture system, thereby opening an avenue of investigation into the repertoire of B lymphocytes that can generate Ab specific for the 2F5 MPER epitope.

I confirmed that in vitro cultures of BL/6 BM cells supported the development of MPER-specific CD B cells using the ELISpot method. CD B cells (5-10%) differentiate into antibody forming cells (AFC) after stimulation with BAFF and LPS (Holl, Haynes et al. 2010). I determined the frequency of R4A- and MPER-antigen specific AFC using biotinylated-peptide reverse ELISpot assays (Verkoczy, Moody et al. 2009). Both R4A- and MPER-specific AFC were present (~0.1-0.4%) in LPS/BAFF-activated CD B cells (Fig.
29B, unselected), data that are congruent with the frequency of *in vitro*-derived MPER-specific B cells measured via tetramer labeling experiments (Fig. 29A). FACS enrichment of MPER-tetramer+ CD B cells substantially increased (~12 fold) the frequency of MPER-reactive AFC (by ELISpot) (Fig. 29B, selected). By comparison, selection of MPER-tetramer+ CD B cells showed little (<2-fold increase) change in the frequency of R4A-reactive AFC (by ELISpot) (Fig. 29B, selected). These data demonstrate that tetramer labeling of B-cell compartments can be used to selectively enrich for antigen-specific cells similar to previous experiments (Scheid, Mouquet et al. 2009).

### 4.1.5 Generation of Hybridomas to recover MPER-reactive Ab

To directly assess whether self- and MPER-reactive B cells were generated inBL/6 and 2F5 HC-KI BM cultures, I activated CD B cells (Fig. 27, bottom) with LPS and BAFF for fusion with NS1 and NS0-bcl2 myeloma cells, respectively, to create hybridomas (Yu, McGraw et al. 2008).

After electrofusion of LPS/BAFF-activated B6 CD B cells with NS1 myeloma cells, cells were plated (~10^4 cells/well; n=3360 wells) in selection media (DMEM+HAT) to establish hybridoma cell lines. We identified 3148 wells (93.7%) that contained cellular growth and screened their culture supernant for reactivity to NIH-3T3 and MPER antigens. We identified culture supernant that were reactive to MPER antigen (n=239 total wells (7.6%); n=18 hybridoma lines established), NIH-3T3 cell antigen(s) (n=264 total wells (8.4%); n=72 hybridoma lines established) and both MPER/NIH-3T3 cell
antigen(s) (n=81 total wells (2.6%); n=8 hybridoma lines established). Based on the individual frequency of antigen reactivity for MPER and NIH-3T3 antigen reactivity, the expected frequency of double-reactive hybridomas that would occur due to random chance can be estimated at 0.64%. The observed frequency (~2.6%) of MPER/NIH-3T3 cell antigen-reactive wells suggested that reactivity to HIV-1 MPER and NIH-3T3 cells was linked. Cells were sub-cloned at 0.3 cells/well and each mAb was re-screened for MPER- and NIH-3T3 antigen reactivity. NIH-3T3 cells are used to detect Abs that react with self-antigens (Holl, Haynes et al. 2010). These hybridoma lines were also screened for reactivity to MPER antigen by ELISA. Each set of hybridomas were grouped into three categories based upon their respective reactivities: NIH-3T3⁺ only, MPER⁻ only or NIH-3T3⁺/MPER⁻. (Table 5 and Fig. 30)
Table 5: Status of hybridoma analysis

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Source</th>
<th>No.s</th>
<th>Sub-cloned</th>
<th>Isotype</th>
<th>Purified Protein</th>
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<tr>
<td>B6CD-3T3</td>
<td>LPS-activated CD B cells from B6 BM</td>
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<td>0</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>B6CD-MPER</td>
<td>LPS-activated CD B cells from B6 BM</td>
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<td>IgM</td>
<td>4</td>
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<tr>
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<td>8</td>
<td>6</td>
<td>IgM</td>
<td>6</td>
</tr>
<tr>
<td>2F5BM-MPER</td>
<td>LPS-activated B cells from 2F5 BC-K1BM</td>
<td>5</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>2F5BM-MPER/3T3</td>
<td>LPS-activated B cells from 2F5 BC-K1BM</td>
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<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>2F5CD-MPER</td>
<td>LPS-activated CD B cells from 2F5 BC-K1 BM</td>
<td>12</td>
<td>7</td>
<td>IgM</td>
<td>3</td>
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<td>IgM</td>
<td>0</td>
</tr>
<tr>
<td>CDER-3T3</td>
<td>Spleen and LN from (6m) DP178-Q16L imm.</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>CD-RAG mice</td>
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<tr>
<td>CDER-MPER</td>
<td>Spleen and LN from (6m) DP178-Q16L imm.</td>
<td>7</td>
<td>3</td>
<td>IgG</td>
<td>0</td>
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<td>CD-RAG mice</td>
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<tr>
<td>CDER-MPER/3T3</td>
<td>Spleen and LN from (6m) DP178-Q16L imm.</td>
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<td>1</td>
<td>IgM</td>
<td>1</td>
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<tr>
<td></td>
<td>CD-RAG mice</td>
<td></td>
<td></td>
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</tbody>
</table>

After electrofusion of LPS/BAFF-activated 2F5 CD B cells with NS0/bcl2 myeloma cells, cells were plated (~10^4 cells/well; n=1440 wells) in selection media (DMEM+HAT) to establish hybridoma cell lines. We identified 1440 wells (100%) that contained cellular growth and screened their culture supernatant for reactivity to NIH-3T3 and MPER antigens. We identified culture supernatant that were reactive to MPER antigen (n=236 total wells (16.4%); n=12 hybridoma lines established), and both MPER/NIH-3T3 cell antigen(s) (n=42 wells (3.9%); n=14 hybridoma lines established). Based on the individual frequency of antigen reactivity for MPER and NIH-3T3 (data not shown), the expected frequency of double-reactive hybridomas that would occur...
due to random chance can be estimated at 1.7%. Again, we observed a “greater than
expected” frequency (~3.9%) of MPER/NIH-3T3 cell antigen-reactive wells. I expanded
cells from each subset to sub-clone, as described above, and isolated mAb for further
characterization (Table 5 and Fig. 30).

Figure 30: Examples of purified hybridoma proteins that bind to NIH-3T3 and
MPER antigen(s).
NIH-3T3 cell ELISA plates were created as previously described (Fig. 20). ELISA plates were coated with MPER-specific capture antigen (DP178-Q16L). Purified 13H11 mAb (orange diamonds) was used as a positive control to detect MPER antigen. B1-8 (♦) mAb was used as a negative control to determine the amount of non-specific IgM Ab reactivity to MPER and NIH-3T3 cells antigens. Column purified hybridoma proteins (IgM-isotype) from 2F5CD-MPER/3T3 #17-C9 (□), #2-D7 (Δ), #5-B6 E3 (○), #5-B6 F3 (●) or B6CD-MPER/3T3 #1011 (▲) clones were screened for antigen reactivity. Bound Ab was identified using a goat anti-mouse IgG or goat anti-mouse IgM detection reagent.

