INTRINSIC MECHANISMS OF REGULATION OF ANTI-Sm B CELL ANERGY

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ABSTRACT

RAMIRO E. DIZ: Intrinsic mechanisms of regulation of anti-Sm B cell Anergy
(Under the direction of Stephen H. Clarke, Ph.D)

One of the fundamental properties of the immune system is its capacity to avoid autoimmune diseases. The mechanisms regulating this process, known as self-tolerance, are many and varied. Self-reactive B cells that are not deleted in the bone marrow are regulated by anergy in the periphery. This mechanism renders self-reactive B cells unresponsive to activating signals. The use of immunoglobulin transgenic mouse models specific for self or neo-self antigens have indicated that anergy encompasses a broad spectrum of complex and multi-factorial states of cell unresponsiveness. The work presented here addresses the regulatory complexity of anergy in anti-Sm B cells.

In this study using a high affinity anti-Sm mouse model, I have identified several mechanisms for anti-Sm B cell anergy. In addition, I have shown that the mechanisms of anergy differ depending on affinity of the B cell receptor (BCR) for Sm and subset identity. High affinity anti-Sm B cells, unlike low affinity anti-Sm B cells, are unable to survive in the presence of competitor B cells. This defect correlates with a BAFF non-responsiveness and increased cell death. Both high and low affinity anti-Sm follicular B cells express signaling competent BCRs, although BCR ligation induces rapid cell death, which correlates with an imbalance of pro and anti-apoptotic protein expression. In contrast, the BCRs expressed by

marginal zone B cells from the high affinity mouse model are defective in signaling suggesting an uncoupling of the signalsome from the BCR on these cells.

Activation by toll-like receptors (TLRs) is also defective in anergic B cells. I find that TLR-induced activation of anergic anti-Sm B cells is regulated by two mechanisms; activation induced cell death and a block in Plasma cell (PC) differentiation. The block in PC differentiation occurs prior to the expression of PC-specific genes. The importance of each mechanism to the regulation of B cells of different subset varies, with the block in PC differentiation not occurring efficiently until after B cells reach maturity.

Together, the data presented provide evidence of the complexity and variety of anti-Sm B cells regulation and provide insight to the mechanisms of B cell anergy.

DEDICATIONS

I would like to dedicate this work to my parents, Martha & Adolfo. They gave me roots and wings and the desire to fly and follow my passions. To them I owe everything I am and everything I will be.

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"Los hermanos sean unidos, esa es la ley primera"

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LIST OF ABBREVIATIONS

Abs	antibodies
Ab	antibody
ASC	antibody secreting cell
Ag	antigen
APC	antigen presenting cell
BAFF-R	B cell activating factor receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
Blimp-1	B lymphocyte induced maturation protein 1
BAFF	B cell activating factor belonging to the TNF family
BM	bone marrow
dsDNA	double-stranded DNA
JNK	c-Jun NH_2-terminal kinase
CIITA	class II transactivator
ERK	extracellular signal-regulated kinase
Fo	follicular (B cell)
GC	germinal center
H	heavy
HEL	hen egg lysozyme
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
L	light

LPS	lipopolysaccharide
MHC	major histocompatibility complex
MZ	marginal zone
M.F.I.	median fluorescence intensity
MAPK	mitogen activated protein kinase
NF-κB	nuclear factor κB
PAMP	pattern associated recognition motif
PALS	periarterial lymphoid sheath
PLC	phospholipase C
PI3K	phosphotidylinositol 3 kinase
PC	plasma cell
pre-BCR	pre-B cell Receptor
RAG	recombination activating genes
sHEL	soluble HEL
sIgM	surface IgM
snRNP	small nuclear ribonuclear protein
Sm	Smith
SLE	systemic lupus erythematosus
ssDNA	single-stranded DNA
TD	T-dependent
TI	T-independent
TLR	toll-like receptor
Tr	transitional

Tg	transgenic
TACI	transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor
TNF	tumor necrosis factor
UPR	unfolded protein response
XBP-1	X-box binding protein 1

CHAPTER 1

INTRODUCTION

The immune system is a complex myriad of cells, organs and tissues working as a dynamic network with the purpose of protecting the body from infection. Understanding the mechanisms through which the immune system distinguishes self from foreign antigens is one of the most confounding and fundamental problems of immunology. This phenomenon, known as self-tolerance, is paramount for an efficient defense against pathogens and prevention of autoimmune diseases. Over the past 20 years, the introduction of genetically modified transgenic mice carrying rearranged B cell receptors has provided insight into the normal physiology and mechanisms of tolerance regulating B cells. Immunoglobulin (Ig) Tg mice have been used to determine mechanisms of tolerance such as central and peripheral deletion, anergy and follicular exclusion, among others. Nonetheless, more and more evidence shows that these mechanisms represent only a fraction of those that are involved in B cell regulation, and that these and other mechanisms vary greatly depending on the characteristics of the antigen and the affinity of the BCR. Therefore, it is of great importance to understand which mechanisms are invoked to induce tolerance and prevent autoimmunity. The work presented in this thesis is an initial characterization of a new Tg mouse model with a high BCR affinity for Smith (Sm) antigen, in which several novel mechanisms of tolerance have been determined.

A. B cell development and maturation

B cell development is initiated in the bone marrow. Following several distinct stages of differentiation in which cells are subjected to various means of regulation or checkpoints, a pool of B cells expressing a diverse BCR repertoire is generated. These cells then migrate to the spleen where, after passing through more checkpoints, they undergo final maturation.

1. Early B cell development

The development of B cells begins in the bone marrow, where haematopoietic stem cells give rise to a B and T cell precursor, or common lymphoid progenitor [1]. The earliest B cell committed precursor is differentiated by the surface expression of B220; the B cell specific splice variant of CD45 [2]. Several stages of differentiation from this early B cell progenitor to the immature B cell expressing surface IgM have been described in detail [1, 3]. Early stages of B cell development have a high dependency on cytokines, such as FLT3 ligand and IL-7, for survival and proliferation [4]. As B cells progress through the different stages, they become less dependent on cytokines and more dependent on signaling, induced via the pre-BCR and BCR complex, for their survival [3, 5, 6, 7].

During early B cell development, multiple V_H, D_H and J_H Ig gene segments are randomly rearranged and assembled in a stepwise manner to generate the final IgM [8]. With the help of recombinases RAG1 and RAG2 and other enzymes, D_H to J_H rearrangement is initiated at the pro-B cell stage, followed by V_H to D_HJ_H rearrangement which generates the IgH or μH chain. The newly formed μH chain associates with a surrogate light chain formed by VpreB and λ5 proteins, and together they are shuttled to the cell surface in association with the signaling proteins Igα and Igβ as the pre-BCR at the large pre-B cell stage [9, 10, 11, 12].

Cells with a productive pre-BCR undergo rapid clonal expansion [13, 14]. This is a critical check point in early development where up to 75 percent of large pre-B cells are eliminated due to failure of the Ig chain to associate with the surrogate L chain. Lack of μH chain, Igα or λ5 blocks B cell development at this stage, demonstrating the importance of the pre-BCR and signals induced by it for further B cell development [3, 5, 6, 15, 16].

In the next stage, (small pre-B) B cells re-express RAG genes and undergo IgL chain rearrangement, V_L to J_L [15, 17]. After productive rearrangement, the new IgL chain associates with the IgH chain and is expressed on the surface of immature B cells. At this stage, B cells undergo another major checkpoint. Cells lacking a functional light chain rearrangement, arrest and undergo cell death, as demonstrated by mice deficient in both κ and λ light chains [18, 19, 20]. At this stage, central tolerance mechanisms test the reactivity of the BCR for self-antigens, and cells that respond positively are eliminated by negative selection [5, 15, 21]. Other mechanisms of regulation occur at this stage, and these will be discussed below. The immature B cells which are not eliminated migrate to the periphery, more specifically to the spleen, and undergo further maturation.

2. Peripheral B cell maturation

2.1 Transitional B cells.

Newly formed immature B cells emigrate from the bone marrow to the spleen where they undergo further selection and maturation to long lived follicular (Fo), marginal zone (MZ) or B1 B cells. These cells are termed transitional (Tr) B cells and are distinguished by surface markers such as IgM^{hi}, IgD^{lo}, $CD23^-$, $CD21^-$ and $AA4.1^+$ ( ). Tr B cells have a short

half-life and most will be eliminated by negative selection – only 20-50% will enter the pool of mature B cells [22, 23].

Several transitional stages prior to full B cell maturation have been described. Originally, Carsetti described these transitional cells and subdivided them into two groups: T1 and T2; the latter expressing high levels of CD21 and CD23 on their cell surface [24, 25]. Later studies have questioned the validity of these subpopulations on the ability to properly distinguish T2 from MZ B cells [25, 26].

More recent work by Allman, determined that transitional cells expressed CD93 (C1qRp), which is recognized by both 493 and AA4.1 monoclonal antibodies [23, 27]. Using this new marker together with CD21, CD23 and IgM, three distinct Tr subpopulations where determined (T1, T2 and T3) which are easily separated from MZ and Fo B cells. All three Tr B cell subpopulations are unable to proliferate in response to anti-IgM and undergo rapid cell death [23, 28]. Here I will refer to Tr B cells as one population defined as $CD23^-$ and $CD21^-$.

2.2 Mature B cells.

Long live mature B cells that derive from Tr cells are further divided into Fo, MZ and B1 subsets [1]. The cells of these subsets are distinguished based on surface markers, anatomical localization and function [29]. Fo B cells have a half-life of 6 to 10 weeks, make up to 70 to 80 percent of all B cells present in the spleen and are also found in lymph nodes [23, 25, 30, 31]. Anatomically, they are found in the lymphoid follicles of the spleen and lymph nodes. They are generally described as IgM^{lo}, IgD^{hi} $B220^{hi}$, $CD21^{int}$, $CD23^{hi}$, $CD93^-$, $CD9^-$ and $CD1d^{lo}$ [32, 33]. Fo B cells are able to respond to T-cell dependent and T-cell independent antigen-driven activation and are involved in primary and secondary responses. Even so, they

are responsible for most of the IgG antibodies produced following an immune response [34]. These cells also undergo somatic hypermutation and affinity maturation in germinal center reactions [35, 36, 37].

MZ B cells make up 5 to 10 percent of B cells in the spleen. They are also long live and self-replenishing. Evidence indicates that recruitment and selection of MZ-B cells occurs on the basis of positive selection via the BCR and likely driven by self antigen [38, 39]. These cells are found in the external boundary of the follicle and the marginal sinus. Blood enters the spleen, exits circulation through the marginal sinus and flows through the marginal zone [40]. Therefore MZ B cells are in a privileged position to detect blood-born pathogens or particulate antigens. It is not surprising then that MZ B cells are the primary line of defense against these pathogens. Upon encountering antigen, MZ B cells are rapidly activated and induce secretion of IgM [29, 34, 38, 41, 42, 43]. MZ B cells express higher levels of complement receptors, MHC molecules and toll-like receptors than Fo B cells, which might explain their ability to respond quickly and allowing them to link the innate and adaptive immune systems [44]. They are also important in Ag presentation to T cells and can act as potent T cell activators [45]. Although MZ B cells are traditionally considered to be important only in T-independent activation, it has been demonstrated in recent years that these cells are also important in T-dependent activation, and that they can undergo class switching and somatic hypermutation [42, 46, 47].

B-1 B cells usually are divided into B1a and B1b subpopulations based on the expression of CD5 on the surface of B1a cells. Together with MZ B cells, B1 B cells are responsible for the production of ‘natural’ antibodies and are recruited and activated early in the response, as another component of the first line of defense to T-independent type 2

antigens. They can be found in small numbers in the spleen of wild type mice, but are mainly found in the peritoneal cavity [48]. They are long lived cells and are able to replenish themselves. Most of these cells have BCRs that grant them autoreactivity, and a preferential usage of VH and VL genes which, taken together, suggest these cells have been positively selected. B1 B cells are produced early in prenatal development from fetal liver stem cells but can also be produced in adult bone marrow [49, 50, 51].

2.3. Fo vs MZ vs B1 B cell fate.

B cell development and maturation is regulated by many intrinsic and extrinsic factors. Although I will not describe every factor here, I will attempt to summarize those most important to the process.

The lack of pre-BCR has already been mentioned as a factor for B cell development in the BM. A lack of the pre-BCR, and therefore a lack of signaling induced by it, block further development of the B cells at the pro-B cell stage [16]. B cells that emigrate to the spleen express a mature rearranged BCR. Work from the Rajewsky laboratory has demonstrated that the lack of a mature BCR, or its subsequent ability to signal (Igα inactivation), was sufficient to induce a loss of peripheral mature Fo B cells; MZ B cells were only slightly affected [52, 53]. These data support the hypothesis that B cells require a "tonic" BCR induced signal for survival. To date, the nature of and the ligand involved in this "tonic" signal remains unresolved.

BCR signaling is not only important in the maintenance of the peripheral mature B cell pool, but also influences the fate of B cells, as it is responsible for dictating into which mature pool the cells will enter [43]. Multiple lines of evidence from different groups using

transgenic and knock-out mice support a hypothesis that BCR signal strength determines or influences whether a B cell will enter the Fo, MZ or B1 pool of cells.

Several pieces of evidence using Tg mice over-expressing positive regulators of BCR signaling (CD19), or eliminating them (CD19, p85 and p110 subunits of PI3K), or lacking negative regulators of BCR signaling (SHP-1, CD22, CD72) suggest that strong BCR signals result in the production of B1 and MZ cells, while low BCR signals generate Fo B cells [38, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66]. More evidence to this effect was recently provided through the use of a mouse expressing LMP2A in varied amounts. LMP2A (a component of the Epstain-Barr Virus) mimics the BCR and induces signaling. B cells that expressed higher levels of LMP2A became B1 cells while cells with lower levels became Fo and MZ B cells [67, 68].

Nonetheless, others have argued that low BCR signaling favors MZ B cell production while stronger signals favors Fo B cell production [33]. The complexity of BCR signaling makes these results difficult to reconcile into a simple model of maturation based solely on BCR signal strength. It is more likely that a combination of the quality and quantity of the signal, the form of the antigen as well as other extrinsic factors, contribute to produce the various cell types. For example, several transcription factors have been shown to be important in the final fate of maturation of B cells. Aiolos deficient mice have strong BCR signal strength but reduced MZ B cells [69]. Similar defects are observed in mice defective in Notch-2 [70, 71]. In addition, extrinsic factors such as cytokines or secreted ligands of the TNF family (such as BAFF; described below) can also alter peripheral B cell maturation. Therefore, peripheral B cell development is a complex multifactor process in which normal B cell development might be shifted to favor one mature subset over another in the presence of a shortage or excess of certain components.

2.4 BAFF and peripheral B cell maturation.

Several independent groups cloned a ligand, belonging to the TNF family, which promoted B cell survival and was subsequently named BAFF (B cell activating factor belonging to the TNF family) [72]. Several other names have been used (TALL-1, THANK, BLyS, etc), but to avoid confusion BAFF will used here [73, 74]. BAFF is produced and secreted by many cell types including monocytes, macrophages, DCs and neutrophils, but it was recently shown that the major source of BAFF is radiation-resistant stromal cells [41, 73, 75, 76].

There are three BAFF receptors: B cell maturation antigen (BCMA), transmembrane activator and cAML interactor (TACI) and BAFF-R (also known as BR3). In addition to BAFF, there exists another ligand known as APRIL (a proliferation-inducing ligand) that binds only to BCMA and TACI. The expression pattern of all three BAFF receptors is highly regulated in B cell development [77]. BCMA is expressed mainly in plasma cells (PC) and pre-PCs and plays a role in PC survival [78, 79, 80]. TACI is expressed in both Fo and MZ B cells. TACI deficient mice have an increased number of peripheral B cells, which lead to the conclusion that TACI acts as a negative regulator of BAFF signaling [81]. Nonetheless, TACI might play a positive role in B cell activation as demonstrated by a defective response to T-independent type 2 antigens [82]. The major evidence of the role BAFF plays as a survival factor for B cells was demonstrated by BAFF or BAFF-R deficient mice in which B cell maturation is impaired following T1 stage of B cell development [83, 84]. Furthermore, excess BAFF promotes enhanced Fo and MZ B cell numbers [85].