Ab was purified from these hybridomas and clones (n=17) were identified that contained reactivity to both MPER- and NIH-3T3 cell antigens (subset shown in Fig. 30A-B). Conversely, the NP-reactive IgM mAb, B1-8, did not bind either MPER- or NIH-3T3 antigens by ELISA (Fig. 30A-B), suggesting that our IgM mAbs generated from CD B cells were specific to MPER and NIH-3T3 cell antigens. Interestingly, the non-neutralizing MPER-reactive mAb, 13H11, only reacts with an MPER antigen that is discreet from any epitope present in NIH-3T3 cells (Fig. 30 A-B). These data confirm that self- and MPER-reactive B cells that are lost during development in BM of BL/6 and 2F5 HC-KI mice were recovered using this culture system.

4.1.6 Reconstituting RAG-1−/− mice with CD cells, but not LN cells, results in serum autoantibody

CD B cells can be used to reconstitute peripheral lymphoid tissues of RAG-1−/− mice (Holl, Haynes et al. 2010), and these CD-RAG mice contain elevated levels of serum autoantibody compared to intact BL/6 controls. Since in vitro B-cell development permitted the formation of auto- (Holl, Haynes et al. 2010) and MPER-reactive cells (Fig. 27-30), we were interested whether CD-RAG mice would provide an experimental
model to study B-cell populations that are normally excluded from the mature repertoire. However, it is formally possible that the lymphopenic environment of RAG-1−/− mice, not changes in the CD B-cell repertoire, supported the formation of autoantibody. Therefore, I tested whether mature, “self-tolerant” B cells isolated from LNs would form autoantibody upon transfer to RAG-1−/− mice.

Peripheral lymphoid tissues of RAG-1−/− mice were repopulated with lymphocytes after transfer of either CD or LN cells (Fig. 31A). As observed in CD-RAG mice, the frequency of splenic MZ-like (IgMhiIgDloCD21hi) B cells was elevated in LN-RAG mice when compared to BL/6 controls (Fig. 31A). The frequencies of mature (IgMloIgDhiCD21lo) and MZ-like B-cell populations present in the LNs of CD-RAG mice were comparable to that of BL/6 mice; however, LN-RAG mice contained an elevated (~3 to 5-fold) frequency of MZ-like LN B cells when compared to either BL/6 or CD-RAG mice (Fig. 31A). We observed a population of B220hiIgMnegIgDneg B cells in the spleen and LNs of both CD- and LN-RAG mice (Fig. 31A), a phenotype consistent with B cells that have undergone class-switch recombination. These data indicate that exposure of B cells to the lymphopenic RAG-1−/− environment may i) alter the surface phenotype of both CD and LN B cells relative to their respective input phenotypes and/or ii) preferentially expand MZ-like and class-switched B-cell compartments. Clearly, the immune system of RAG-1−/− mice is compromised due to the lack of T- and B lymphocytes. This lymphocyte-deficiency could lead to an increase in microbial burden; therefore, the expansion of
both MZ and class-switched B cells may be the result of CD and LN cell populations responding to pathogen stimulation.

Figure 31: RAG-1−/− mice reconstituted with CD B cells, but not LN B cells, results in serum autoantibody.
Non-adherent BL/6 BM cells were cultured to generate CD B cells for injection into B6.RAG<sup>−/−</sup> mice as previously described (Holl, Haynes et al. 2010). BL/6 LN cells were isolated for transfer into B6.RAG<sup>−/−</sup> mice (LN-RAG). (A) At 6 wk post-transfer, spleen and LN cells from BL/6 (top), CD-RAG (middle) or LN-RAG (bottom) mice were labeled with mAbs to B220, IgM, IgD, CD93 and CD21. Flow diagrams were pre-gated on live, single, B220<sup>+</sup> cells and were representative of each mouse analyzed (n=5 per group). Sera from each experimental group were collected via retro-orbital eye bleeding at 6 wk post-transfer. (B) Sera samples were diluted (1:160) and used to labeled *C. luciliae* substrate slides. After overnight washing, Ab bound to cells was detected using rat anti-mouse IgG-FITC Ab. All images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope with an exposure time of 300ms at 400x magnification. Representative examples of strong (++), weak (+) and no (-) DNA binding activity are presented. Scale bar equals 20 μm for all images. (C) Concentrations of serum IgG were determined using anti-mouse IgG-specific ELISAs including standard curves. Each sera sample (1:160 dil) was screened for reactivity to DNA by immunofluorescent microscopy with a fixed exposure time (300ms) at 400x magnification. Each group contained multiple mice (n=5) that were screened independently.

Next, I detected DNA autoantibody in the serum (1:160 dilution) of BL/6, CD- and LN-RAG mice using *Crithidia luciliae* direct immunofluorescence assay (Gilkeson, Pippen et al. 1995) by dividing the observed binding into strong (++), weak (+) and no (-) reactivity (representative images for each category in Fig. 31B). BL/6 sera samples contained only weak (1/5) or no (4/5) reactivity to DNA (Fig. 31C). In contrast, most (4/5)CD-RAG serum samples contained strong reactivity to DNA (Fig. 31C), while most LN-RAG samples (3/5) showed no reactivity to DNA (Fig. 31C). To ensure that differences in serum DNA Ab was not the result of unequal IgG reconstitution, I directly compared the ability of CD and LN B cells to reconstitute serum IgG using ELISA (Fig. 31C). I observed that both CD- and LN-RAG mouse serum contained similar (~1.5 mg/ml) amounts of IgG to that of BL/6 controls (Fig. 31C). These data support my
conclusion that the B-cells repertoire formed *in vitro* is qualitatively different from the mature, peripheral B-cell repertoire of BL/6 mice, indicating that the CD-RAG animal model can be used to study B-cell populations that are normally excluded from the mature repertoire.

### 4.1.7 CD-RAG mice mount robust GC responses after immunization with MPER antigen

CD B cells contained MPER-specific populations (Fig. 27 and 29) and were able to reconstitute lymphocyte-deficient mice with a unique repertoire of B cells ([Holl, Haynes et al. 2010](#)) and Fig. 31); therefore, I tested whether CD-RAG mice could respond to immunization with MPER peptide antigens precipitated in aluminum sulfate (alum). I harvested the spleen and mesenteric LNs (MLN) of immunized and control mice (d16 post-immunization) and determined the frequency of germinal center (GC) B cells (B220<sup>hi</sup>GL-7<sup>hi</sup>) within the total B220<sup>+</sup> population (Fig. 32A and 32B). Additionally, I saved a portion of each tissue to confirm the presence of GC structures for histological analysis (Fig. 32C and 32D).

In BL/6 mice, immunization with MPER antigen did not significantly increase (1° p=0.80; 2° p=0.52) the frequency of MLN GL-7<sup>hi</sup> B cells when compared to naïve animals (Fig. 32A). Furthermore, BL/6 mice did not form robust splenic GC responses after MPER antigen immunization (Fig. 32B), indicated by small increases (1° p=0.04; 2° p=0.10) in the frequency of GL-7<sup>hi</sup> B cells and confirmed by histological analysis (Fig. 32C). I conclude that BL/6 mice do not elicit robust GC responses upon MPER-peptide
immunization due to the loss in frequency of MPER-specific mature B cells demonstrated previously (Fig. 25).