The signaling pathways induced by BAFF that lead to B cell survival have been complicated by the fact that most cells express multiple BAFF receptors. Several pathways

known to be activated by BAFF include: Akt, ERK, Pim2, NF-κB1 and 2, etc [86, 87, 88, 89]. Woodland et al. have recently proposed a model in which Akt signaling inhibits Bim upregulation (a pro-apoptotic protein) by inhibiting Foxo3a and by increasing the expression of Mcl-1 which blocks Bim expression as well [77]. In addition, ERK activation by BAFF has been proposed to inhibit Bim [90]. The NF-κB pathways activated by BAFF induce upregulation of Bcl-x_L, A1 and Bcl2, which act as pro-survival molecules inhibiting apoptosis [91]. In section B.3 we will further discuss the role of BAFF in B cell activation.

B. B cell activation

B cell activation results in antibody production and secretion that is necessary for an efficient immune response against foreign pathogens. B cells sense the environment via their BCRs, as well as through other receptors such as toll-like receptors. Ligation of these receptors induces a complex signaling pattern that activates transcription factors, induces gene expression changes and ultimately results in B cell proliferation, antibody secretion or cell death. The context in which the B cells are activated, as well as the balance between the signaling pathways, dictate the final outcome of activation. In this section, I will review the signaling pathways induced by BCR, TLRs and BAFF ligation, and their effects on the biology of B cells.

1. BCR induced activation

1.1. BCR induced signaling pathways

The B cell receptor is composed of a membrane Ig associated non-covalently with the Igα/Igβ heterodimers containing an immunoreceptor tyrosine-based activation motif

(ITAM). Antigen-induced aggregation of the BCR complex results in phosphorylation of the ITAMs, and recruitment and activation of protein-tyrosine kinases (PTKs), which include Lyn, Btk and Syk, and leads to the formation of the BCR signalosome [92]. BCR ligation also induces mobilization of Igα/Igβ into the 'lipid rafts' where there is a higher concentration of PTKs. BCR cross-linking leads to the phosphorylation of many interconnecting downstream signaling pathways, but four major pathways can be described [93].

Syk induces phosphorylation of BLNK (SLP-65) which acts as an adaptor molecule and provides a platform for other effector molecules that in turn activate several diverging signaling pathways [94]. BLNK phosphorylation is essential for activation of Bruton's tyrosine kinase (Btk) and phospholipase C (PLC) γ2 [95]. PLCγ2 recruitment leads to the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of Ca^{2+}, which, via the calmodulin-dependent serine phosphatase calcineurin, induces NFAT translocation to the nucleus. Transcription factor NFAT plays a mayor role in B cell survival and differentiation [96]. DAG formation activates protein kinase C (PKC), which, in synergy with other pathways, activates the NF-κB signaling pathway [93].

BCR-Ag engagement induces the recruitment and activation of the Ras/Raf/MEKK cascade via BLNK and Bam32 [97, 98]. This pathway leads to the activation of ERK. A downstream target of ERK activation is c-Myc (a proto-oncogene) which induces B cell proliferation [99]. However, ERK activation plays other roles in cell survival and differentiation. Other members of the mitogen-activated protein kinase family – JNK and p38 – are also activated by BCR ligation, and lead to the phosphorylation of multiple transcription factors.

PTK Lyn acts as both a positive and negative regulator of BCR induced signaling. Lyn exerts its positive role in activation via association with CD19, leading to the activation

of Phosphoinositide-3 kinase (PI3K), among other pathways [100]. A major downstream target of PI3K is Akt, or protein kinase B. Akt is involved in cellular survival pathways, where it directly and indirectly inhibits apoptotic processes [101].

As I will address in section D, changes in the ability to signal following BCR ligation could prevent autoreactive B cells from being activated and therefore maintain tolerance. Nonetheless, changes in BCR signaling that lead to enhanced B cell survival might rescue autoreactive B cells that would normally be eliminated from the peripheral repertoire.

1.2. Cell death regulation by BCR

B cell death or survival is largely decided by members of the Bcl-2 family of proteins. Pro-survival members include Bcl-2, Bcl-X_L, MCL-1 and A1, while pro-apoptotic members include BAX, BAK and BH3-only proteins Bim, Bad and Bid. A balance between the expression and interaction of pro- and anti-apoptotic proteins dictates the fate of B cells. These proteins are regulated by both transcriptional and post-transcriptional mechanisms. The pro-apoptotic proteins described here usually mediate cell death via the mitochondrial pathway [102, 103, 104, 105].

The initiating factor of B cell death is thought to be the activation of BH3-only proteins. These proteins are thought to sequester anti-apoptotic proteins or directly activate BAX or BAK, although disagreements remain about the initiating events. The subsequent event is the oligomerization of BAK and BAX on the outer membrane of the mitochondria, which results in the permeabilization of the membrane, and the release of cytochrome c from the mitochondria to the cytoplasm. Released cytochrome c is then able to bind to apoptotic-protease-acting factor 1 (Apaf1), which activates and oligomerizes caspase-9, forming the

apoptosome. Caspase-9 activation is paramount in the induction of B cell death and leads to caspase-3 activation as well. Pro-survival proteins like Bcl-2, Bcl-x_L and MCL-1 are thought to oppose cell death by binding and sequestering BAX and BAK, thereby inhibiting their oligomerization [106, 107, 108, 109]. Overexpression of Bcl-2 and Bcl-X_L result in reduced BCR-induced negative selection and an accumulation of B cells in Tg mice [110, 111, 112]. B cells lacking BAX and BAK are refractory to apoptosis induction by BCR stimulation. Moreover, an accumulation of transitional and Fo B cells is observed in mice lacking both BAX and BAK, suggesting there could be a defect in negative B cell selection [113].

Upon activation, BH3-only proteins interact with pro-survival proteins releasing BAX and BAK which are then able to oligomerize and induce cell death. Of the BH3-only proteins, Bim is a key player in the induction of B cell death, and its deletion results in enhanced B cell survival and autoimmunity. Bim can bind to all pro-survival proteins whereas other pro-apoptotic proteins are more specific and only bind a few members. As mentioned above, these proteins are regulated by multiple mechanisms. Bim expression is induced following BCR ligation, but BCR ligation also induces ERK signaling that results in Bim phosphorylation, which leads to its degradation. In addition, ubiquitination of Bim might also lead to its regulation [114, 115].

In addition to ERK, other signaling pathways such as Akt and NF-κB have been implicated in the regulation of apoptosis. BAD, a BH3-only protein is a direct target of Akt. In its unphosphorylated state, BAD induces apoptosis, but, when phosphorylated by Akt, it binds to 14-3-3 protein inhibiting its binding to Bcl-2 [116]. Furthermore, Akt activation can inhibit apoptosis via BAD-independent mechanisms. These include increased expression of

MCL-1 and NF-κB activation. NF-κB signaling induces the expression of pro-survival proteins such as Bcl-2, Bcl-X_L and A1 [106].

It is important to note that not only are the signaling pathways important in dictating B cell survival or death, but also the quality of the signal. For example, transient ERK activation promotes BCR induced apoptosis, while sustained ERK signaling results in proliferation and survival.

2. TLR induced activation

Toll-like receptors (TLRs) are members of the toll/interleukin-1 receptor superfamily. To date, more than 10 members of this superfamily have been described. TLRs recognize a variety of ligands, known as Pathogen-Associated Molecular Patterns (PAMPs), which include ligands from viruses, bacteria, fungi and parasites. TLRs are important in innate immunity, but also play a vital role in adaptive immunity and T-dependent B cell activation [117, 118, 119].

B cells express a variety of TLRs, including TLR3, TLR4, TLR7 and TLR9 among others. In B cells, lipopolysacharide (LPS) is recognized by both the TLR4/MD2 and RP105/MD1 complex (a TLR-like receptor) [120]. The downstream effects of TLR engagement by B cells are not yet fully understood. TLRs can induce, independently or as co-stimulants, B cell proliferation, class switching and differentiation to antibody secreting cells. This activation is independent of BCR specificity and therefore has the potential to activate autoreactive B cells. Much of the work on TLR induced activation of B cells has focused on TLR4, TLR9 and, to a lesser extent, TLR3 and TLR7 [121].

TLR engagement induces intracellular signaling pathways, leading to multiple downstream effectors and physiological events. The proximal events following TLR ligation vary depending on which TLR is engaged. TLR4 ligation induces association with several adaptor proteins. MyD88 is a central adaptor used by most TLRs. MyD88 associates with members of the interleukin-1 receptor associated kinases (IRAKs) family, which induces activation of MAPKs pathways as well as NF-κB pathway via TRAF6. A MyD88-independent pathway exists for some TLRs, including TLR4. This pathway is initiated by Trif and TRAM adaptor proteins, which lead to other downstream events, and demonstrate overlapping roles with the MyD88 pathway as they are also involved in the activation of NF-κB and MAPKs [122]. Additionally, RP105 has been shown to signal in B cells via CD19's activation of Lyn and Vav, which subsequently lead to PI3K activation [123].

As mentioned above, these pathways lead to polyclonal B cell proliferation, survival and differentiation to PCs. TLR engagement can also lead to rescue from apoptosis, activation and PC differentiation of transitional B cells, which are normally induced to undergo cell death following BCR engagement. This can have a profound impact on B cell tolerance as I will discuss later.

3. BAFF role in B cell activation

The role of BAFF in B cell development has been extensively examined. It has been demonstrated that BAFF plays an important role in B cell activation following BCR or TLR engagement, and also in PC maintenance. BAFF exerts its influence on B cell activation in two ways; first by inducing and enhancing B cell survival, and secondly by enhancing B cell metabolism and cell cycling (although BAFF alone does not induce proliferation) [124, 125].

Several lines of evidence indicate that BAFF enhances B cell survival following activation by upregulating Bcl-2, Bcl-X_L and A1 via NF-κB (p50 and p52) signaling [89, 126]. Nonetheless, others have obtained contradicting results generating a controversy on the true importance of these anti-apoptotic factors, and if they are indeed upregulated by BAFF. Recent work by Thompson's group suggests that B cell survival induced by BAFF is as a result of Akt activation. Their work indicates that Akt induces the expression of MCL-1, a pro-survival protein, which binds and inhibits Bim. Furthermore, Akt phosphorylates and inhibits FOXO3, a transcription factor required for Bim expression. ERK activation by BAFF can also lead to Bim phosphorylation and degradation. Regardless of the induction or lack thereof of pro-survival proteins, BAFF appears to target and inhibit Bim, therefore enhancing B cell survival following activation [77].

BAFF stimulation does not induce cell cycling, but instead upregulates and sets the machinery involved in B cell proliferation. Furthermore, BAFF enhances cell metabolic activity and cell size. All these physiological changes appear to work concurrently to induce a more robust and quicker response following B cell activation [127]. Therefore, BAFF acts not only as a survival factor, but also plays an important role in B cell activation. As would be predicted by these functions, BAFF has a major impact on B cell tolerance.

C. Plasma cell differentiation

B cell activation induces proliferation, survival or cell death. Productive B cell activation by appropriate stimuli (BCR + T cell help, TLR stimulation, etc) can result in B cell differentiation to PCs. The end result of PC differentiation is the production and secretion of antibodies by B cells to induce an effective humoral immune response [128].

PC differentiation is a complex and highly regulated process involving multiple transcription factors leading to terminal differentiation and antibody secretion. Several key transcription factors have been identified in PC differentiation, including B lymphocyte-induced maturation protein 1 (BLIMP-1/prdm1), which has been described as the master regulator of PC differentiation. Enforced expression of Blimp-1 alone induces PC differentiation, while deletion of Blimp-1 inhibits B cells from becoming PCs. Blimp-1 exerts its regulatory control primarily by repressing gene expression, although some genes have been shown to be induced by Blimp-1 [128, 129].

Nonetheless, Blimp-1 is not the only important protein in PC differentiation, and the process remains far from been completely understood. As an example, multiple PC intermediate stages (plasmablast) have been described, but it is yet not known how these stages are related to one another; if they are intermediates of a single pathway leading to PCs or if they result in different types of PCs. Furthermore, efforts are ongoing to determine which factors lead to the generation of short versus long-lived PCs [130, 131, 132, 133, 134].

Expression of Blimp-1 inhibits B cell proliferation by inhibiting c-Myc and inducing p18 (CDK inhibitor), consistent with the non-proliferative nature of PCs [127, 135]. Blimp-1 also inhibits PAX5 (BSAP), a transcription factor key in maintaining B cell identity. Additionally, Blimp-1 inhibits BCL6, a repressor and key regulator of Blimp-1 itself. BCL6 is highly expressed in B cells found in germinal centers where affinity maturation and selection occurs. Once BCL6 is downregulated, Blimp-1 can induce PC differentiation. In addition to BCL6, data suggests that PAX5 can also regulate Blimp-1 expression. Decreases in PAX5 or BCL6 independently can lead to Blimp-1 upregulation. PAX5 is known to repress the expression of several proteins involved in antibody secretion, such as XBP-1, J chain and

IgH and IgK. XBP-1 is critical in controlling the response to endoplasmatic reticulum stress or unfolded protein response (UPR), which occurs during antibody production. Although PAX5 downregulation by Blimp-1 is sufficient for the induction of XBP-1, other factors may act in synergy to maximize XBP-1 expression [128, 129, 136].

IRF-4 (Interferon regulatory factor 4) is another transcription factor expressed in PCs. IRF-4 deficient mice lack detectable serum Ig, which indicates its importance in PC differentiation. IRF-4 is expressed in B cells expressing CD138 (pan PC marker) and Blimp-1. Its role is not completely understood but it is known to activate both κ and λ light chain enhancers [137, 138].

The external factors leading to Blimp-1 expression are not yet fully understood. TLRs, IL-2 + IL-5, and IL-6 induce Blimp-1 expression but it is uncertain if this occurs as a direct consequence of or because of the downregulation of BCL-6.

D. Mechanisms of B cell Tolerance

B cells produced in the bone marrow (BM) express a variety of BCR specificities due to the reshuffling and combination of different elements of the IgH and IgL chains [6]. This wide repertoire of BCRs is critical to ensure an efficient immunological response to the multitude of bacterial and viral pathogens. Nonetheless, this process also produces B cells harboring BCRs with specificities for self or auto-antigens. Some reports suggest that as much as 50 to 70 percent of B cells produced in the BM are self-reactive [139, 140]. If unchecked, B cells could induce disease following activation, as has been shown in multiple diseases which are characterized by the presence of auto-antibodies, specific to a self-antigen. Nevertheless, most individuals (and mice) show no signs of autoimmune disease because

there are mechanisms in place that delete, silence or control autoreactive B cells and inhibit their activation. This regulation is known as tolerance [141].

Thanks to the advent of genetically modified mice, in particular, transgenic mice bearing rearranged Ig genes specific for self or neo-self antigens, several of these mechanisms have been identified and our understanding of tolerance has greatly improved, even though much remains unknown [142, 143, 144, 145]. One major observation is that there are multiple mechanisms involved in tolerance, and that they can act alone or in combination to maintain tolerance. Unknown is what triggers one mechanism over another in order to maintain tolerance. It has been proposed that the strength of the BCR signal and the form of the antigen play a key role in dictating which particular mechanism of tolerance is induced.

Studies using Ig-Tg models have established a hierarchy of mechanisms influencing the fate of auto-reactive B cells.

Receptor Editing

Receptor editing is a process by which self-reactive, immature B cells in the BM upregulate RAG genes and undergo secondary rearrangement of the Ig in order to modify their BCR specificity [146, 147, 148, 149, 150, 151]. Rearrangement occurs primarily to modify the light chain, although it can modify the heavy chain as well [149, 152, 153, 154]. It is thought that rearrangement is the first mechanism to act upon autoreactive B cells, and that this occurs at a high frequency in non-transgenic B cells as well [146, 155, 156].

Several Ig-Tg models have been instrumental in demonstrating B cell receptor editing as a mechanism of tolerance. In the anti-dsDNA model (VH3H9 and VH3H9/56R), which expresses a heavy chain Ig from a hybridoma derived from MRL/lpr autoimmune mice, B

cells that exit the BM have preferentially edited their light chain and there are relatively few IgL chains that can be used to form a non-DNA binding BCR [157, 158]. This process is exemplified when VH3H9 mice were crossed to the Vκ4 light chain Tg mice, in which most B cells expressed the heavy chain, but not the light chain [147]. Receptor editing can also be observed in the anti-H-2K^{k,b} mice where cells continue to express RAG genes and undergo, in some cases, light chain rearrangement [149]. When these B cells are unable to receptor edit (RAG -/- or JH -/-), they are eliminated by apoptosis, a process known as central deletion (below) [159].

One interesting consequence of receptor editing has been the generation of dual BCR expressing B cells. In some cases the expression of a non-autoreactive BCR is thought to dilute the autoreactive BCR and therefore allow some "autoreactive" B cell to survive and mature. An example of this is seen in the VH3H9/56R where some B cells express both kappa and lambda light chains, particularly the MZ B cells [160].

Central Deletion

Central deletion is the way in which autoreactive B cells are physically eliminated from the immune repertoire before exiting the BM and migrating to the periphery. Central deletion is thought to occur if receptor editing has been incapable of eliminating BCR autoreactivity.

The role of central deletion as a mechanism of tolerance was determined using Ig-Tg mice. Initial evidence came from the anti-H-2K^{k,b} model where B cells expressed a BCR specific for H-2K ^{k} or ^{b}. When the transgene was crossed to H-2K^{k,b} expressing mice, there was a marked reduction in peripheral B cell numbers compared to control mice expressing

H2K^{d} [144]. Other systems where central deletion plays a significant role in maintaining tolerance have also been described. Double transgenic mice expressing anti-lysozyme Ig (anti-HEL) and membrane bound HEL resulted in the complete elimination of B cells in the BM [161]. This contrasts the model in which HEL is secreted and anti-HEL cells are rendered anergic (see below).

The VH3H9 Ig paired to several light chains results in BCRs capable of binding dsDNA. Although anti-dsDNA B cells are absent in the VH3H9 Tg mice, it is thought that this phenomenon is due to receptor editing. When the ability to edit is inhibited (RAG -/-), anti-dsDNA cells are centrally deleted [159]. It is thought that induction of programmed cell death following BCR engagement is the main mechanism responsible for the elimination of autoreactive B cells in the BM.

Peripheral Deletion

There is evidence that autoreactive B cells can be deleted in the periphery. This was first demonstrated using anti-H-2K^{k,b} Ig Tg mice that also express the H-2K^{b} molecule only in the liver [162]. B cells in these mice were able to exit the BM and were eliminated in the periphery. In addition, in non-Tg mice, BCR stimulation induces program cell death in transitional, newly migrated B cells, suggesting that transitional B cells in the periphery remain sensitive to antigen-induced apoptosis [23, 24, 26, 163]. Defects in transitional B cell signaling following BCR ligation in New Zealand black mice, result in increased Bcl-2 expression leading to a break in tolerance and autoimmunity. Recent findings by Kumar et al. demonstrates how the expression of the Ly108.2 isoform derive from lupus-prone

NZM2410/NZW strain of mice when expressed in anergic HEL B cells (model described below) resulted in reduced B cell death and break in tolerance [164, 165, 166].

Anergy

Autoreactive B cells that are not centrally or peripherally deleted, or undergo receptor editing, can still be regulated by a process that renders them functionally impaired to being activated by antigen or TLR ligands and unable to differentiate into PCs. This state of unresponsiveness is described as anergy, and was originally described by Nossal et al. It is clear that autoreacitve B cells can be rendered anergic by multiple mechanisms and that functional and phenotypical differences exist between the different types of anergic B cells [143, 167, 168, 169, 170, 171, 172].

Initial studies using anti-HEL B cell Tg mice, in which HEL Ag was expressed as a soluble protein (MD4xML5 mice) by Goodnow et al., laid the foundations for future understanding of B cell anergy. His work, and others that followed in the HEL model, as well as in other Ig-Tg B cell models, identified key features that would be used to categorize and determine anergic B cells. These features include inactivation or attenuation of BCR signaling, reduced membrane Ig expression, and reduced half-life. These features were also observed in the dsDNA B cell model (cells expressing $\lambda 1$ or $\lambda 2$ light chains associated to the VH3H9 heavy chain), as well as in the Ars/A1 model. In the latter model, B cells expressed normal levels of surface IgM, yet they had a reduced half-life and defective BCR signaling. Ars/A1 B cells are specific for the arsonate hapten but also cross-react with a low affinity to self antigen (ssDNA) [142, 143, 168, 173, 174].

In contrast, other B cell models in which autoreactive B cells are functionally impaired and unable to secrete antibodies do not share the characteristics described for the anti-HEL, anti-dsDNA and Ars models. B cells in the ssDNA (VH3H9/Vκ8), anti-insulin and the anti-Sm (2-12H, 2-12H/Vκ8) mouse models are able to mature, have a normal half-life, normal levels of surface IgM and are able to induce signaling following BCR stimulation [168, 171].

A recent study by Cambier's group suggests the T3 cells described by Allman et al., which express CD93 (C1qRp), are in fact anergic cells and not an intermediate developmental stage. These T3 cells (An1, anergic population 1), share the same characteristics of classic anergic B cells described above. These An1 B cells are not only found in MD4xML5 mice, but in the Ars1/A1 and anti-dsDNA, as well as in non-Tg mice. Interestingly, T3 or An1 B cells are not found in the anti-insulin or anti-Sm (2-12H/Vκ8) mice, where B cells are also anergic [175]. These differences point to alternative mechanisms leading to anergy. Possible factors dictating which type of anergic state is induced could be the affinity of the BCR, or the form of the antigen. In the particular case of the Ars1 and HEL models, blocking Ag binding (or adding a non-cross linking hapten) resulted in CD93 downregulation and the ability to signal following BCR stimulation. These data suggest that constant antigen engagement maintains anergy in these B cells, and that anergy is reversible. It is not clear whether anergic B cells in other models are or are not constantly engaging Ag.

BCR uncoupling or desensitization has been described as another possible mechanism by which BCR signaling is inhibited. Constant Ag engagement by the BCR results in physical separation of the Igα/Igβ signaling component of the BCR from the IgM Ag binding component of the BCR, which consequently inhibits BCR signaling. The augmented

expression of negatively regulating proteins is an alternative mechanism by which BCR signaling is inhibited in some autoreactive B cells [176]. Examples of these are CD5 and SHP-1 [177]. When bred to the CD5 deficient mouse, anti-HEL B cells are able to break tolerance. Furthermore, SHP-1 is chronically activated in some anergic B cells (Ars1), and when it is deleted, these cells can become activated. SHIP-1 deletion also results in autoimmunity, consistent with its role in B cell tolerance [178].

Alterations of protein expression induced by self-antigen *via* the BCR can also result in B cell anergy without affecting BCR proximal signaling events. These proteins play important roles as effectors or transcription factors required for survival, proliferation and PC differentiation. In the anti-insulin model, B cells are able to signal following BCR ligation, but do not proliferate, and subsequently die. It has been shown that NFAT expression (TF involved in B cell survival) in these anergic B cells is lower than in non-Tg B cells, and is not translocated to the nucleus following BCR signaling [170]. The HEL model of anergy is another example of this. Here, the overexpression of c-Myc (TF involved in proliferation and survival), results in B cells that are no longer anergic and are able to secrete [179]. These examples (and others) suggest that anergy can be attained not just by modifying proximal events of BCR signaling but also by affecting TF involved in proliferation and survival.

B cell activation by microbial and viral infections can precipitate the onset of autoimmunity. Several studies have demonstrated that TLR engagement can lead to the activation of autoreactive B cells and induce Ab secretion. In addition, research has shown the critical role of TLRs for the onset of autoimmunity in autoimmune-prone mice [180]. The loss of TLR9 in lupus-prone mice, for example, leads to the inhibition of anti-dsDNA and anti-chromatin Abs, but has no effect on the anti-Sm response. In some cases (specifically

TLR9 in dsDNA), TLR activation can act simultaneously and synergize BCR activation resulting in a break in tolerance [181, 182, 183].

Interestingly, in some instances, TLR activation of anergic B cells does not result in Ab secretion. TLR4 and TLR9-induced activation of anergic anti-HEL B cells is blocked or reduced in vitro. In this particular case, chronic antigen engagement leading to high basal levels of phosphorylated ERK inhibits TLR induced Ab secretion [184, 185]. When these cells are treated with ERK inhibitors, their ability to secrete Ab in response to TLR activation is restored, but as I will show (Chapter 4) this mechanism is not involved in regulating other anergic B cells.

Although all the mechanisms of anergy described so far involve intrinsic modifications most likely induced by antigen, a recent report by Vilen's group suggests that extrinsic factors can help maintain autoreactive B cells in an anergic state. The report showed that IL-6 secreted by dendritic cells repressed anergic B cells and rendered them unable to respond to TLR stimulation [186]. Other factors such as TNFα and CD40L play similar roles. This mechanism of repression would block bystander activation of autoreactive B cell during the course of an immune response and therefore protect from initiation of autoimmunity.

Overall, it is clear that multiple mechanisms result in B cell anergy. It is more likely that several of the mechanisms described here (among others) act jointly to inhibit activation of autoreactive B cells. Which factors and stimuli are responsible for inducing these mechanisms is still an area of important research.

Follicular exclusion

In contrast to the mechanisms of tolerance described above, autoreactive B cells can be eliminated in the periphery by more indirect means. First, as described in the MD4xML5 model, anergic anti-HEL B cells that are able to form normal follicles are, however, unable to migrate into the follicle in the presence of competitive non-Tg B cells. They remain instead in the B/T interface and are quickly eliminated within a few days. For this elimination to occur, both non-Tg B cells and antigen are required [143, 187]. The inability to enter the follicle is attributed to low expression of CXCR5, a chemokine receptor required for normal B cell migration into the follicle. It was originally argued that the inability to enter the follicle prevented these cells from accessing B cell survival factors within the follicle. However, even if anergic anti-HEL B cells are allowed to enter the follicle they are still eliminated [188].

One possible explanation is that with reduced numbers of self-reactive B cells, antigen is increased, relatively, to the number of self-reactive B cells present. HEL is now more readily available for the self-reactive B cells which could result in increased BCR occupancy and, consequently, increased BCR signaling resulting in B cell deletion. This hypothesis still remains to be resolved, but it has been recently demonstrated that survival factors are indeed involved in this process, particularly BAFF. It is thought that chronic antigen engagement induces Bim upregulation which would lead to apoptosis of the autoreactive cell. BAFF acts by preventing Bim-induced apoptosis. Evidence to support this hypothesis includes elevated BAFF levels in the MD4 x ML5 Tg mice as compared to non-Tg mice. Furthermore, BAFF injection resulted in partially rescue of transferred anergic anti-HEL B cells [189].

Anergic anti-dsDNA B cells are also unable to enter the follicle when non-Tg B cells are present. In this case it is thought that the defect is due to the presence of regulatory T cells. Lack of T cell suppression as well as T cell help were required for these cells to break tolerance and migrate into the follicle [167, 190]. More recently, FcγRIIb has been shown to be required for anti-DNA B cell follicular exclusion as well as maintenance of tolerance [191].

E. Anti-Sm B cells (2-12H Tg)

2-12H transgenic mouse models

One of the antigens that is a frequent target of the immune system in the autoimmune disease Systemic lupus erythematosus (SLE) is the ribonucleoprotein Sm [192, 193]. Antibodies against Sm are also present in autoimmune MRL/lpr mice, which are used as a mouse model of SLE [194]. The prevalence of anti-Sm positivity in these mice is of 25% [195, 196]. To study the regulation and activation of anti-Sm B cells, a rearranged, unmutated heavy chain of a MRL/lpr hybridoma was used to create an Ig Tg mouse, named 2-12H [197]. The 2-12 heavy chain has the ability to pair with a variety of light chains, and bind Sm, ssDNA, or both. Consistent with this, the vast majority of the 2-12H B cells have BCRs specific for Sm. Interestingly, antibody serum levels against Sm were not significantly different from non-Tg littermates. Originally, it was thought that most of the Sm binding B cells (approximately 30%) were transitional B cells and had a short half-life, suggesting that anti-Sm B cells were eliminated by peripheral deletion [197]. Although peripheral deletion occurs, as will be shown in Chapter 2, it was later determined that many of these anti-Sm B cells were mature MZ B cells.

Anti-Sm B cell activation and regulation

Many anti-Sm B cells from non-autoimmune 2-12H Tg mice are not normally activated *in vivo*, but when crossed to the MRL/lpr background, anti-Sm B cells are activated and secrete Ab [198]. The 2-12H Ig transgene in this autoimmune background increases the prevalence and accelerates the onset of anti-Sm secretion. These results demonstrate that the tolerance mechanisms regulating anti-Sm B cells are either overcome or defective in this autoimmune setting.

Additional data suggests that 2-12H B cells can overcome tolerance if changes in BCR signaling strength occur. 2-12H Tg mice crossed to the CD19 Tg mice, in which B cells had higher expression of CD19, showed increased levels of anti-Sm serum. In contrast, 2-12H CD19ko mice showed no signs of B cell activation, but had instead a population of anti-Sm B cells with a mature phenotype [199]. In another instance, 2-12H B mice deficient for CD22 (a negative regulator of BCR signaling) had similar characteristics to the 2-12H CD19Tg mice. These data suggest that changes in the BCR signaling strength can lead to 2-12H anti-Sm B cell activation [199]. One possible explanation for these observations is that increased CD19 levels or decreased CD22 levels result in a lower threshold of BCR activation, as compared to normal 2-12H B cells and that the normal level of self ag can now induce activation. Another possibility is that these changes had an impact on entry of the anti-Sm B cells into the mature B cell subsets. As an example, increased BCR signaling possibly led to an increase in anti-Sm MZ B cells, and as these cells are easier to activate than Fo B cells, this resulted in an increase in anti-Sm Ab serum levels.

Anti-Sm B cell activation could also occur by increasing the Ag load to a level above that needed to exceed the B cell activation threshold. Sm, together with other nuclear

antigens, is found in the surface of apoptotic cells. Injection of apoptotic cells into 2-12H Tg mice resulted in a transient increase in serum anti-Sm. Similarly, defects in apoptotic cell clearance induce an anti-Sm response. The Mer receptor is involved in the clearance of apoptotic cells by macrophages and the loss of function by this tyrosine kinase receptor results in a defect on the clearance of apoptotic bodies. An increase in serum anti-Sm Abs was observed when the 2-12H Ig transgene was crossed to the Mer deficient background [200]. Data suggests that the cells activated are MZ and B1 B cells.

Nonetheless, anti-Sm B cells maintain tolerance in the normal background. In part, it is believed that some anti-Sm B cells in the 2-12H Tg mouse are anergic. Findings using the 2-12H/Vκ8 double Tg mouse (see below) supports this hypothesis [171]. Furthermore, 2-12H B cells express higher levels of CD5 (a negative regulator of BCR signaling) than non-Tg B cells (data not published). CD5 expression was shown to be important in maintaining anergy in the anti-HEL mouse model. In addition, it has been demonstrated by Culton et al. that a particular population of pre-PC cells are blocked from differentiation to PCs in the 2-12H Tg mouse, but not in non-Tg or 2-12H MRL/lpr mice. These $CD138^+$ prePCs were unable to upregulate the BAFF receptor BCMA important for pre-PC survival and were eliminated. Moreover Blimp-1 was not upregulated, suggesting that regulation occurs prior to that point [201].

These results taken together suggest that the affinity of the BCR for the antigen and the amount of antigen present can have a major impact on the maintenance of anti-Sm tolerance. This also suggests that there are several mechanisms of tolerance acting on these cells. Because 2-12H Tg mice have a variety of BCR affinities, it is difficult to completely address these questions. It is possible that the BCR affinity for Sm could determine which, if

any, mechanism of tolerance is set in place. In order to test this, the 2-12H Ig transgene has been crossed to light chain Tg mice. Using this strategy we can analyze anti-Sm B cell populations bearing the same affinity, which will vary depending on the light chain used.

Anti-Sm Double Tg mouse models

A double transgenic mouse was generated using the 2-12H heavy chain and the Vκ8 light chain. This combination resulted in B cells bearing a BCR with a low affinity for Sm. In this double Tg mouse, anti-Sm Fo B cells were present, but MZ and B1 B cells were absent. These cells were long lived, expressed normal levels of surface IgM but were anergic, as they were unable to secrete following LPS stimulation. Unexpectedly, these cells were able to compete with on non-Tg B cells for entry into splenic follicles [171]. Although this double Tg model shed light on the regulation of anti-Sm B cells it represented only one clonotype and one affinity for Sm. Therefore, I generated a second double Tg mouse line, combining the 2-12H transgene with a Vκ4 transgene. 2-12H/Vκ4 B cells bind to Sm with a higher affinity than 2-12H/Vκ8 B cells allowing me to examine the influence affinity on regulation of anti-Sm B cells. Furthermore, 2-12H/Vκ4 Tg mice also present a mature population of MZ B cells which provided me the opportunity to address regulation of anti-Sm MZ B cells without the confounding influence of diverse clonotypes or affinities.