In contrast, immunization of CD-RAG mice with MPER antigen significantly increased ($1^\circ$ p=0.01; $2^\circ$ p=0.05) the frequency of MLN GL-7$^{hi}$ B cells (Fig. 32A) and initiated robust splenic GC reactions (Fig. 32B). Histological analysis of spleen samples from these immunized CD-RAG mice confirmed that GL-7$^{hi}$ B cells were organized into GC structures (Fig. 32C-32D). Compared to BL/6 controls, CD-RAG mice contained significantly elevated (MLN: $1^\circ$ p<0.01; $2^\circ$ p=0.01 and Spl: $1^\circ$ p=0.01; $2^\circ$ p=0.05) frequencies of GL-7$^{hi}$ B cells after each immunization with MPER antigen (Fig. 32A and 32B). Combined, my data demonstrate that CD-RAG mice mount robust GC responses to MPER antigen immunization and I correlate these observations with the recovery of MPER-reactive B cells 	extit{in vitro}.
Figure 32: CD-RAG mice form GC responses after immunization with MPER peptide

BL/6 (○) and CD-RAG (●) mice were immunized (ip) with 10 μg DP178-Q16L (MPER) peptide in alum. (A) MLN and (B) spleen cells were harvested at d16 after 1-2
immunizations. Cells were labeled with mAb to B220, IgM, IgD, TCRβ and GL-7. The percent of B220hiGL-7hi B cells of total B220hi cells was determined by flow cytometry. Each group contained multiple mice (n=6-12) compiled over multiple (n=2-4) independent experiments. Significant differences (*; p ≤ 0.05 and **; p ≤ 0.01) between groups were determined by (two-tailed) Student’s t-test. 5 µm sections of spleen from BL/6 and CD-RAG mice at d16 after (C) primary or (D) secondary immunizations were labeled with mAbs to B220-AF350 (blue), TCRβ-PE (red) and GL-7-FITC (green). FITC signal was amplified using anti-FITC-AF488 Ab. Scale bar equals 50µm for all images. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope at 200x magnification.

### 4.1.8 Induction of MPER-reactive IgG Ab in CD-RAG Mice

Historically, MPER-specific serum Ab is poorly elicited after administration of HIV vaccines (Eckhart, Raffelsberger et al. 1996; Coeffier, Clement et al. 2000; Ferrantelli and Ruprecht 2002; Derby, Kraft et al. 2006). CD B cells reconstitute peripheral lymphoid tissue, organize into follicles and form GCs (Fig. 31 and Fig. 32); however, the capacity to generate antigen-specific serum Ab had not been tested. I tested whether CD-RAG mice were capable of forming antigen-specific IgG to NP-CGG and compared this response to the generation of gp41 MPER-reactive Ab. The serum of naïve and antigen-immunized mice was collected and antigen-specific serum Ab was quantified by ELISA containing standard curves.

Immunization of BL/6 and CD-RAG mice with NP-CGG/alum elicited a large increase (~100- and 30-fold, respectively) in NIP-specific serum IgG Ab compared to naïve animals (Fig. 33A). NIP-specific serum IgG of CD-RAG mice was ~3-fold less than
elicited in BL/6 mice (Fig. 33A), indicating that CD-RAG animals are capable of mounting a B-cell response to antigen immunization that is proportional to their level of cellular reconstitution.

B cells modify their B-cell receptor in the GC reaction resulting in the preferential expansion of high affinity clones, a process termed affinity maturation (AM) (Jacob, Kassir et al. 1991; Jacob, Kelsoe et al. 1991); while low affinity clones are eliminated by apoptosis (Rajewsky 1996). I measured AM in NP-CGG immunized mice by determining serum IgG reactivity to NIP₅-BSA. While serum from immunized BL/6 mice contained NIP₅-reactive IgG (~30% of NIP₂₅), serum from immunized CD-RAG mice showed no evidence for AM (Fig. 33A).
Figure 33: CD-RAG mice contain MPER-specific serum IgG after immunization

(A) BL/6 (n=5) and CD-RAG (n=5) mice were immunized (ip) with 10 μg NP13-CGG in alum. Serum was harvested at day12 post-immunization. ELISA plates were coated with either NIP19-25-BSA or NIP3-BSA capture antigens. NIP-specific Ab was detected using goat anti-mouse IgG Ab. Purified H33Lγ1 (IgG) mAb was used to generate a standard curve to calculate antigen-specific serum Ab concentration. These results are from 2 independent experiments. (B) BL/6 (n=12-15) and CD-RAG (n=17-20) mice were immunized (ip) 1-2 times with 10 μg MPER peptide in alum. Serum was harvested at d16 post-immunization. ELISA plates were coated with MPER-specific capture antigen and bound Ab was identified using a goat anti-mouse IgG detection reagent. Purified 13H11 mAb was used to generate a standard curve to calculate antigen-specific serum Ab concentration. These results are pooled from 3-4 independent experiments.
Significant differences (*; p < 0.05, **; p < 0.01) between groups were determined by (two-tailed) Student’s t-test.

Prior to immunization, sera from many naive animals did not contain a detectable amount of MPER-reactive IgG Ab; however, some naïve BL/6 mice (4/13) and CD-RAG mice (11/18) contained MPER-specific IgG (~0.5 μg/ml) that was near the limit of detection by ELISA (Fig. 33B). If these low amounts of MPER Ab represent real binding, these data are consistent with CD-RAG mice containing higher levels of serum autoantibody (Holl, Haynes et al. 2010), a potential source of MPER-reactive Ab. After primary immunization of BL/6 mice with MPER peptide, I could detect a significant increase (p<0.01) of MPER-specific serum IgG; however, the average amount of Ab was low (~0.7 μg/ml) (Fig. 33B). After secondary challenge of BL/6 mice with MPER peptide, the level of antigen-specific IgG did not significantly increase (~2-fold) over primary challenge (Fig. 33B), indicating the humoral memory to this MPER peptide was not formed.

In contrast, primary immunization of CD-RAG mice resulted in significantly (p<0.01) more (~3 μg/ml) MPER-specific IgG serum Ab than was formed in immunized BL/6 mice (Fig. 33B). CD-RAG mice that received secondary immunization significantly (p<0.01) increased (~10-fold) the amount of MPER-specific IgG (~25μg/ml) over primary challenge (Fig. 33B). This robust expansion of MPER-specific IgG suggests that CD-RAG mice had formed humoral memory to the MPER peptide during the initial
immunization. These data demonstrate that the humoral immune response to this HIV-1
gp41 MPER peptide antigen can be restored in mice when the constraints of B-cell
tolerance have been relaxed.
5. Identification of a Conserved Cellular Antigen Recognized by 2F5 mAb

The demonstration that HIV-1 gp41 MPER-reactive B cells are lost during B-cell development initiated studies to identify potential self-antigen(s) that could mediate this induction of tolerance. Preliminary studies attempted to identify antigens from whole cell extracts (WCE) of NIH-3T3 cells that were specifically recognized by 2F5 mAb (Liu and Holl, unpublished results). These studies identified several 2F5-specific antigens, including a prominent 50-54 kDa protein, using denaturing/reducing SDS-PAGE and western blot analysis (Liu Y, unpublished data).