In chapter 2, I present evidence arguing that high affinity anti-Sm B cells from 2-12H/Vκ4 transgenic mice are anergic but, unlike low affinity 2-12H/Vκ8 B cells, are unable to compete with non-Tg B cells and are eliminated. This elimination correlates with a minimal responsiveness to the B cell survival factor BAFF. It there for suggests that the affinity of the BCR for Sm will dictate if a cell will be able to compete or will be eliminated.

In chapter 3, I will show evidence that the BCRs of 2-12H and 2-12H/Vκ4 anti-Sm Fo B cells signaling competent. Nonetheless these cells undergo rapid cell death unable to proliferate. This defect correlates with an imbalance in pro and anti-apoptotic proteins. However, while 2-12H MZ B cells signaled efficiently in response to BCR ligation, 2-12H/Vκ4 MZ B cells are defective. They also show no sign of *in vivo* activation which lead me to conclude that 2-12H/Vκ4 MZ B cells are anergic while 2-12H MZ are not, and are regulated by BCR uncoupling.

I also assessed the ability of these cells to respond to TLR stimulation. Anti-Sm B cells are able to proliferate but are however defective in antibody secretion. I demonstrate in chapter 4 that the antibody secretion defect observe regulated by a combination of activation-induced cell death and a block in PC differentiation, which is dependent on the B cell subset identity. Furthermore, 2-12H/Vκ4 B cells display the highest degree of regulation, compared to 2-12H B cells, suggesting once again that the BCR affinity is able to influence the degree of regulation. These mechanisms are ERK-independent which suggests the existence of an ERK-independent pathway in the regulation of PC differentiation by anergic B cells contrasting the anergic anti-HEL B cells observations.

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CHAPTER 2

Regulation of anergic anti-Sm B cells by decreased responsiveness to the B cell survival factor BAFF

ABSTRACT

Anergy is an important mechanism for the regulation of autoreactive B cells. In the presence of naïve B cells, anergic B cells of some specificities are unable to enter the follicle and undergo rapid cell death due to a requirement for higher than normal concentrations of the survival factor BAFF. Previous analysis of low affinity anti-Sm B cells from 2-12H/Vκ8 transgenic mice differ from this paradigm. Low affinity anti-Sm B cells are neither blocked from entry into the follicle, nor short-lived even in the presence of competitor non-autoreactive B cells. I hypothesize that the BCR affinity for self-antigen influences the mechanisms regulating survival and competition abilities of anti-Sm B cells. Herein I present evidence that high affinity anti-Sm B cells from 2-12H/Vκ4 transgenic mice are also divergent from this paradigm, and are regulated differently than low affinity anti-Sm B cells. 2-12H/Vκ4 B cells belong to the follicular (Fo), marginal zone (MZ), and B-1 subsets. Like low affinity anti-Sm B cells, high affinity anti-Sm Fo and MZ B cells are non-responsive to toll-like receptor (TLR) activation and thus anergic. They can enter the follicle, even in the presence of competition, likely due to elevated CXCR5 levels. However, unlike low affinity anti-Sm B cells, high affinity anti-Sm B cells are eliminated from the B cell repertoire in the presence of competitor B cells, which correlates with a minimal responsiveness to the B cell

survival factor BAFF. Thus, affinity for Sm determines the responsiveness to a key B cell survival factor.

INTRODUCTION

The regulation of autoreactive cells is paramount to prevent the development of autoimmune diseases. Multiple mechanisms of tolerance have been described, which ensure that autoreactive B cells are inhibited from reaching maturity or/and differentiating to plasma cells (PCs). These mechanisms include receptor editing and central deletion in the BM [1, 2, 3, 4]. Autoreactive B cells that can escape to the periphery are subject to regulation by peripheral deletion and anergy [3, 5, 6]. Which mechanism is employed to regulate the autoreactive B cells of any given specificity is not fully understood, but is influenced by multiple factors, including the form and amount of Ag, when antigen is encountered, and BCR affinity.

Immunoglobulin transgenic mouse models have been powerful tools for understanding the mechanisms of B cell anergy [3, 7]. In the case of anergic anti-HEL B cells, these cells are normally arrested from development to mature Fo or MZ B cells, express low levels of membrane IgM and have a short half-life. Other models, including the anti-dsDNA and the Ars/A1 models, display similar characteristics [8, 9, 10]. It has been recently determined that transitional 3 (T3) B cells also share these characteristics and therefore it is thought that they are anergic cells in non-transgenic mice [11]. However, not all anergic B cells share these characteristics. For example, anti-ssDNA and anti-insulin B cells differentiate to mature Fo and MZ B cell, have normal half-lives and express normal BCR levels, but are nevertheless defective in PC differentiation [12, 13, 14]. It is therefore important to understand what causes these differences and whether the mechanisms of anergy differ among autoreactive B cells that exhibit these various anergic phenotypes.

Anergic B cells are refractive to activation, but because they persist in the repertoire, they could escape tolerance leading to autoimmunity. In the presence of non-Tg B cells, anti-

HEL and anti-dsDNA anergic B cells are concentrated at the B/T interface [12, 15, 16]. Anti-HEL are eliminated faster due to an increased requirement for BAFF [17, 18]. BAFF a TNF family member, is required for B cell survival and maturation [19, 20, 21, 22, 23, 24]. It provides a key survival signal through its ability to downregulate the pro-apoptotic protein Bim and by upregulate the pro-survival factor Mcl-1 [22]. Thus, in addition to defects in ability to respond to activating signals, anergic B cells are highly susceptible to elimination from the normal B cell repertoire through defects in the ability to respond to critical survival signals.

We have previously described the anti-Smith (Sm) 2-12H Tg mice. Sm is a ribonucleoprotein targeted by the immune system in systemic lupus erythematosus. In these mice, 30-50 % of B cells bind Sm and are mature Fo, MZ, and B-1 B cells [25]. Despite the large number of anti-Sm B cells, serum anti-Sm levels are not different from those in non-Tg mice. Multiple regulatory mechanisms contribute to anti-Sm B cell tolerance. 2-12H bearing B cells can express diverse light chain genes, generating B cells with a wide range of Sm binding abilities and likely accounts for their presence as Fo, MZ and B-1 B cells. This range of Sm binding ability generates anti-Sm B cells that are likely to be regulated differently. 2-12H Tg mice have a higher turn over rate compared to wild-type mice, suggesting that some anti-Sm B cells are peripherally eliminated [25]. Some anti-Sm B cells are activated to a pre-plasma cell stage before quickly being eliminated and others are regulated by anergy [26]. Anergy was demonstrated by using 2-12H mice that carry a Vκ8 L chain transgene and produce low affinity anti-Sm B cells [27]. Anergy is maintained by B cell intrinsic and extrinsic mechanisms. Thus, while these cells are intrinsically poor responders to LPS, chronic antigen engagement has reprogrammed their response to the pro-differentiation cytokines, IL-6, CD40L, and TNFα repress further LPS responsiveness [28].

To determine whether anti-Sm B cells of higher affinity are similarly regulated, we generated an anti-Sm B cell model that generates B cells of higher affinity than our previously described 2-12H/Vκ8 Tg mice. These mice carry the 2-12H transgene along with a Vκ4 transgene. Although 2-12H/Vκ4 B cells are anergic, they differ from low affinity 2-12H/Vκ8 B cells in that, while low affinity B cells are limited to differentiation to the Fo B cell stage, high affinity anti-Sm B cells differentiate to multiple B cell subsets. In addition, high and low affinity anti-Sm B cells differ in ability to compete with non-autoimmune B cells for survival; the latter are effective competitors and have a long half-life even in the presence of a majority of non-autoreactive B cells, whereas the former are not competitive and are eliminated in the presence of competitor non-autoreactive B cells. The inability of high affinity anti-Sm B cells to compete for survival is not due to an inability to enter B cell follicles, but is likely due to a minimal responsiveness to BAFF. These data suggest that BCR affinity determines the competitive ability of autoreactive B cells regulating responsiveness to BAFF.

MATERIALS AND METHODS

Mice

Anti-Sm 2-12H Tg mice and Vκ4 Tg mice have been described previously [29, 30]. 2-12H/Vκ4 double Tg mice were generated by crossing 2-12H with Vκ4 Tg mice. 2-12H Tg mice are on a mixed background of C57BL/6 and CB17. Vκ4 Tg mice are on the CB17 background. 2-12H/Vκ4 Cκ-/- double Tg mice were also generated to confirm allelic exclusion. No phenotypic or functional differences were observed between the 2-12H/Vκ4 and the 2-12H/Vκ4 Cκ-/- double Tg mice. Offspring carrying transgenes were identified by tail genomic DNA as described previously [25]. All animal protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Flow Cytometry

Single-cell suspensions of splenocytes were prepared and RBCs were lysed using an RBC lysis solution (1 M Tris, 0.15 M ammonium chloride, and 0.1 M EDTA). All staining was done in RPMI 1640 medium (HyClone) containing 2.0% bovine calf serum (HyClone) as previously described (Qian). FcRs were blocked with mAb 2.4G2 for 15 min at 4°C. Cells were analyzed at the University of North Carolina Flow Cytometry Facility (Chapel Hill, NC) using a FACSCalibur (BD Biosciences) or CyAn (Dako). Analysis was performed using Summit software (Dako). The Abs specific for IgM, IgMa, B220, CD21, CD23, CD9, CD1d and CXCR5 were obtained from BD Biosciences. These Abs were biotinylated or conjugated to allophycocyanin, PE, FITC, PE-Cy7, APC-Cy7, or PerCP. For the identification of anti-Sm B cells we used Sm (SMA-3000; Immunovision, Springdale, AR) that was biotinylated in our laboratory as described previously (Santulli-Marotto). BAFF receptors were detected

using anti-TACI (TNFRSF13B), anti-BAFF R (TNFRSF13C) and anti-BCMA (TNFRSF17) from R&D Systems.

BrdU labeling

Mice were given 0.5 mg/ml 5-bromo-2_-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) with 1 mg/ml dextrose in their drinking water continuously for 4 wk. At different time points mice were sacrificed and spleens cells were prepared for flow cytometry as described above. Staining for BrdU was done as described previously using anti-BrdU-FITC (BD Biosciences) [27, 31].

Cell purification

Splenic B cells were isolated by negative selection using IMag™ Streptavidin Particles Plus (BD Biosciences) following manufacturers protocol for magnetic labeling and separation. Cells were incubated for 40 min with a cocktail of biotinylated antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD11b and anti-CD49b (BD Biosciences)). Cells were washed thoroughly and incubated with Streptavidin DM particles and then separated *via* magnet. B cells were 92–98% pure as determined by flow cytometry.

Stimulation

Purified B cells were ($1x10^6$/ml) were cultured in 24 well plates in complete RPMI containing 10% FCS, L-glutamine, penicillin, streptomycin, and 2-mercaptoethanol. Cells were stimulated with 10 μg/ml LPS or 1 μg/ml CpG (InvivoGen) for 5 days. rIL-6 was added

at day 0 as described previously [28]. Supernatants were collected and Ab secretion determined by ELISA.

For BAFF survival assay, B cells were cultured as described above and stimulated with indicated amounts of recombinant BAFF (PeproTech 310-13). To determine spontaneous cell death purified B cells were cultured in complete RPMI for the indicated times. Cell death was determined by flow cytometry staining cells with either 7AAD (BD Biosciences) or LIVE/DEAD dead cell stain kit (Probes-Invitrogen).

ELISAs

Quantitation of anti-Sm Abs, total IgM and IgMa in mouse serum and supernatants was done by ELISA as previously described [25]. For anti-Sm Ab detection 96-well flat-bottom polyvinylchloride plates were coated with Sm protein (Immunovision) over night in borate-buffered saline. All washes were performed with borate-buffered saline. Plates were blocked with borate-buffered saline, 0.5% BSA, and 0.4% Tween 80 for a minimum of 1 h before adding the samples. All samples were analyzed in triplicate. To develop the assays we used either goat anti-mouse IgM-alkaline phosphatase (AP; Southern Biotechnology) or anti-IgMa-biotin followed by streptavidin-AP (BD Biosciences). IgM was quantitated by adding sera or supernatant to plates coated with polyclonal goat anti-mouse IgM (Southern Biotechnology) and detected with either biotinylated anti-IgM^a or anti-IgM^b (BD Biosciences), followed by streptavidin-alkaline phosphatase (Southern Biotechnology). Assays were developed with 1 mg/ml *p*-nitrophenyl phosphate (Sigma, St. Louis, MI) in 0.01 M diethanolamine and the OD_{405} determined. Purified mouse IgMa (TEPC 183; Sigma-Aldrich) was used as a standard for total IgM and IgMa ELISAs.

BM Chimeras

Hematopoietic stem cells from 2- to 3-mo-old 2-12H/Vκ4 Tg mice and non-Tg littermates were isolated as described previously [27, 32].Hemopoietic stem cells, which exclude the Hoechst dye, were sorted using a MoFLo high speed sorter (Cytomation). Sorted cells from both strains were mixed in a 1 to 1 ratio in PBS and were injected via tail vein into non-Tg littermate mice that had been lethally irradiated (2 doses of 500 rads 4hs apart) 24 h previously. Analysis of recipients was performed at least 3 months after reconstitution. To determine the degree of chimerism, BM cells were stained to determine the ratio of immature ($CD21^-$, $CD23^-$, IgM^+) IgMa *vs* IgMb B cells.

Adoptive Transfer

Approximately 5 X 10^6 purified splenic B cells from both 2-12H/Vκ4 Tg and non-Tg mice were labeled with CFSE (Molecular Probes) and transferred i.v. into non-Tg littermate mice. Spleen cells were analyzed by flow cytometry 3 and 7 days after transfer. CFSE positive cells were assessed for IgMa or IgMb (2-12H/Vκ4 or non-Tg) expression to determine cell numbers.

For immunofluorescence, 1 x 10^7 purified splenic 2-12H/Vκ4 B cells were transferred i.v into non-Tg littermate mice.

Immunofluorescence

Freshly isolated spleens were embedded in Tissue-Tek OCT (Sakura Finetek) and snap frozen. Six-micrometer spleen sections were prepared and fixed for 5 min in acetone:MeOH at -20°C before staining. Sections were blocked for 1 h with Superblock

Blocking buffer in PBS (1:1 ratio) containing 2.4G2 anti-FcR Ab. Slides were rinsed and stained at room temperature for 2hs with anti-CD3-PE, anti-B220-APC and anti-IgMa-biotin (BD Biosciences) diluted in blocking buffer. IgMa staining was revealed by

streptavidin Alexa 488 (Molecular Probes). Stained slides were rinsed with PBS and coverslips mounted in FluorSave mounting media (Calbiochem). Analysis was performed using a digital deconvolution microscope (Intelligent Imaging Innovations (3I)). Images were collected and analyzed usingSlidebook software (3I).

Statistical analysis

The Student *t* test was used to assess the significance of the differences between groups. A value of $p < 0.05$ was considered significant.

RESULTS

2-12H/Vκ4 anti-Sm B cells are better Sm binders, generate all B cell subtypes and are long lived

To determine how affinity for Sm affects the regulation of anti-Sm B cells, we combined the 2-12H transgene with a Vκ4 transgene (Fig 1A-B). The relative Sm binding ability of 2-12H/Vκ4 Tg B cells was compared to 2-12H and 2-12H/Vκ8 anti-Sm B cells by calculating the ratio of the anti-Sm to IgM mean fluorescence intensities (Fig. 1B). 2-12H/Vκ4 B cells had the highest ratio, which suggests the 2-12H/Vκ4 BCR possesses a higher affinity for Sm. The potential of 2-12H/Vκ4 B cells to differentiate into different B cell subtypes was assessed by flow cytometry. Cells were gated on $B220^{+}$ IgM^{+} B cells. In addition to transitional (Tr) B cells ($CD23^{-}$ $CD21^{-}$), 2-12H/Vκ4 mice also had mature follicular (Fo) B cells ($CD23^{+}$ $CD21^{+}$) and a significant population (~12 %) of marginal zone (MZ) B cells ($CD23^{+/-}$ $CD21^{high}$), which also expressed CD9 and higher levels of CD1d (Fig. 1A and data not shown). This contrasts with 2-12H/Vκ8 mice in which MZ B cells were essentially absent, but was similar to 2-12H Tg mice [27]. The number of Fo, MZ and Tr B cells were similar between 2-12H and 2-12H/Vκ4 mice, but the number of Fo B cells was about half of that in non-Tg mice (Fig. 1C). Analysis of peritoneal cells showed that 2-12H/Vκ4 mice also generated B1 B cells based on a phenotype of $CD5^{+}$ $CD23^{-}$ and $CD11b^{+}$ (data not shown).

To determine whether 2-12H/Vκ4 B cells were regulated to prevent spontaneous activation, we assessed IgM^{a} and anti-Sm antibody levels in serum. Total serum IgM levels were similar in 2-12H/Vκ4 and 2-12H mice, but significantly lower than in non-Tg mice. IgM^{a} levels were lower than in 2-12H Tg mice, but their anti-Sm IgM^{a} levels were similar

(~2ug/ml). Thus, 2-12H/Vκ4 B cells have not differentiated to antibody secreting cells (ASCs).

Autoreactive B cell turnover rates are often shorter than those of non-autoreactive B cells, and therefore, we determined the turnover rate of 2-12H/Vκ4 B cells by BrdU incorporation [33]. As previously reported, 2-12H mature B cells showed a significantly higher turnover rate than non-Tg mature B cells. Surprisingly, the turnover rate of 2-12H/Vκ4 mature B cells was no different than that of control non-Tg B cells, which would suggest that high Sm-binding B cells are not peripherally deleted in the absence of competition (Fig 1E). Thus, 2-12H/Vκ4 B cells, despite expressing a monoclonal BCR, differentiate to all B cell subsets, are not spontaneously activated, and have a normal half-life.

2-12H/Vκ4 anti-Sm B cells are anergic

To assess the functionality of 2-12H/Vκ4 anti-Sm B cells, we determined their ability to become ASCs following LPS and CpG stimulation. Purified B cells were stimulated *in vitro* for 6 days and IgM secretion levels in the supernatant measured by ELISA. As seen in Fig. 2A, 2-12H/Vκ4 B cells showed a marked reduction in ability to secrete Ab following LPS stimulation compared to non-Tg B cells, secreting on average 78% less than the control cells. 2-12H B cells showed a lesser degree of regulation and secreted on average 38% less than control B cells. Similar defect in Ab secretion were observed following CpG stimulation Fig 2B. 2-12H and 2-12H/Vκ4 B cells secreted on average 46% and 58% less than control respectively. Thus, 2-12H/Vκ4, and to a lesser degree, 2-12H anti-Sm B cells are hypo-responsive to mitogenic stimulation, indicating functionally non-responsivness (anergy).

We recently showed that IL-6 represses LPS-induced Ab secretion by 2-12H/Vκ8 anti-Sm B cells and other autoreactive B cells, but not naïve B cells [28]. To determine whether higher affinity anti-Sm B cells are also repressed by IL-6, 2-12H/Vκ4 B cells were stimulated with LPS in the presence or absence of IL-6, and antibody (Ab) secretion measured by ELISA. As shown in Fig. 2C, IL-6 reduced Ab secretion by ~60% compared to the untreated LPS stimulated cells. Thus, as with low affinity anti-Sm B cells, IL-6 represses the activation of the higher affinity 2-12H/Vκ4 B cells.

Competition with non-Tg B cells regulates high Sm binding 2-12H/Vκ4 B cells survival.

Autoreactive B cells can be regulated by competition for survival factors with non-autoreactive B cells. In an environment that contains a majority of non-autoreactive B cells, anti-HEL B cells are unable to access BAFF, a potent B cell survival factor required for B cell survival in the periphery, and consequently have a shorter half-life than when in a monoclonal anti-HEL B cell environment [15, 16]. Surprisingly, low affinity 2-12H/Vκ8 anti-Sm B cells were unaffected by the presence of a majority of non-Tg B cells [27]. To determine whether high affinity anti-Sm B cells are competitively disadvantaged, we generated bone marrow chimeras and performed B cell transfers into non-Tg littermates.

To create bone marrow chimera mice, hematopoietic stem cells were sorted from bone marrow of non-Tg and 2-12H/Vκ4 mice as previously described [27, 32]. Cells from mice of the two strains were mixed at a 1:1 ratio and injected i.v. into lethally irradiated non-Tg littermates. Control mice were injected with non-Tg or 2-12H/Vκ4 stem cells or received no cells. Three months later, we analyzed 4 chimeric mice. To establish the degree of chimerism, the ratio of immature ($B220^+IgM^+CD21^-$) IgM^b (non-Tg) vs IgM^a (2-12H/Vκ4)

expressing B cells in the bone marrow was determined by flow cytometry. As shown in Fig. 3A (left panel) the ratio of IgM^a/IgM^b immature B cells was approximately one, reflecting the input stem cell ratio. However, the $IgM^b:IgM^a$ ratio was markedly higher in among mature B cells in the spleen, averaging ~8 fold. Control mice that received non-mixed stem cells had normal numbers of splenic B cells (either IgM^a or IgM^b) (data not shown). The majority of 2-12H/Vκ4 B cells in the chimera spleen were mature Fo mature B cells indicating that the anti-Sm B cells that populated the spleen were able to mature. Thus, high affinity anti-Sm B cells are unable to compete with non-Tg B cells and are presumably eliminated.

Splenic B cell transfers into non-irradiated non-Tg littermates confirmed the non-competitiveness of 2-12H/Vκ4 B cells. Purified non-Tg and 2-12H/Vκ4 B cells were mixed at a 1:1 ratio, labeled with CFSE, and transferred i.v. into non-Tg littermates. The spleens were analyzed 3 and 7 days later. Spleen cells were stained with anti-B220 and IgM^a. CFSE cells were gated and IgM^a B cell numbers determined, as shown in Fig. 3B. As expected, fewer non-Tg B cells were seen in the spleen at day 3 than were transferred, due either to elimination or to trafficking to sites other than the spleen. However, the numbers of non-Tg B cells remained high at day 7. In contrast, the number of 2-12H/Vκ4 B cells present in the spleen at day 3 was significantly lower than seen for non-Tg B cells, and the number decreased significantly by day 7. The difference in cell number was not due to cell division of non-Tg B cells after transfer, since there was no dilution of CFSE in B cells of either population at day 3 or day 7 (data not shown). Taken together, the B cell transfer and bone marrow chimera experiments show that in contrast to low affinity anti-Sm B cells, high Sm binding 2-12H/Vκ4 B cells are not competitive in a polyclonal environment.

2-12H/Vk4 B cells were not follicularly excluded.

Anergic B cells of other specificities, such as anti-HEL and anti-dsDNA B cells, are similarly defective in survival in the presence of a normal non-Tg repertoire [9, 12]. This defect correlated with an inability to access B cells follicles and therefore these cells were found at the T/B boundary due to a decrease in CXCR5 expression, a chemokine responsible for B cell migration into the follicle. To determine whether 2-12H/Vκ4 B cells were similarly regulated, we measured CXCR5 expression levels. High affinity anti-Sm B cells expressed normal to high levels of CXCR5 as compared to non-Tg B cells (Fig. 4A). This was seen on both Fo and MZ B cells. Although expression levels were normal, it is possible that the receptors were not functional, or that other receptors could still impede the migration of 2-12H/Vκ4 B cells to the follicles. Therefore, $1x10^7$ 2-12H/Vκ4 B cells were transferred i.v. into non-Tg littermates and their ability to enter B cell follicles by immunohistochemistry. As shown in Fig. 4B, 24 hours after transfer 2-12H/Vκ4 B cells were distributed throughout the follicles. Thus, the inability to compete with non-autoreactive B cells is independent of the ability to access B cell follicles.

2-12H/Vκ4 anti-Sm B cells are defective in BAFF-mediated cell survival.

To investigate the mechanism for the non-competitiveness of 2-12H/Vκ4 B cells, we assessed spontaneous cell death in the absence of any stimuli *in vitro*. As shown in Fig. 5A, anti-Sm B cells from either 2-12H or 2-12H/Vκ4 mice died significantly faster than non-Tg B cells, and 2-12H/Vκ4 more so than 2-12H B cells. Thus, in the absence of any stimuli, anti-Sm B cells undergo accelerated cell death.

Since BAFF plays an important role in B cell survival and anergic anti-HEL B cells require greater amounts of BAFF to survive, we tested the ability of 2-12H/Vκ4 B cells to be rescued by BAFF *in vitro*. 2-12H/Vκ4, 2-12H, and non-Tg splenic B cells were cultured in increasing amounts of BAFF for 72hs and the percentage of live cells determined. Both 2-12H and 2-12H/Vκ4 B cells showed partial rescue compared to unstimulated cells, but their survival rates were still significantly lower than non-Tg B cells (Fig. 5B). 2-12H/Vκ4 B cells displayed the highest death rate, with only an average of 15% of the cells surviving at 3 days, compared to 60% and 30% for non-Tg and 2-12H B cells, respectively. Thus anti-Sm B cells are responding poorly to BAFF, which is not overcome by increased BAFF levels.

2-12H/Vκ4 anti-Sm B cells are defective in BAFF signaling.

The inability to survive following BAFF stimulation could be due to a defect BAFF receptor expression. We determined the expression pattern of the three known BAFF receptors in anti-Sm B cells, BAFF-R, TACI, and BCMA (Fig. 5C). The expression level of BAFF-R was elevated on 2-12H and 2-12H/Vκ4 Fo B cells and more so on MZ B cells compared to the corresponding B cells of non-Tg mice. On the other hand, in comparison to non-Tg B cells TACI levels were significantly reduced on 2-12H and 2-12H/Vκ4 Fo and MZ B cells. No expression difference in BCMA was observed between non-Tg and 2-12H and 2-12H/Vκ4 B cells of either subset. Thus, 2-12H/Vκ4 anti-Sm B cells express normal or slightly higher levels of BAFF-R and lower than normal TACI levels suggesting that the minimal response to BAFF is not due to a defect in receptor expression, pointing to a defect in BAFF signaling.

DISCUSSION

Previous and current findings show that high and low affinity anti-Sm B cells are outwardly normal. In the absence of competition they show no sign of central deletion or developmental arrest, have a normal turn-over rate, express normal levels of surface IgM, and differentiate to a mature B cell stage, high affinity to Fo, MZ, and peritoneal B-1, and low affinity to Fo (Fig. 1 and data not shown) [27]. Neither high nor low affinity B cells exhibit evidence of receptor editing based on the lack of lambda expressing B cells (data not shown). Nevertheless, they are anergic, indicated by an inability to secrete antibody in response to LPS or CpG (Fig. 2A).

The ability of anti-Sm B cells to differentiate to a mature B cell stage and remain anergic contrasts with anergic anti-HEL B cells. Anergic anti-HEL B cells normally arrest at the transitional 2 and 3 stage and undergo cell death [11]. However, Oliver et al. have shown that if cell death is blocked by a deficiency in the pro-apoptotic protein Bim, differentiation to the Fo B cell stage occurs and anergy is abrogated suggesting an association between the ability to reach the mature B cell stage and loss of anergy [34]. The analysis of 2-12H/Vκ8 and 2-12H/Vκ4 B cells indicates that differentiation to a mature B cell stage and anergy are separately regulated rather than causal. The differences between the tolerizing antigens in these two systems are substantial and likely contribute to the differences in association of anergy and maturation. For example, HEL is a soluble protein antigen, whereas Sm is present on the outer leaflet of the plasma membranes of apoptotic cells, which would increase the avidity of the BCR/Ag interaction [35]. In addition, Sm is a component of a ribonucleoprotein that contains RNA, a potential ligand for TLR7. In fact, TLR7 is required for spontaneous development of an anti-Sm response in autoimmune mice [36, 37]. Thus, in

addition to chronic BCR signals, anti-Sm B cells may receive chronic TLR signals, which may have a different outcome on the mechanism of tolerance than a chronic BCR signal alone. Crosstalk between the BCR and TLR signaling pathways occurs, since chronic BCR signaling abrogates TLR responsiveness through an ERK-mediated molecular switch [38]. However, the reciprocal has not been tested, and thus it will be of interest to determine the effect of chronic TLR signaling on tolerance induction.

Despite differentiation to a mature B cell, high affinity anti-Sm B cells are not competitive with non-Tg B cells for survival *in vivo*. The competitive loss of 2-12H/Vκ4 B cells occurs in the spleen, since non-Tg and 2-12H/Vκ4 B cell numbers are equal in bone marrow chimeric mice (Fig. 3). The few splenic 2-12H/Vκ4 B cells present in the bone marrow chimeras are Fo mature B cells suggesting that the competitive loss occurs at a mature B cell stage. Thus, as with anergy induction, differentiation to a mature B cell and competitive elimination are controlled separately. Studies of the mechanism of competitive elimination for anergic anti-HEL B cells have revealed that the B cell maturation and survival factor BAFF is limiting for anergic B cells, and is responsible for their competitive elimination. Anergic anti-HEL B cells require higher BAFF levels for survival and thus when cell numbers are low, as in anergic anti-HEL transgenic model, the amount of BAFF per anergic anti-HEL B cell is sufficient for survival. However, when the number of B cells is high, as in chimeric mice, the amount of BAFF per B cell is lower and below the threshold for anergic B cell survival [17, 18]. A similar mechanism may underlie the competitive elimination of 2-12H/Vκ4 anti-Sm B cells. However, that 2-12H/Vκ4 B cells are largely minimal response to BAFF in an *in vitro* survival assay seemingly contradicts this explanation. The percentage of surviving 2-12H/Vκ4 B cells in the presence of BAFF does

not increase beyond 15-20%, even at concentrations as high as 900 ng/ml. In contrast, anti-HEL B cell survival reaches 50-60% survival with just 100 ng/ml of BAFF. Thus, BAFF may not be the limiting factor for 2-12H/Vκ4 survival, since even high BAFF concentrations may be insufficient to restore *in vivo* survival. Although we cannot exclude that the small survival advantage afforded by BAFF may be sufficient to ensure a normal life span to 2-12H/Vκ4 B cells, anti-HEL B cells, which are more responsive to BAFF *in vitro*, have only a half-life of one-week. Thus, another factor may control 2-12H/Vκ4 B cell competitiveness. One major difference between the HEL and the Sm models is the form of the antigen. While HEL is in a soluble form Sm is most likely found on the surface of apoptotic cells or phagocytosing cells. This difference could result in a continuous binding of antigen by HEL B cells while anti-Sm B cells might have sporadic encounters Sm rather than continuous interaction. Another difference between HEL and Sm autoantigens that may be relevant in this regard is the association of the Sm autoantigen with TLR ligands. Thus, anti-Sm B cells may receive chronic TLR signaling, which could inhibit the BAFF survival pathway.

The ability of 2-12H/Vκ4 B cells to become mature Fo and MZ B cells, whether subject to competition or not, implies a responsiveness to BAFF. BAFF is required for Fo and MZ B cell differentiation, and B cell differentiation arrests at a transitional stage in BAFF-deficient mice. Thus, 2-12H/Vκ4 B cells must be competent to receive BAFF differentiation signals. Oliver et al. originally demonstrated independent regulation of BAFF-induced differentiation and survival and that B cells are more sensitive to BAFF for differentiation than for survival [34]. Thus, our findings with 2-12H/Vκ4 B cells predicts that the defect in BAFF responsiveness is with only the survival pathway, such as the Akt and

Pim2 pathways leading to upregulation of the anti-apoptotic protein Mcl-1 and the NF-κB activation pathway [22, 39, 40]. We are currently testing these possibilities.