The 2F5 mAb recognizes the minimal epitope, ELDKWA, of HIV-1 gp41 MPER (Song, Sun et al. 2009). Pre-treatment of 2F5 mAb with peptide reagents that contain the ELDKWA motif is able to block the neutralization activity of 2F5 mAb in HIV-1 pseudovirus assays (Shen, Parks et al. 2009). The genomes of many organisms have been sequenced in their entirety, allowing for detailed analysis of conserved genetic homology throughout phylogeny. Surprisingly, NCBI blast search (http://www.ncbi.nlm.nih.gov/BLAST) of the human and murine proteome for this ELDKWA amino acid sequence identified only one protein, Kynureninase/ L-kynurenine hydrolase (KYNU; 52 kDa in mice and humans) (Liu Y, unpublished results). Furthermore, this ELDKWA motif present in KYNU is conserved through most vertebrate (Table 6) and invertebrate species (Yang G, unpublished results).
Table 6: Kynureninase from many species contains the HIV-1 2F5 MPER Epitope: ELDKWA

<table>
<thead>
<tr>
<th>Species</th>
<th>Motif</th>
<th>Posn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>... YLE EELDKWAK IAAY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Pan troglodytes</td>
<td>... YLE EELDKWAK IAAY ...</td>
<td>290-285</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>... YLE EELDKWAK MGAY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>... YLE EELDKWAK IGAY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Ailuropoda melanoleuca</td>
<td>... YLE EELDKWAK MGAY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>... YLE EELDKWAK MGAY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Oryctolagus cuniculus</td>
<td>... YLE EELDKWAK MGY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Equus caballus</td>
<td>... YLE EELDKWAK MGGY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Monodelphis domestica</td>
<td>... YLE EELDKWAK MGY ...</td>
<td>90-95</td>
</tr>
<tr>
<td>Omithorhynchus anatinus</td>
<td>... YLE EELDKWAK MGAF ...</td>
<td>90-95</td>
</tr>
</tbody>
</table>

KYNU is an enzyme of the kynurenine metabolic pathway that leads to the production of nicotinamide adenine dinucleotide (NAD+) via the degradation of the essential amino acid tryptophan (Khan, Forouhar et al. 2007). KYNU is a pyridoxal-phosphate dependent enzyme that cleaves Kynurenine into anthranilic acid (De Ravin, Zarembter et al.) and also produces 3-hydroxykynurenine (to produce 3-hydroxyanthranilic acid) and other (3-arylcarbonyl)-alanines (Costantino 2009).

In part, the kynurenine pathway regulates a diverse array of physiological functions. Some research indicates that the kynurenine pathway mediates neurotoxicity associated with several inflammatory neurological diseases, including AIDS dementia complex, Alzheimer’s disease, Huntington’s disease, multiple sclerosis and Parkinson’s disease (Vamos, Pardutz et al. 2009). Elevated concentrations of kynurenic acid are cytotoxic to neonatal neuronal cells in vitro (Eastman and Guilarte 1989) or when
administered to the brain (Nakagami, Saito et al. 1996). 3-hydroxykynurenine and one of its metabolites, quinolinic acid, can induce epilepsy-like symptoms by interacting with NMDA-receptors (Stone and Perkins 1981).

5.1 The 2F5 mAb Binds to Purified KYNU

After recognizing that KYNU carries the ELDKWA nominal peptide epitope of 2F5 mAb, I directly tested the ability of 2F5 to bind recombinant human KYNU and compared this binding to 2F5-mediated recognition of HIV-1 gp41 MPER antigen by ELISA. I used anti-KYNU IgG Ab as my positive control to detect KYNU antigen.

KYNU was readily detected using anti-human KYNU Ab; however, this anti-human KYNU Ab did not bind HIV-1 gp41 MPER antigen (Fig. 34). 2F5 mAb detected both KYNU and MPER antigen at comparable levels (Fig. 34). These data suggest that 2F5 mAb may recognize the ELDKWA motif that is shared by KYNU and the MPER peptide; however, the anti-human KYNU IgG Ab clearly binds to KYNU at epitopes other than the ELDKWA motif.
Figure 34: 2F5 mAb binds to purified KYNU

ELISA plates were coated with either Kynureninase (top) (0.5μg/ml; 4°C, o/n) or DP178-Q16L (bottom) (5μg/ml; 4°C, o/n) antigen. After wash and blocking (PBS w/ 0.5% BSA, 0.1% Tween-20; 2hr, 25°C), 2F5 (●), polyclonal huIgG (○), goat anti-human kynureninase IgG Ab (♦) or no Ab (◊) were added to the ELISA plates (2hrs, 25°C). Bound IgG Ab was detected with either anti-human IgG-HRP or anti-goat IgG-HRP Abs (1hr, 25°C).

5.1.1 MPER peptide and gp140 competitively inhibit 2F5 binding to KYNU

Formally, 2F5 mAb could interact with an epitope(s) of KYNU that is distinct from the ELDKWA motif. I tested whether 2F5 mAb utilized the ELDKWA combining site to bind both KYNU and MPER antigens. I incubated 2F5 mAb with increasing molar amounts of MPER antigens, gp140 and MPER peptide, and determined whether 2F5
bound KYNU and/or gp140 by ELISA. I utilized the R4A peptide to determine if inhibition by MPER antigens was specific.

Figure 35: MPER antigens competitively inhibit 2F5 mAb binding to KYNU

ELISA plates were coated with either KYNU (top) (1μg/ml; 4°C, o/n) or JRFL (bottom) (1μg/ml; 4°C, o/n) antigen. After wash and blocking (PBS w/ 0.5% BSA, 0.1% Tween-20; 2hr, 25°C), 2F5 mAb (250 ng/ml) was mixed with increasing molar concentration of either JRFL (♦), DP178-Q16L (◊) or R4A (□) peptide and added to the ELISA plates (2hrs, 25°C). Bound IgG Ab was detected with anti-human IgG-HRP Ab (1hr, 25°C).

2F5 mAb detected both KYNU and HIV-1 gp140 antigen at similar levels in the absence of competitive antigen (Fig. 35). The addition of an “irrelevant” antigen, R4A peptide, did not reduce the binding of 2F5 mAb to either KYNU or gp140 antigens (Fig.
35). In contrast, the addition of soluble gp140 antigen reduced (up to ~90%) the binding of 2F5 mAb to both KYNU and gp140 (Fig. 35). Similar to gp140, the addition of soluble MPER peptide antigen reduced (up to ~50%) the binding of 2F5 mAb to both KYNU and gp140 (Fig. 35). These data strongly suggest that 2F5 mAb binds to the same ELDKWA motif that is shared by both KYNU and HIV-1 MPER of gp41.