Despite the anergy and competitive elimination of anti-Sm B cells, high affinity anti-Sm cells, like low affinity anti-Sm B cells, are not excluded from entry into the follicle [27]. Follicular entry of these cells is likely due to high CXCR5 expression, the receptor for the follicular chemokine CXCL13/BLC that is present in follicles and important for B cell retention in the follicle [41, 42]. We observed no change in CCR7 levels (data not shown), the receptor for CCL19/ELC and CCL21/SLC expressed in T cell areas. Changes in the relative levels of these two chemokine receptors are responsible for B cell migration to the T cell area following antigen stimulation [43]. Within hours of stimulation CCR7 levels increase leading to the B cell migration toward T cell areas, even without a concurrent decrease in CXCR5 levels. A shift in the balance of CXCR5 and CCR7 expression is responsible for the follicular exclusion of anergic anti-HEL B cells [16], although in contrast to 2-12H/Vκ4 B cells, by a decrease in CXCR5 expression leading to their retention at the T-B boundary

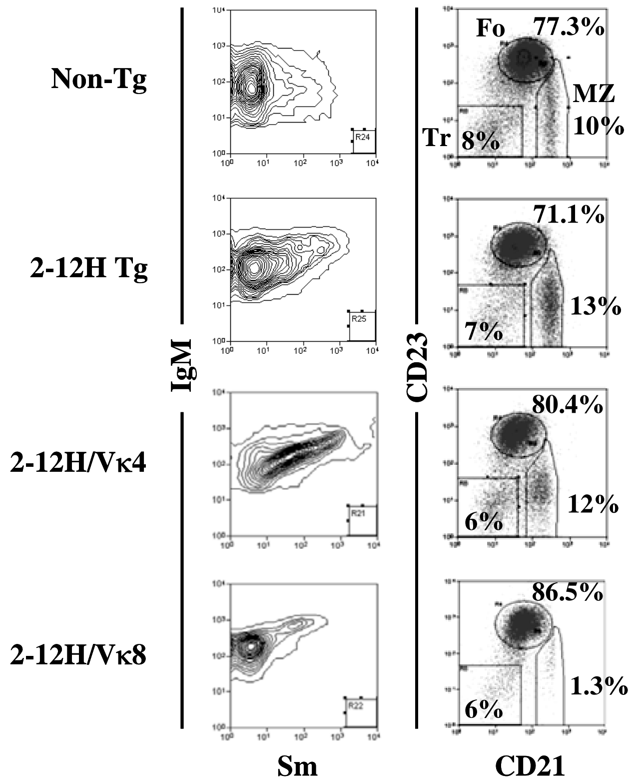
Overall, our findings reveal multi-layered regulation of anti-Sm B cells. First, affinity for Sm is a determining factor, which must be between that of the 2-12H/Vκ8 BCR and below that of the 2-1H/Vκ4 BCR. Second, B cells above a certain threshold are competitively eliminated before becoming mature B cells, and third, above an affinity threshold they are regulated by anergy. That some high affinity anti-Sm B cells escape competitive elimination in the bone marrow chimeric mice and differentiate to the mature Fo B cell stage highlights the importance of redundancy in the regulation of high affinity anti-Sm B cells. An important aspect of the anergy of 2-12H/Vκ4 B cells is that they are not activated by either LPS or CpG and are repressed by IL-6 produced by dendritic cells or

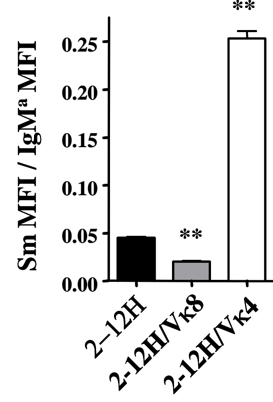
macrophages (Fig. 2). Thus, recruitment into a compartment with greater exposure to these TLR ligands will not result in inappropriate activation.

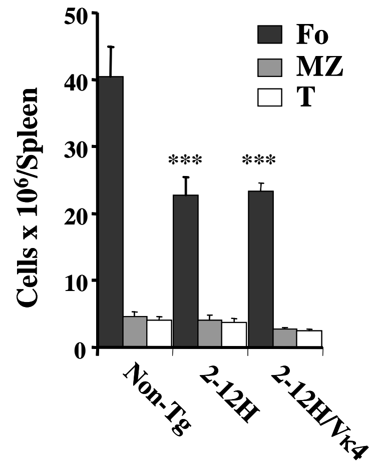
The findings reported here indicate that B cells specific for a self-antigen targeted in mouse and human SLE are regulated by a form of anergy different in multiple respects from that exhibited to neo-self antigens such as HEL. These differences likely reflect important differences in the nature and concentration of antigen pointing to the importance of understanding the complex interplay of signals through multiple receptors on the B cell, particularly BCR, BAFF-R, and TLRs, given the importance of both BAFF and TLR signaling to B cell autoimmunity [44, 45].

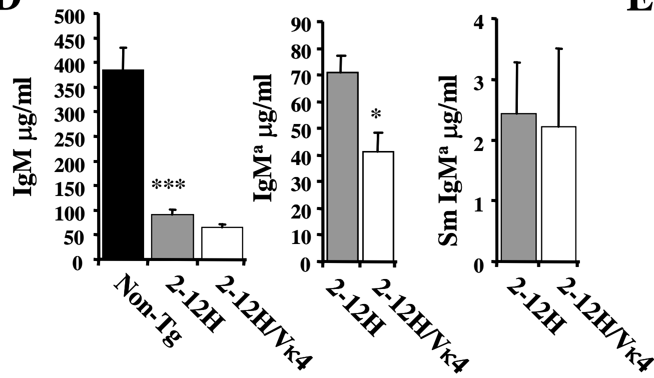
Figure 1. Phonotypical and functional analysis of splenic anti-Sm B cells from 2-12H and 2-12H/Vκ4 mice.

A, Flow cytometry analysis of splenic anti-Sm B cells subsets. Cells were gated using expression of CD21 and CD23 to define T1, FO, and MZ B cells as indicated in the *top row*. B, Relative BCR affinity assessment between the 2-12H, 2-12H/Vκ8 and 2-12H/Vκ4 anti-Sm B cells. C, Splenic B cell numbers were determined by flow cytometry for the mice indicated. All mice were 2–5 mo of age (n=9). D, Analysis of IgM and transgene-encoded Abs in the sera of anti-Sm B cells by ELISA. Total IgM, left, total IgM^a middle and total anti-Sm IgM^a (n=6 each). E, Mature Fo Anti-Sm B cell half-life was calculated by BrdU incorporation. * Significant difference ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).


A
Non-Tg
2-12H Tg
2-12H/Vκ4
2-12H/Vκ8
IgM
Sm
CD23
CD21
Fo
77.3%
MZ
10%
Tr
8%
71.1%
13%
7%
80.4%
12%
6%
86.5%
1.3%
6%


B
Sm MFI / IgMª MFI
**
**
2–12H
2-12H/Vκ8
2-12H/Vκ4


C
Cells x 10⁶/Spleen
Fo
MZ
T
***
***
Non-Tg
2-12H
2-12H/Vκ4


D
IgM µg/ml
IgMª µg/ml
Sm IgMª µg/ml
***
*
Non-Tg
2-12H
2-12H/Vκ4

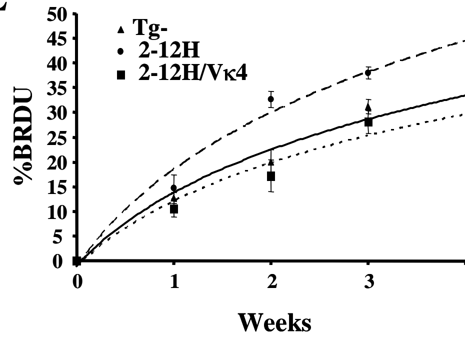

E
%BRDU
Tg-
2-12H
2-12H/Vκ4
Weeks

Figure 2. 2-12H/Vκ4 anti-Sm B cells are unresponsive to TLR stimulation and repressed by IL-6.

A, Purified B cells were stimulated *in vitro* with 10μg/ml LPS for 6 days, and supernatants were analyzed by ELISA for total IgM. B, same as A, using 1μg/ml CpG. C, Purified B cells were stimulated with LPS in the presence or absence of rIL-6. IgM secretion was determined by ELISA. * Significant difference (p<0.05), ** (p<0.01) and *** (p<0.001).

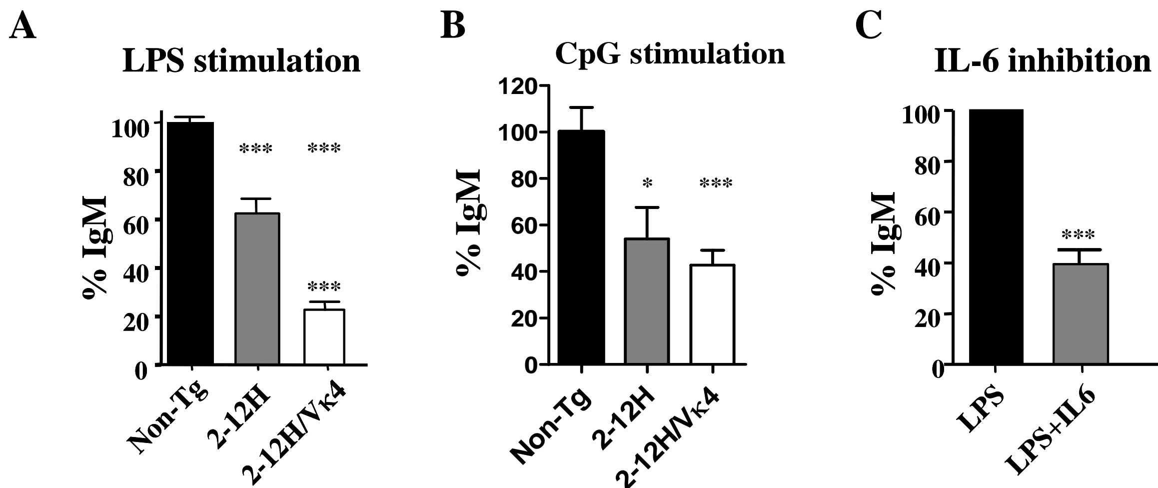


Figure 3. Anti-Sm 2-12H/Vκ4 B cells are poor competitors in the presence of non-Tg B cells.

A, BM chimeras were analyzed by flow cytometry. *Left panel* Degree of chimerism obtained in the BM of the chimeric mice. Cells were gated on $CD21^-$ IgM^+ to determine the percentage of newly formed immature B cells derived from each strain. *Right panel* P percentage of IgM^a (2-12H/Vκ4) or IgM^b (non-Tg) B cells present in the spleen (n=4). B, Purified 2-12H/Vκ4 and non-Tg B cells were labeled with CFSE, mixed together and transferred into non-Tg littermates. 3 and 7 days later number of B cells remaining were calculated to determine loss of cells.

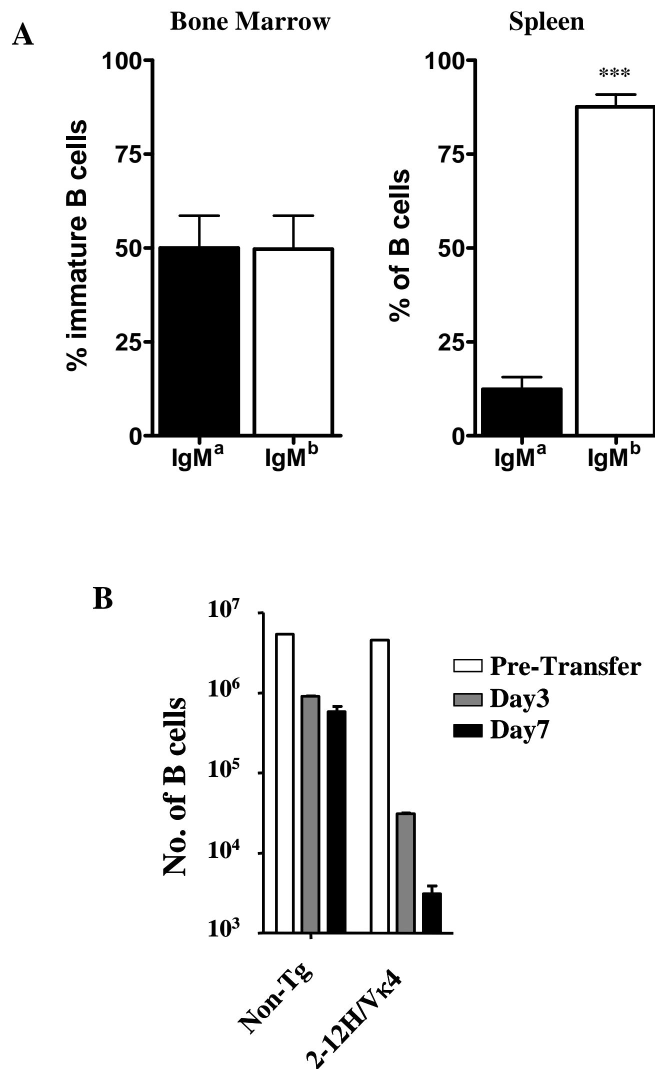
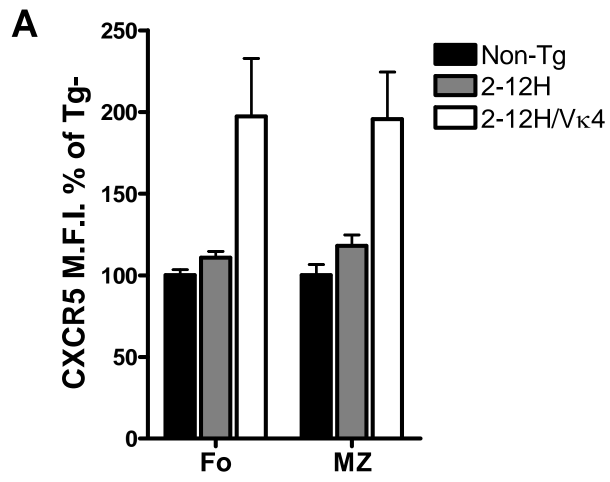


Figure 4. Anti-Sm 2-12H/Vκ4 B cells are not excluded from the follicle and express higher levels of CXCR5 than control B cells.

A, Flow cytometric analysis of CXCR5 expression on Fo and MZ B cells. B, 2-12H/Vκ4 B cells were purified and transferred into control littermates to determine their ability to enter the B cell follicle in the presence of competitor B cells. Blue (B220-APC), Red (CD3-PE), Green (IgM^a Bio-StAv AL488). *Top left* control spleen from 2-12H/Vκ4 mouse.