5.2 2F5 Binds the Native, Soluble Form of KYNU

The accessibility of Abs to bind their cognate epitope(s) varies for antigens in their native and denatured conformations. Antigenic epitopes may be linear (continuous) or conformational (discontinuous). Linear epitopes are determined by i) cross-reactivity with anti-protein Ab or ii) induction of Ab that cross-react with the parental protein (Saha and Raghava 2006). A conformational epitope is composed of several disparate sequences that are spatially contiguous. These sequences form a compact accessible region of the protein when properly folded (Saha and Raghava 2006). The linear ELDKWA peptide motif is present in KYNU; however, this linear epitope may be inaccessible to Ab recognition when KYNU is in its native configuration. Presumably, recombinant human KYNU is purchased in its native conformation as this enzyme has specific activity during in vitro assays. In my ELISA assays thus far, adsorption of KYNU to ELISA plates may promote partial and/or complete denaturation of KYNU, resulting in exposure of “internal” epitopes for Ab recognition. I tested
whether 2F5 mAb binds soluble KYNU to determine if the ELDKWA motif is accessible in the native conformation of KYNU.

For soluble KYNU assays, 2F5 mAb or purified human IgG Ab were adsorbed on ELISA plates. KYNU was added to Ab-coated wells at varying concentration. Other wells received biotinylated MPER peptide to ensure that adsorption of 2F5 mAb to ELISA plates did not destroy the capacity for antigen recognition. After washing away excess antigen, the presence of KYNU was detected using the anti-human KYNU IgG Ab. For adsorbed antigen assays, KYNU, MPER peptide or BSA were coated on ELISA plates and assayed as previously described (Section 5.1).

![Figure 36: 2F5 mAb binds soluble KYNU](image)

ELISA plates were coated with 2F5 mAb (♦) or polyclonal huIgG (●) (2µg/ml; 4°C, o/n). After wash and blocking (PBS w/ 0.5% BSA, 0.1% Tween-20; 2hr, 25°C), increasing amounts of human KYNU (0.9ng/ml to 2µg/ml) was added to the ELISA plate. Other wells were directly coated with KYNU (◊) (2µg/ml to 0.9ng/ml; 4°C, o/n). Goat anti-human KYNU IgG Ab was added to detect KYNU (2hrs, 25°C) and anti-goat IgG-HRP was used to detect bound goat Ab (1hr; 25°C).
As observed previously, anti-human KYNU IgG Ab detected adsorbed KYNU in a concentration-dependent manner (Fig. 36). Plate-bound 2F5 mAb captured soluble KYNU (Fig. 36) and biotinylated MPER peptide (data not shown). I could not detect KYNU in wells that were coated with human IgG Ab, indicating that the 2F5-KYNU interaction was specific (Fig. 36). These data demonstrate that 2F5 mAb recognizes soluble KYNU protein and, presumably, does not require KYNU denaturation for Ab binding. These data suggest that soluble KYNU antigen could interact with the BCR of developing B lymphocytes and generate “tolerizing” signals without requiring protein degradation and/or denaturation.

5.3 The MPER-reactive, Non-neutralizing mAb, 13H11, Does Not Bind KYNU

Rarely, HIV-1 gp41 MPER-reactive Abs have been recovered from mice after immunization with MPER peptide/adjuvants (Alam, Scearce et al. 2008); however, these Abs are unable to neutralize primary isolates of HIV-1 (Alam, Scearce et al. 2008). HIV-1 gp41 Abs are divided into two clusters (Xu, Gorny et al. 1991; Gorny and Zolla-Pazner 2000). Cluster I Abs are non-neutralizing and react with the immunodominant region of gp41 (amino acids 579 to 613) while cluster II Abs react with gp41 MPER amino acids 644 to 667. Cluster II Abs can be either non-neutralizing (Gorny and Zolla-Pazner 2000) or neutralizing (e.g. 2F5) (Muster, Steindl et al. 1993).
I tested whether the non-neutralizing cluster II mAb, 13H11, reacted with KYNU antigen and compared this reactivity to 13H11-mediated recognition of HIV-1 gp41 MPER antigen by ELISA. I used anti-KYNU IgG Ab and 2F5 mAb as my positive control to detect KYNU and gp41 MPER antigen, respectively. Instead of using the small linear peptide epitope of gp41 MPER, I screened 2F5 and 13H11 mAbs on HIV-1 gp140 (JRFL) antigen, a construct that contains most of gp120 and gp41 in their native structure.

Figure 37: MPER-reactive, non-neutralizing mAb, 13H11, does not bind KYNU
ELISA plates were coated with either KYNU (top) (1µg/ml; 4°C, o/n) or JRFL (bottom) (1µg/ml; 4°C, o/n) antigen. After wash and blocking (PBS w/ 0.5% BSA, 0.1% Tween-20; 2hr, 25°C), 2F5 (●), 13H11 (○) or goat anti-human KYNU IgG Ab (♦) were added to the ELISA plates (2hrs, 25°C). Bound IgG Ab was detected with either anti-human IgG-HRP, anti-mouse IgG-HRP or anti-goat IgG-HRP Abs (1hr, 25°C).

Again, KYNU was readily detected using anti-human KYNU Ab; however, this anti-human KYNU Ab did not bind HIV-1 gp140 (Fig. 37). 2F5 mAb detected both KYNU and HIV-1 gp140 antigen at comparable levels (Fig. 37). While 13H11 bound gp140 at levels similar to 2F5, I did not detect any binding of 13H11 to KYNU (Fig. 37). I correlate this lack of 13H11 binding to KYNU with the lack of 13H11 binding to NIH-3T3 cell demonstrated previously (Fig. 30A). These data indicate that non-neutralizing Ab responses (e.g. 13H11) elicited by MPER antigen(s) may not cross-react with self antigen(s), suggesting that the development of these MPER-reactive B cells are not influenced by tolerance mechanisms. Conversely, neutralizing Ab responses (e.g. 2F5) elicited by MPER antigen(s) demonstrate cross-reactivity with self antigen(s) and induce mechanisms of B-cell tolerance.
6. Discussion

6.1 In vitro B-cell Development

6.1.1 Advantages of stromal cell-independent B cell culture system

B-lymphocytes arise from CLP present in BM of adult mice (Kondo, Weissman et al. 1997). The BM stromal compartment consists of heterogeneous cell populations that confound the investigation of B-cell development (Dorshkind 1990) by providing distinct developmental niches for B-cell progenitors (Nagasawa 2006; Pereira, An et al. 2009). Many investigators have discovered the utility of in vitro culture techniques to identify factors that are required for mouse and human B-lymphopoiesis (Fluckiger, Sanz et al. 1998; Ray, Stoddart et al. 1998; Hess, Werner et al. 2001). Some B-cell culture systems use sequential conditions that require the addition or removal of factors to promote B-cell maturation (Claudio, Brown et al. 2002; Luo, Maarschalk et al. 2009). I have described a stromal-independent culture system that supports the survival, proliferation and differentiation of virtually all BM B-cell developmental stages. While IL-7 is a key cytokine required for the development and survival of T cells (reviewed in (Bradley, Haynes et al. 2005)), I did not observe the development of any early T-cell compartments (DN-DP) (data not shown) as previous research demonstrated that T-cell development, in vitro, requires stromal cells that express Notch ligands (Schmitt, de Pooter et al. 2004). Compared to stromal-dependant techniques, the clear advantages of
this culture system is i) the ability to easily scale the size of cultures to suit experimental requirements and ii) the ability to control the cytokine composition of culture conditions.

All CD B cells express low levels of CD5 (Fig. 10), raising the possibility that these cultures preferentially support B-1 B cell development from fetal-like progenitors (Dorshkind and Montecino-Rodriguez 2007). We think this possibility is unlikely. First, B-1 B-cell progenitors preferentially develop in the fetus and hematopoietic reconstitution by adult BM inefficiently supports the generation of CD5⁺ B cells (Hayakawa, Hardy et al. 1985; Hardy and Hayakawa 2001). Although small numbers of hematopoietic stem cells (HSC) with fetal characteristics can be found in the BM of very young (≤2 - 4 wks) mice (Bowie, McKnight et al. 2006; Kikuchi and Kondo 2006), the B-cell culture experiments used BM cells from significantly older (>6 wks) BL/6 mice, well after the establishment of adult hematopoiesis.

Second, although B-1 B cells are maintained in adult mice by self-renewal (Hayakawa, Hardy et al. 1986), the potential for self-renewal from the few Ig⁺ cells in IL-7 cultures (Fig. 8) seems unlikely. The very low numbers of Ig⁺ cells following two rounds of IL-7 culture (Table 1) make it improbable that all - or even many - of the maturing Ig⁺ present in BAFF cultures descend from Ig⁺ CD5⁺ B cells. I note that all immature and transitional CD B cells express CD93 (Fig. 8), a marker of developmentally immature B cells but not the progeny of dividing, mature B
lymphocytes (McKearn, Baum et al. 1984). It is, therefore, implausible that self-renewal by B1 cells contributes significantly to the Ig⁺ CD B cell populations.

Finally, CD B cells show substantial evidence of Tdt expression (Fig. 10E) and activity (Table 4); whereas, Tdt expression is low or absent during fetal B-cell development (Feeney 1990; Li, Hayakawa et al. 1993), further indicating that CD B cells do not contain a substantial population of B1 B cells.

6.1.2 Utility towards investigation of the autoimmune repertoire

During development, self-reactive B cells are tolerized by apoptosis, anergy, or receptor editing (reviewed by Goodnow, Cyster et al. 1995). These tolerizing processes have been examined in various transgenic mouse lines that express BCR for authentic (Nemazee and Burki 1989; Erikson, Radic et al. 1991) or neo-self-antigens (Hartley, Crosbie et al. 1991). These experimental models defined immature and T1 B cells as the targets of tolerizing apoptotic signals by developmental blockade (Hartley, Cooke et al. 1993) and identified anergy (Adams, Basten et al. 1990) and receptor editing (Gay, Saunders et al. 1993; Tiegs, Russell et al. 1993) by characterization of B-cell populations that escape apoptotic deletion. This culture system may provide an experimental platform to systematically re-introduce components present in BM (e.g. cytokines, cell populations) and directly measure their influence on B-cell tolerance. Some in vitro experiments have been performed to investigate mechanisms of B-cell tolerance using HEL antigen(s) and IL-7⁺ BM cultures (Tze, Baness et al. 2000; Tze, Hippen et al. 2003;
HEL-specific BCR Tg+ B cells were induced to undergo “de-differentiation” and “receptor editing”, defined as the loss of surface IgM, increased expression of genes associated with earlier (pro- and pre-B) developmental stages and the initiation of endogenous LC rearrangement (Tze, Baness et al. 2000; Tze, Hippen et al. 2003; Tze, Schram et al. 2005).

However, it is difficult to explain how a transgenic BCR can undergo “receptor editing”, which is defined as the cis-replacement of a functional primary LC rearrangement. It is reasonable to assume that stimulation with HEL antigen results in BCR internalization leading to the reduction of PI3K activity and the induction of Foxo1-regulated gene expression, such as Bim and RAG1/2 (Srinivasan, Sasaki et al. 2009). Induction of Bim, a pro-apoptotic member of the Bcl-2 family, would promote the death of cells unless surface Ab expression is restored to increase PI3K activity, thereby decreasing Foxo1 transcriptional activity and Bim expression (Srinivasan, Sasaki et al. 2009). The induction of RAG1/2 expression after the loss of BCR expression may represent an attempt to restore surface Ab expression to promote survival; however, this process does not require the “recognition” of self-antigen as a driving force for secondary LC gene replacement, only that surface Ab density be decreased.

Clearly, factors that promote B-cell survival, such as Bcl-2 over-expression, alter B-cell tolerance and result in autoimmunity (Nisitani, Tsubata et al. 1993; Lang, Arnold et al. 1997). BAFF Tg mice contain high levels of rheumatoid factors, circulating immune
complexes, anti-DNA Abs and immunoglobulin deposition in the kidneys (Mackay, Woodcock et al. 1999). These cultures support B-cell maturation with BAFF at 20ng/ml, a concentration that is ~5-10 times greater than the BL/6 serum BAFF concentration (Cain D, unpublished data). It remains unclear whether elevated BAFF-R signaling is required, in vitro, for self-reactive B-cell survival or that specific “death signals” are absent.

Apoptotic thymocytes marked for clearance are rapidly removed by the mononuclear phagocyte system (Surh and Sprent 1994). The possibility remains that autoreactive B-cell development would be abolished in the presence of phagocytes. However, autoreactive B cells survive in vivo and facilitate serum Ab reconstitution after transfer to RAG1−/− mice (Fig. 17).

Serum BAFF levels were measured in B-lymphopenic animals, RAG-1−/− and μMT mice, and we determined the concentration to be ~400-500ng/ml, roughly 100-fold higher than normal mice (D. Cain, data not shown). Would pre-treatment of RAG1−/− recipient animals with TACI-Ig, thereby lowering systemic BAFF availability, favor B-cell reconstitution of non-autoreactive B cells at the expense of autoreactive clones? Future experiments should also determine i) any correlation between the concentration of BAFF in reconstituted RAG−/− and the persistence of autoantibody over time, and ii) whether enhanced GC responses are controlled by BAFF concentration. These observations predict that B-cell activity and autoimmunity could be modulated by controlling BAFF availability in the organism and, in fact, BAFF is a current target for
therapeutic intervention in autoimmune disorders (Pelletier, Thompson et al. 2003; Pranzatelli, Tate et al. 2008).