A
CXCR5 M.F.I. % of Tg-
250
200
150
100
50
0
Fo
MZ
Non-Tg
2-12H
2-12H/Vκ4

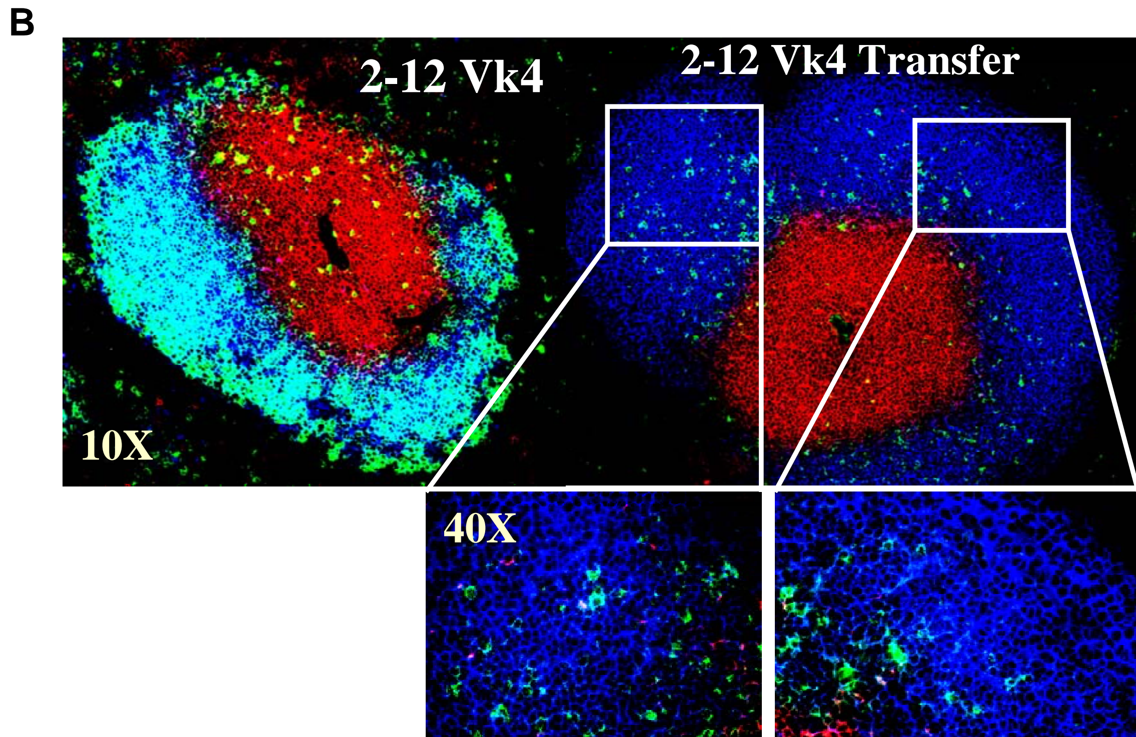
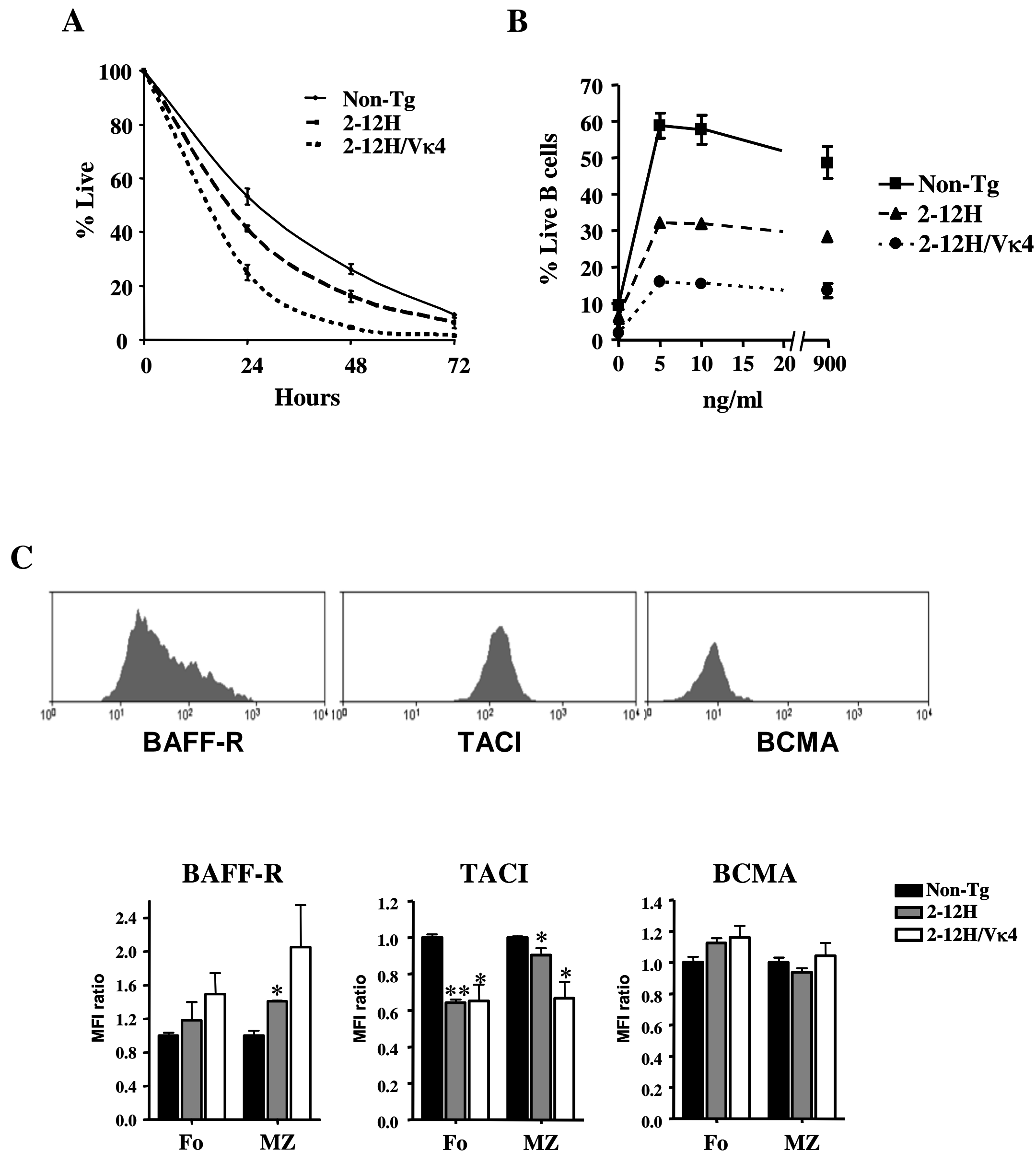

B
2-12 Vk4
2-12 Vk4 Transfer
10X
40X

Figure 5. Spontaneous cell death and BAFF responsiveness and receptor expression.

A, Purified B cells were cultured for the indicated times at which time they were collected and cell death analyzed. B, Purified B cells were cultured in the absence or presence of increasing concentrations of BAFF for 72hs later at which time cell death was assessed by flow cytometry. C, *Top pane,l* representative histograms showing staining for the there different BAFF receptors. *Bottom panel,* Compiled data normalized to non-Tg control. * Significant difference ($p<0.05$), ** ($p<0.01$)



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CHAPTER 3

An uncoupling of the BCR from its signalsome regulates high affinity anti-Sm marginal zone but not follicular B cells.

ABSTRACT

Multiple mechanisms of tolerance regulate self-reactive B cells. Encounter with self-antigen can lead B cells to a state of anergy, normally characterized by cell unresponsiveness to BCR stimulation or other activating stimuli. In chapter 2, I described mice (2-12H/Vκ4) transgenic for B cell receptors (BCRs) specific for Sm, a ribonucleoprotein antigen targeted in systemic lupus erythematosus (SLE). In these mice, anti-Sm mature B cells are anergic and Ab secretion is severely impaired, more so than 2-12H B cells. Anti-Sm 2-12H Fo B cells are anergic, but MZ B cells are functional. I hypothesize that both 2-12H/Vκ4 Fo and MZ B cells are regulated and anergic. To test this hypothesis, I determined the ability of the BCR on cells of each subset to signal in response to BCR ligation. I find that the BCRs of 2-12H and 2-12H/Vκ4 anti-Sm Fo B cells signal following BCR ligation, as measured by general protein tyrosine phosphorylation and phosphorylation of the BCR signaling intermediates Syk and ERK. However, while 2-12H MZ B cells signaled efficiently in response to BCR ligation, 2-12H/Vκ4 MZ B cells exhibit a weak protein tyrosine phosphorylation response and little increase in Syk and ERK phosphorylation. BCR ligation of 2-12H/Vκ4 Fo an MZ B cells induces rapid cell death, which can be explained by an

imbalance in pro- and anti-apoptotic proteins that favors apoptosis. 2-12H/Vκ4 MZ B cells are also unable to respond to endogenous self antigens, since in contrast to 2-12H MZ B cells, they do not constitutively generate anti-Sm pre-plasma cells and are not activated by an increase in apoptotic cell load. Thus, we conclude that 2-12H/Vκ4 MZ B cells are anergic due to a significant uncoupling of the BCR from its signalsome.

INTRODUCTION

Immune tolerance is paramount for the prevention of autoimmune diseases. B cells developing in the bone marrow are subject to several mechanisms of tolerance to prevent the differentiation of functional self-reactive B cells. Many autoreactive B cells undergo differentiation and are regulated by anergy characterized by a state of non-responsiveness to BCR-mediated activation [1, 2, 3, 4, 5, 6]. In some cases, anergic B cells have low BCR levels, a short life span, are excluded from the follicles, and are unable to induce a normal BCR signaling cascade [7, 8, 9, 10]. Recently, anergic B cells have been shown to arrest at a transitional 3 stage (although thought to be incorrect and renamed An1) of differentiation and are not mature Fo B cells [11]. However, other anergic B cells have BCRs that are capable of signal transduction, have normal BCR levels, and normal life spans [11, 12, 13, 14]. These contrasting characteristics could be explained by differences in the form of the self-antigen, as the level of self-antigen, when antigen is encountered during differentiation, or the affinity of the BCR.

Why some anergic B cells are unable to tranduce a BCR signal is still unclear. One possibility is that chronic antigen engagement of the BCR results in a physical dissociation of the Igα and Igβ signaling components of the BCR from Ig, thereby preventing antigen from initiating signal transduction [15, 16, 17, 18]. However, this cannot be the only mechanism, as in the case of anergic anti-insulin and anti-ssDNA B cells, signal transduction (cell phosphorylation levels) occurs normally following BCR ligation [9, 12]. Partial defects in signal transduction could result in B cell anergy and explain the differences between anergic B cells of different specificities. For example, anergic anti-insulin B cells are defective in Ca^{2+} mobilization and NF-AT expression and translocation to the nucleus [12]. In addition, the

balance between pro and anti-apoptotic protein expression is an important factor regulating B cells survival [19, 20, 21, 22, 23, 24]. Anti-HEL B cells deficient in the pro-apoptotic protein Bim are able to respond to stimulation and are not anergic [25]. The expression of pro- and anti-apoptotic proteins is regulated by multiple pathways such as NF-κB and Akt [26, 27, 28, 29].

Previous analyses of B cell anergy have generally considered B cells as a single homogeneous population, since in most cases the self-reactive B cells belonged to a single subset. However, some autoreactive B cells are selected into multiple B cell subsets, and the B cells of each have different requirements for activation [30, 31]. This is clearly the case for B cells specific for the self-antigen Sm [32, 33, 34]. Sm is a ribonucleoprotein that is frequently targeted by the immune system in systemic lupus erythematosus (SLE). Its presence in human SLE is associated by renal disease and a poor prognosis [35, 36]. Analysis of anti-Sm B cells in 2-12H Tg mice indicates that anti-Sm B cells are present as transitional, follicular (Fo), marginal zone (MZ), B-1, and pre-plasma cells (pre-PCs), and that these cells are regulated differently. Whereas, 2-12H Fo B cells are anergic, anti-Sm MZ and B-1 B cells are functional and can be activated by self-antigen [33, 37, 38]. In addition, some anti-Sm B cells are activated to become pre-PCs before undergoing apoptosis [32]. Thus, for some autoreactive B cells regulation is determined by their subset identity.

In the previous chapter, we demonstrated that anti-Sm B cells mice carrying the 2-12H transgene and a Vκ4 L chain transgene are selected into mature Fo and MZ B cell subsets, but in contrast to 2-12H Tg mice, are not activated by toll-like receptor (TLR) agonists, and therefore are anergic. To understand the mechanism for anergy of anti-Sm B cells, and how these Fo and MZ B cells differ, I have investigated the ability of these cells to transduce a signal through their BCRs. I find that Fo and MZ B cells differ in their ability to

signal transduce through their BCRs. Since the BCRs of Fo and MZ B cells are identical in these mice, these data indicate that B cell subset influences the mechanism of anergy.

MATERIALS AND METHODS

Mice

Anti-Sm 2-12H Tg mice and 2-12H/V*k*4 double Tg mice have been described previously (Chapter 2) [34]. *Mer*^{kd} mice were obtained from Dr. G. Matsushima (University of North Carolina at Chapel Hill) and bred with 2-12H Tg and Vκ4 Tg mice. 2-12H/V*k*4 *Mer*^{kd} Tg mice were subsequently generated by crossing 2-12H *Mer*$^{kd+/-}$ Tg and Vκ4 *Mer*$^{kd+/-}$ Tg mice.

Flow Cytometry

Single-cell suspensions were prepared and analyzed as described in chapter 2. The Abs specific for IgM, B220, CD21, CD23 and CD138 were obtained from BD Biosciences.

For intracellular staining of pro and anti-apoptotic proteins, after the indicated times, cells were fixed with 2% PFA (Electron Microscopy Sciences) and permeabilized with BD Perm/Wash™ buffer (BD Biosciences) containing saponin. Anti-Bim (poly Rab) and anti-Bax (Ms IgG1 6A7) were obtained from BD Biosciences and Bcl-X_L PE (SC-8392) was purchased from Santa Cruz Biotechnology. Anti-Rabbit Alexa647 and anti-Ms IgG1 Alexa488 were obtained from Probes-Invitrogen.

Cell purification

Splenic B cells were isolated by negative selection using IMag™ Streptavidin Particles Plus (BD Biosciences) following manufacturers protocol for magnetic labeling and separation as described in Chapter 2. B cells were 92–98% pure as determined by flow cytometry.

In vitro stimulation

Purified B cells were ($1x10^6$/ml) were cultured in 24 well plates in complete RPMI containing 10% FCS, L-glutamine, penicillin, streptomycin, and 2-mercaptoethanol. For proliferation analysis B cells were labeled with CFSE (Probes-Invitrogen) at a concentration of 1 μM for 10 min at room temperature.

Cells were stimulated with 10 μg/ml of $F(ab')_2$ fragment goat anti-mouse IgM (Jackson Immunoresearch) or left unstimulated. For survival and proliferation analysis cells were stimulated for 72hs then washed and stained with LIVE/DEAD dead cell stain kit (Probes-Invitrogen). To analyze pro and anti- apoptotic protein expression B cells were cultured for the indicated times.

Cell signaling by Flow Cytometry

Cell signaling was determined by flow as previously described Nolan [39, 40, 41]. Briefly, B cells were purified and placed in RPMI with 2%FCS. Cells were left at 37°C for 30 min and the stimulated with 10 μg/ml of $F(ab')_2$ fragment goat anti-mouse IgM (Jackson Immunoresearch). At indicated times; cells were fixed with 2 % PFA for 15 min. Cells were then permeabilized for 30 min with ice cold MeOH. Cell were then washed with PBS containing 2%FCS and blocked with mAb 2.4G2 for 15 min. Cells were then stained with B220, CD21 and CD23 (BD Biosciences) and with either anti-phospho-ERK(p44/42) Ab (Cell Signaling Technology), anti-phospho-Syk or anti-phosphotyrosine (BD Biosciences) for 1 hr. Phosphorylation levels were determined by flow cytometry.

ELISAs

Quantitation of anti-Sm Abs, total IgM and IgMa in mouse serum was done by ELISA as previously described in Chapter 2

Statistical analysis

The Student *t* test was used to assess the significance of the differences between groups. A value of $p < 0.05$ was considered significant.

RESULTS

Anti-Sm B cells fail to proliferate and undergo cell death in response to BCR cross-linking.

High affinity anti-Sm 2-12H/Vκ4 B cells appear to be anergic. They are unable to survive in the presence of non-Tg B cells, probably due to a defective responsive to the pro-survival factor BAFF, and unable to secrete normal levels of antibody following LPS stimulation. B cell anergy is also characterized by an inability to proliferate in response to BCR ligation and an inability of the BCR to transducer a signal following ligation [2]. To determine whether 2-12H/Vκ4 anti-Sm B cells are responsive to BCR crosslinking, we assessed the ability of anti-IgM to induce these B cells to proliferate. Enriched splenic B cells from non-Tg, 2-12H, and 2-12H/Vκ4 mice were CFSE labeled and cultured *in vitro* for 72hs in the presence of anti-IgM. Cells were then harvested, and cell death and proliferation were determined by flow cytometry analysis. While non-Tg B cells underwent several cycles of proliferation, 2-12H and 2-12H/Vκ4 B cells underwent little if any proliferation (Fig. 1A). Moreover, most 2-12H and 2-12H/Vκ4 B cells underwent cell death upon anti-IgM stimulation (Fig. 1B). Thus, 2-12H and 2-12H/Vκ4 B cells are unable to proliferate and undergo cell death in response to BCR cross-linking.