Recently, B-cell tolerance in humans has been investigated by expressing IgH and IgL rearrangements from single immature, transitional, and mature B cells and determining the frequencies at which these Ab reacted with self-antigens (Wardemann, Yurasov et al. 2003; Wardemann, Hammersen et al. 2004). This in vitro culture technique allows for the systematic analysis of autoreactive B-cells removed from the primary repertoire by recovering cells reactive to common autoantigens and identifying their V_H and V_κ/λ pairs. In humans, autoreactive Ab frequencies decline with increasing developmental maturity by virtue of apoptotic loss and receptor editing (Wardemann, Yurasov et al. 2003; Wardemann, Hammersen et al. 2004), even when cells were recovered from peripheral sites (Meffre, Schaefer et al. 2004; Tsuiji, Yurasov et al. 2006). Similarly, analysis of 3H9 Tg+ mouse BM cells revealed that IgM+ B cells did not co-label with an anti-idotypic 3H9 Ab, suggesting that 3H9 B cell-specificities were lost no later than the immature/transitional stage(s) (Gay, Saunders et al. 1993). Furthermore, Ig heavy chain gene replacement occurs in some 3H9 HC-KI B cells as a mechanism of receptor editing (Chen, Nagy et al. 1995). Since DNA-reactive 3H9+ B cells were readily recovered in our culture system, mechanisms that promote tolerance of developing lymphocytes can be investigated using this model by the manipulation of antigens, cell populations and/or survival factors.
6.2 Investigation of the Tolerance Hypothesis

6.2.1 Utility of in vitro culture system towards investigation of the tolerance hypothesis

The inability to mount robust B-cell responses to some MPER antigens appears to be phylogenetically conserved from rodents to humans (Graham 2002; Letvin, Barouch et al. 2002). Recent work has characterized the Ab response to HIV-1 gp41 in patients whose serum contains moderate-to-high virus neutralizing activity (Pietzsch, Scheid et al.). Interestingly, these mAbs were unable to compete for the binding of 2F5 and 4E10 Ab to their respective MPER epitopes (Pietzsch, Scheid et al.), illustrating the rarity of humoral responses to the MPER region of the gp41 envelope antigen. Haynes et al. has reported that these rare Abs (2F5 and 4E10) are, in fact, poly-reactive and cross-react with highly conserved self antigens (Haynes, Fleming et al. 2005). The “tolerance hypothesis” represents an explanation for this state of non-responsiveness to gp41 MPER antigen(s).

The “tolerance hypothesis” predicts B-cell compartments that are enriched for self-reactive lymphocytes might contain elevated frequencies of HIV-1 MPER-reactive cells. The MZ B-cell compartment is a natural reservoir of auto-reactive B cells in mice (reviewed in (Lopes-Carvalho and Kearney 2004)), suggesting that MPER-reactive B cells may accumulate in the MZ B-cell compartment. Our data indicate that the MZ B-cell compartment was not enriched for MPER-tetramer+ B cells (Fig. 25), supporting the
conclusion that MPER-reactive cells are efficiently deleted or undergo receptor editing as previously demonstrated (Verkoczy, Diaz et al.).

Furthermore, our data indicate that B cells reactive to this linear peptide epitope of the MPER can be recovered in mice that contain frequent autoreactive B cells. In the infrequent instance that MPER-specific Ab responses are formed against HIV-1 gp41 in humans, it is only after months (10-20) of chronic HIV-1 infection, antigen exposure and induction of autoantibody (Shen, Parks et al. 2009), further indicating that the relaxation of B-cell tolerance may promote the induction of MPER Ab responses. However, the induction of MPER Ab responses after viral reservoirs have been established do not control viral load (Shen, Parks et al. 2009), since humoral immunity is not effective for clearing intracellular pathogens.

Unfortunately, the origin of these B-cell clones, 2F5 and 4E10, cannot be traced as they were created from pooled samples of peripheral blood from HIV-infected individuals. Because the frequency of autoreactive Ab declines with increasing developmental maturity (Wardemann, Yurasov et al. 2003; Wardemann, Hammersen et al. 2004), the frequency of MPER-reactive human B cells will sequentially decrease as B cells mature. Did the donor(s) that formed 2F5 and 4E10 have a pre-disposition to autoimmune diseases? Alternatively, it is possible that this donor(s) contains mutation(s) within the self-antigen(s), potentially KYNU, that 2F5 and/or 4E10 recognizes, effectively removing the constraints of B-cell tolerance on this Ab repertoire.
Currently, the study of MPER-reactive Ab has yielded their physical structure, neutralizing capacity and antigen reactivity. The 2F5 and 4E10 Ab contain long, hydrophobic complementarity-determining region-3 (CDR3) structures that are similar to many human Ab shown to be deleted in the BM (Meffre, Schaefer et al. 2004). In addition to the MPER region of HIV-1 gp41, 2F5 and 4E10 Abs show significant affinity to highly conserved self-antigens, such as cardiolipin and phosphatidylserine (PS). Previously, investigators have shown that anti-PS-reactive B-cell clones are deleted in BM due to receptor editing (Li, Jiang et al. 2003). While neutralizing Ab to the MPER are poorly formed after infection or immunization, HIV-reactive non-neutralizing Ab fair much better, apparently to the detriment of the host. Alam et al. recently reported that non-neutralizing anti-gp41 Ab can mask the binding sites of protective, neutralizing anti-HIV-1 Ab (Alam, Scearce et al. 2008). Combined, these data indicate that a narrow balance must be found between measures required to elicit the appropriate humoral response and the potential for equally harmful autoimmunity.

Normally, the GC reaction must balance AM and the elimination of newly formed self-reactive B cells that arise via SHM (Han, Zheng et al. 1995). Therefore, it is somewhat surprising that both 2F5 and 4E10 exhibit the hallmarks of maturing through the GC reaction (AM) yet retain significant reactivity to phylogenetically conserved self-antigens. It is possible that B cells rarely survive the pressures of immune tolerance during the mutation of Ab that elicits both autoreactivity and broadly neutralizing HIV-
1 activity. We have provided a direct test of the notion that MPER peptides mimic self antigens and that B cells reactive to these epitopes are tolerized in the BM transitional compartments. It would appear that in mice the mature peripheral B-cell pool is purged of MPER-reactive cells that would be recruited to the GC reaction upon immunization. This B-cell transfer model may work simply by increasing the frequency of cells that are available to initiate this difficult path to protection. Regardless of whether the immune system has exerted selective pressure on HIV-1 or this antigen mimicry is a random phenomenon, future vaccine strategies need to account for these observations to promote robust B-cell responses against the HIV-1.

### 6.3 Implications of Identifying KYNU as a Self-ligand of 2F5 mAb

Combined, my data provides strong evidence that immunological tolerance prevents the formation of 2F5-like Ab. The self-antigen(s) that mediates the selection of 2F5+ B-cell development has not been identified; in contrast, previous studies utilized BCRs of known specificity to self-antigens (MHC and DNA) to demonstrate that expression of autoantibody resulted in the induction of immunological tolerance. A priori, self-antigens must be present in humans and mice that contain the HIV-1 2F5 epitope. I and my colleagues have identified one candidate molecule, KYNU, which contains the core ELDKWA epitope. 2F5 mAb binds to KYNU at comparable levels to HIV-1 MPER reagents and these MPER reagents can be used to inhibit 2F5-binding to KYNU, suggesting that 2F5 mAb binds an epitope that is shared between KYNU and
gp41 MPER. The identification of KYNU as a potential autoantigen permits new avenues of experimentation to i) confirm that 2F5+ B-cell development is under control of immunological tolerance, ii) create novel mouse models to identify populations of B cells that are progenitors to 2F5-like B cells, and iii) determine whether patients that support 2F5-like Ab responses carry KYNU mutations that might reduce tolerance to the ELDKWA motif.

6.3.1 Generation of KYNU-deficient mice

The 3-83 transgenic mouse expresses an Ab that reacts with some (Kb and Kk) MHC class I molecules, but not others (Kd) (Ozato, Mayer et al. 1980). 3-83+ B cells develop normally on an H-2d background (Nemazee and Burki 1989; Russell, Dembic et al. 1991); however, when 3-83 Tg mice are genetically crossed to mice that express MHC class I products which can act as a ligand for 3-83, B-cell tolerance occurs (central or peripheral) and is dependent upon the site of initial antigen exposure (Russell, Dembic et al. 1991). These data demonstrate that self-reactive B cells can develop in mice that lack their cognate autoantigen. The creation of KYNU-deficient mice could provide an experimental model to directly test whether the observed defect in 2F5+ B-cell development is due to reactivity with KYNU antigen.

Clearly, B-cell development is impaired in BM of 2F5 HC-KI mice (Verkoczy, Diaz et al.) but their development can be recovered by culturing 2F5 HC-KI BM cell in vitro (Fig. 27 and 30), presumably absent many self-antigens. I hypothesize that genetic
crossing of 2F5 HC-KI alleles to the KYNU−/+ background would result in enhanced 2F5 HC-KI B-cell development, thereby confirming that KYNU is the self-antigen that mediates 2F5+ B-cell tolerance. If enhanced 2F5 HC-KI B-cell development was not observed, other candidate self-antigens that mediate this B-cell tolerance would have to be explored. In fact, 2F5 mAb reacts with several antigens present in WCE of NIH-3T3 cells (Y. Li and Y. Liu, unpublished results), indicating that complete rescue of 2F5+ B-cell development may be unlikely.

Beyond advancing our knowledge of B-cell tolerance, KYNU−/+ mice may provide an experimental model to identify the early compartments of B lymphocytes that generate Ab specific for the 2F5 MPER epitope. If 2F5+ B-cell tolerance was relaxed, MPER-reactive B cells may be identified and isolated using MPER tetramer reagents and used to generate hybridoma cell lines. These studies would identify which V(D)J gene segments of the IgH and Igκ/λ loci are preferentially utilized, if any, to form MPER-reactive Abs.

Presuming that 2F5+ B-cell development was enhanced in the absence of KYNU, KYNU−/+ mice would provide a valuable experimental model to test whether novel antigen/adjuvant reagents elicit robust amounts of MPER reactive Ab. This would represent a significant advance in HIV-1 vaccine biology as there has been little success at eliciting anti-MPER responses (Eckhart, Raffelsberger et al. 1996; Coeffier, Clement et al. 2000; Derby, Kraft et al. 2006). These experiments should include immunization at
multiple anatomical locations to determine which site(s) results in i) high titer Ab
formation, ii) formation of humoral memory, and iii) differences in mucosal and
systemic Ab localization.

6.3.2 Natural mutations of KYNU

In rare instances, spontaneous genetic mutation has resulted in almost complete
protection of individuals against HIV-1 infection. CCR5 is a human beta chemokine
receptor that is encoded by the CCR5 gene (Samson, Labbe et al. 1996). Two chemokine
receptors, CCR5 and CXCR4, function as viral coreceptors, but CCR5 appears to be the
dominate coreceptor for viral transmission during natural infection (Anderson and
Akkina 2007). In humans, the CCR5 gene is located on the short (p) arm of chromosome
3 (NCBI gene map). Some individuals have inherited the “Delta 32” mutation resulting
in the deletion of the CCR5 gene and homozygous carriers of this mutation are resistant
to HIV-1 infection (Samson, Libert et al. 1996). We hypothesize that rare individuals may
contain natural mutations in their KYNU genetic loci that permits the formation of
protective MPER Ab responses.

Rarely, infection with HIV-1 does elicit 2F5-like Ab responses in patients (Shen,
Parks et al. 2009). I propose that the KYNU genetic loci in some portion of these patients
may contain mutation in the conserved 2F5 ELDKWA motif, thereby removing the
selective pressure on 2F5-like B-cell development. Some individuals, long term non-
progressors (LTNP), are persistently infected with HIV-1, but do not develop signs of
disease for many (>12) years (Cao, Qin et al. 1995). Individual LTNP can be distinguished based upon control of viral load and the elicitation of specific immune responses against HIV-1 (Easterbrook 1994). Some LTNP's generate a strong and broad set of HIV-specific humoral and cell-mediated responses that delay the progression to AIDS (Cao, Qin et al. 1995). Does mutation in self-antigens (e.g. the ELDKWA portion of KYNU) contribute to the capacity of an individual to become a LTNP?

6.4 Concluding Remarks

B-cell development in mice and humans share many fundamental aspects that promote the generation of a diverse repertoire of antigen-specific lymphocytes. Mechanisms of B-cell tolerance that limit autoimmunity can purge up to half of the initial BCR repertoire and, as a consequence, remove some portion of foreign antigen-reactive lymphocytes. Obviously, many microbial antigens are not expressed by host organisms and, consequently, highly immunogenic. On the other hand, antigenic similarities do exist as evidenced by the capacity of some infections to elicit autoimmunity. Many mechanisms are used by pathogens to subvert immune recognition, but complete understanding of how HIV-mediated B-cell dysregulation occurs in vivo requires further investigation. The “tolerance hypothesis” as a mechanism to subvert neutralizing B-cell responses is supported by i) the demonstration that broadly neutralizing HIV-1 Abs exhibit cross-reactivity to self-antigens, ii) the recovery of MPER IgG Ab responses under conditions of relaxed B-cell tolerance, iii) the loss of
2F5 HC-KI and MPER-tetramer+ B-cell development in the BM, and *iv* a lower frequency of HIV-1 infection in some autoimmune patients.

The identification of novel self-antigens that influence humoral immunity to microbial pathogens is just beginning. Interestingly, the frequency of pathogens that utilize host-antigen mimicry as a form of immune evasion is difficult to estimate. Many pathogenic organisms (*e.g.* Influenza) are eliminated by immune responses directed against unique foreign antigens, thereby masking the ineffective immune responses directed against the shared, conserved antigen structures. Is this phenomenon unique to HIV-1 infection? The rarity of B cells that react to highly conserved portions of Influenza hemagglutinin suggests that viral mimicry of self-antigens is more wide-spread than previously appreciated.
References


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Biography

Thomas Matthew Holl was born in Watertown, New York USA on January 26th, 1976. Matt began his academic career at Jefferson County Community College in Watertown, New York where he studied the field of Criminal Justice. Matt obtained his Bachelors’ degree in Microbiology at the University of Pittsburgh in Pittsburgh, Pennsylvania in May 1999. Since graduating from Pittsburgh, Matt has co-authored research publications in the field of Immunology and has received a Young/Early Career Investigator Recognition award from the Bill and Melinda Gates Foundation in April 2010.


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