Signaling transduction is normal in anti-Sm B cell following anti-IgM cross-linking

The lack of anti-Sm cell proliferation and consequential cell death following BCR crosslinking could be due to defective signal transduction that uncouples the BCR from downstream signaling pathways [11, 15, 16, 18]. We assessed the phosphorylation of several key proteins to determine whether anti-Sm B cells could invoke normal signal transduction following BCR stimulation [42]. We assessed general cell phosphorylation levels (p-Tyr), the

phosphorylation of an early member of the signaling pathway induced by BCR (p-Syk) and of a downstream member (p-Erk). Fo, transitional, and MZ B cells differ in the robustness of protein phosphorylation following BCR cross-linking, and therefore spleen purified B cells that vary in the representation of the these three B cell subsets could lead to inaccurate interpretations of BCR signaling ability [31]. Therefore, we examined the ability of B cells from each subset to respond separately using flow cytometry, as described by Nolan et al. [39, 40, 41]. Enriched B cells were stimulated with anti-IgM at10 μg/ml, a dose used to determine signaling defects in other anergic B cells, and protein phosphorylation was assessed over time. To control for experimental variations, non-Tg B cell phosphorylation was used as a control for each time point. Non-Tg B cells were mixed with either 2-12H or 2-12H/Vκ4 B cells at a 1:1 ratio and subjected to anti-IgM stimulation. 2-12H and 2-12H/Vκ4 B cells were distinguished from non-Tg B cells by IgM^a staining. In addition to staining with anti-phosphoprotein antibodies, cells were stained with anti-CD21, CD23 and B220 to distinguish B cell subsets (Fig. 2A). General Tyr and Syk phosphorylation was similar in magnitude and duration in Fo B cells from mice of all three mouse lines in response to anti-IgM (Fig. 2B top and middle left panel). Erk phosphorylation was similar in non-Tg or 2-12H/Vκ4 B cells, but consistently greater in 2-12H Fo B cells (Fig. 2B, bottom left). In striking contrast, 2-12H/Vκ4 MZ B cells consistently exhibited diminished general p-Tyr, p-Syk, and p-ERK levels after anti-IgM stimulation (Fig. 2B, right panels). 2-12H and non-Tg MZ B cells showed a similarly strong phosphorylation following anti-IgM stimulation. These data suggest that the BCR on 2-12H/Vκ4 MZ B cells is largely uncoupled from the BCR signalsome. This is not the case for 2-12H/Vκ4 Fo B cells indicating that BCR signal impairment in these cells likely involves a discrete branch of the BCR signaling cascade.

Thus, impaired BCR signaling by 2-12H/Vκ4 Fo and MZ B cells occurs through different mechanisms, despite the expression of identical anti-Sm BCRs.

We also assessed phosphorylation prior to stimulation to determine whether there was any increase in the basal levels, as noted with other autoreactive B cells [43]. p-Tyr and p-Syk levels were significantly higher in 2-12H and 2-12H/Vκ4 Fo B cells compared to non-Tg Fo B cells (Fig. 2C). However, p-ERK levels were similar in Fo B cells from mice of all three strains (Fig. 2C). In contrast, basal phosphorylation levels in 2-12H/Vκ4 MZ B cells were not significantly different from non-Tg MZ B cells. These data suggest that the BCRs of 2-12H and 2-12H/Vκ4 Fo B cells may constitutively engage antigen resulting in a high basal phosphorylation level of Syk and other proteins. However, this constitutive signal does result in high Erk phosphorylation. In addition, these data indicate that the 2-12H/Vκ4 MZ B cells are not constitutively signaling, consistent with the uncoupling of the BCR from its signalsome indicated above.

Anti-Sm B cells up-regulate pro-apoptotic proteins following BCR activation

Bim and Bax, two pro-apoptotic proteins, have been shown to regulate B cell induced cell death, while Bcl-X_L plays a positive role in B cell survival [19, 23, 24]. Thus, a shift in the balance of pro- and anti-apoptotic proteins to favor apoptosis in anti-Sm B cells following BCR stimulation could explain the cell death induced by anti-IgM stimulation. To examine this possibility purified B cells were stimulated with anti-IgM (10 μg/ml) for 16 and 40 hrs, and protein expression measured by intracellular flow cytometry. Expression levels of Bim and Bax were similar in the absence of stimulation in B cells from all three strains, while Bcl-X_L levels in 2-12H and 2-12H/Vκ4 B cells were somewhat lower than in non-Tg B cells

(Fig. 3). At 16 hs post-stimulation, Bim and Bax levels had increased in 2-12H and 2-12H/Vκ4 B cells and increased further by 40 hrs post-stimulation. However, Bim and Bax levels did not change in non-Tg B cells. In contrast, there was no change in $Bcl\text{-}X_L$ levels in 2-12H and 2-12H/Vκ4 B cells at 16 hrs and only a modest increase by 40 hrs that resulted in levels similar to those in non-Tg B cells (Fig. 3). Thus, BCR crosslinking of anti-Sm B cells from both 2-12H and 2-12H/Vκ4 mice induces a shift in the balance of pro- and anti-apoptotic protein expression in favor of apoptosis, consistent with their inability of these cells to survive after BCR cross-linking (Fig. 1).

2-12H/Vκ4 B cells were defective in response to endogenous autoantigen in vivo

We recently described a population of pre-PCs that are characterized by intermediate levels of the CD138 ($CD138^{int}$) [32]. They appear to differentiate in response to antigen stimulation, since they are not generated in the absence of antigen and are reduced in number in mice with defective BCR signal transduction. Anti-Sm pre-PCs are regulated to prevent differentiation to the antibody secreting cell (ASC) stage in non-autoimmune mice, but not in autoimmune mice. To determine whether the anti-Sm B cells of 2-12H/Vκ4 mice can differentiate to the $CD138^{int}$ pre-PC stage in response to endogenous autoantigen, we determined the frequency of anti-Sm pre-PCs in these mice by flow cytometry. We observed a ~4-fold reduction in the frequency of $CD138^{int}$ pre-PCs in the spleens of 2-12H/Vκ4 mice compared to 2-12H and non-Tg mice (Fig. 4). Thus, activation of 2-12H/Vκ4 B cells by endogenous autoantigen is impaired, consistent with the defects in BCR signaling identified by anti-IgM stimulation.

The inability to respond to endogenous autoantigen was further tested by introducing a defect in apoptotic cell clearance into 2-12H/Vκ4 mice. Apoptotic cells expose Sm antigen on their surface and induce an anti-Sm response in non-Tg and 2-12H mice. Moreover, mice with a defect in the *Mer* gene (*Mer*^{kd}) have a macrophage defect in the phagocytosis of apoptotic cells, which results in a mild lupus-like disease [44, 45, 46] and the constitutive production of anti-Sm antibodies in 2-12H mice [33]. To determine whether higher levels of apoptotic cell antigens resulting from a defect in *Mer*^{kd} could induce activation of 2-12H/Vκ4 B cells, we generated 2-12H/Vκ4/*Mer*^{kd} mice. As shown in Fig. 5, 2-12H/Vκ4 mice with and without the *Mer*^{kd} mutation had similar frequencies of Fo, MZ and Tr B cells, indicating that *Mer*^{kd} had not grossly affected differentiation to the Fo and MZ B cell subsets. Serum was collected from mice at 6 months of age and IgM and anti-Sm levels determined by ELISA. As shown in Fig. 5B, 2-12H/Vκ4 and 2-12H/Vκ4 *Mer*^{kd} mice did not differ in IgM or anti-Sm antibody levels. Thus, unlike 2-12H anti-Sm B cells, the anti-Sm B cells of 2-12H/Vκ4 mice were not activated by defective clearance of apoptotic cells.

DISCUSSION

In this study we have established that anti-Sm B cells are regulated in part through a defect in BCR signaling. Anti-Sm B cells mature to the Fo and MZ B cell stages in both 2-12H and 2-12H/Vκ4 mice and they have comparatively normal half-lives. However, we show here that 2-12H/Vκ4 Fo and MZ B cells undergo cell death in response to anti-BCR stimulation rather than proliferate, as occurs with non-Tg B cells. The failure to respond to anti-BCR stimulation reflects an *in vivo* defect in activation. 2-12H/Vκ4 mice have few anti-Sm pre-plasma cells, which are antigen dependent, and the Mer^{kd} defect does not induce a chronic anti-Sm response. In contrast, 2-12H mice have a large anti-Sm pre-plasma cell population and the Mer^{kd} mutation induces a chronic anti-Sm response [32, 33]. This defect in BCR-mediated activation is in addition to the inability to secrete normal levels of Abs to LPS and CpG by anti-Sm B cells of both strains (Chapter 2), and thus, anergy in anti-Sm B cells affects both TLR and BCR activation. These characteristic are similar to those displayed by other anergic systems, such as the anti-HEL and anti-dsDNA, although B cells of both specificities also show defects in B cell development and activation. In contrast, anti-ssDNA B cells, which do not undergo developmental arrest, are able to proliferate following anti-BCR stimulation [9]. Anti-insulin B cells show the greatest similarity to 2-12H/Vκ4 anti-Sm B cells. They differentiate to Fo and MZ B cells, but are unable to undergo BCR induced proliferation [12, 13]. Thus, there are multiple forms of anergy, and which is exhibited by a given B cell is dependent on the form of the antigen and affinity of the BCR for the antigen.

The failure of anti-Sm Fo B cells to undergo proliferation following anti-IgM stimulation is not due to a general uncoupling of the BCR from its induced intracellular signal, as has been suggested for B cells of other self-specificities [18]. This is evident from

general p-tyrosine induction following anti-IgM treatment, and most importantly, by levels of phosphorylated Syk and Erk, an early and intermediate event in the signaling cascade, respectively, that equals or exceeds the levels in non-Tg B cells. Thus, 2-12H/Vκ4 Fo B cells are able to initiate the BCR signaling cascade indicating that, rather than a general uncoupling of the BCR from the signalsome, anergy is mediated by disruption of one or more signaling pathways from the BCR. An example of this is seen in anti-insulin B cells where BCR induced phosphotyrosine is normal, but there is a defect in IP3 metabolism and a reduced basal NFATc1 expression and activation.

Pathways leading to proliferation and survival are likely disrupted in anti-Sm Fo B cells of 2-12H and 2-12H/Vκ4 mice, although the failure to proliferate could be secondary to the inability to deliver a survival signal. In support of this latter view, the few surviving 2-12H and 2-12H/Vκ4 B cells after IgM stimulation have undergone one or more divisions, as well as have some cells prior to apoptosis, (Fig. 1A) suggesting that the anti-Sm BCR is able to signal B cell proliferation. Cell death appears to be mediated by the pro-apoptotic proteins Bim and Bax, since anti-IgM stimulation induces an increase in these molecules in anti-Sm B cells, but not non-Tg B cells. Bim expression in particular has a significant role in BCR induced cell death, since Bim-deficient B cells are refractory to activation induced cell death following anti-IgM stimulation [47, 48], and anergic anti-HEL B cells that lack Bim undergo maturation to the Fo B cell stage and break tolerance [25]. In addition to alteration of pro-apoptotic protein production, anergic anti-Sm B cells also exhibited altered production of the anti-apoptotic protein Bcl-X_L that might have counterbalanced the effects of Bim and Bax. Bcl-X_L binds pro-apoptotic proteins inhibiting their function. The anti-Sm BCR may be unable to activate phosphoinositol-3-kinase (PI3K) leading to activation of the Akt survival

pathway and a failure to inhibit the upregulation of pro-apoptotic gene expression [28, 29]. Furthermore, the effect of defective PI3K activation can be observed in CD19KO B cells which are defective in proliferation and survival following BCR stimulation probably due to defective Akt and Ca^{2+} activation [49, 50]. Alternatively, a failure of NFκB activation could lead to a lack of Bcl-X_L upregulation, leaving the cells unable to be rescued from BCR induced death [27]. Whatever the upstream defect may be, it is clear from these data that anti-Sm Fo B cells are intrinsically wired to undergo cell death following BCR engagement.

The biochemical mechanism of regulation of anti-Sm MZ B cells from 2-12H/Vκ4 mice is different from that for Fo B cells from these mice. 2-12Vκ4 MZ B cells show reduced overall protein phosphorylation, as well as reduced levels of phosphorylated Syk and Erk compared to non-Tg and 2-12H MZ B cells (Fig. 2). These data suggest that the BCR on anti-Sm MZ B cells of 2-12H/Vκ4 mice is uncoupled from the signalsome. Thus, anti-Sm MZ and Fo B cells are regulated at different levels in the BCR signaling cascade. This difference may be inherent in the cell type (Fo vs. MZ) rather than a function of affinity, since the 2-12H/Vκ4 Fo and MZ B cells have identical BCRs. However, we cannot rule out that MZ B cells by virtue of their location have greater access to antigen than Fo B cells leading to regulation at different levels in the signaling pathway.

Interestingly, 2-12H anti-Sm MZ B cells are not uncoupled from the signalsome, since they show similar protein phosphorylation to non-Tg MZ B cells, including Syk and Erk, suggesting that they are functional. Indeed, apoptotic cells, which expose Sm on the outer leaflet of their plasma membranes, induce an anti-Sm response in 2-12H mice [33], and concomitantly induce a loss of anti-Sm MZ B cells (Kara Conway, and S.H.C., unpublished observation). In addition, 2-12H MZ B cells are significant precursors to the large anti-Sm

pre-plasma cell pool (Kara Conway and S.H.C., unpublished observation) that is present in non-autoimmune mice suggesting that they are continually activated [32]. The evidence indicating that 2-12H MZ B cells have not uncoupled their BCR from the signalsome is consistent with evidence suggesting that 2-12H MZ B cells are functional. The difference between 2-12H and 2-12H/Vκ4 MZ B cells is solely with the L chain. L chain use by 2-12H/Vκ4 MZ B cells is fixed in contrast to diverse L chain use by 2-12H MZ B cells. Single cell sequencing identified over 20 different L chains expressed by anti-Sm MZ B cells (Suzanne McCray and S.H.C., unpublished observation). Thus, affinity for Sm is likely the determining factor for the difference in regulation between the MZ B cells of these two mouse strains.

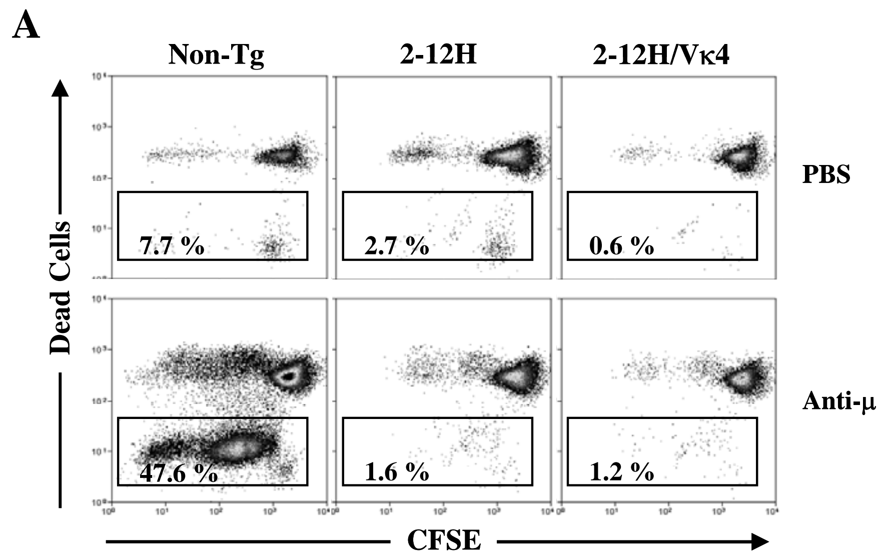
ERK signaling is proposed to be a molecular switch to prevent ASC differentiation by anergic B cells upon TLR stimulation [51]. The basal phosphorylation level of ERK in anergic anti-HEL B cells is elevated compared to naïve anti-HEL B cells preventing differentiation to ASCs by CpG or LPS stimulation and inhibition of ERK phosphorylation restores responsiveness to CpG and LPS [51, 52]. The block in LPS and CpG activation by 2-12H/Vκ4 B cells (Chapters 2 and 4) is unlikely to be mediated by this mechanism, since 2-12H/Vκ4 Fo B cells do not exhibit elevated basal ERK phosphorylation levels. Basal phosphorylated Syk levels and general protein phosphorylation levels are elevated in 2-12H/Vκ4 Fo B cells confirming chronic stimulation through the BCR. MZ B cells do not show elevated general protein phosphorylation or Syk and ERK phosphorylation consistent with the evidence that their BCRs are uncoupled from the signalsome. The conclusion that high basal ERK phosphorylation is not involved is reinforced by the observation that inhibition of ERK phosphorylation does not restore responsiveness to LPS or CpG (Chapter 4). Thus, the ERK

molecular switch mechanism for blocking TLR-mediated activation may not be a generalizable phenomenon and may be dependent on any number of variables, such as the affinity/avidity of the BCR, antigen concentration, and coincident signals through other receptors.

In summary, antigen activation of anti-Sm B cells from 2-12H/Vκ4 mice is defective both in vitro and in vivo, but that biochemical mechanism is different for anti-Sm Fo and MZ B cells. The BCRs on anti-Sm Fo B cells are signaling competent, but are defective in delivering a cell survival signal to block antigen activated cell death caused by the upregulation the pro-apoptotic proteins Bim and Bax. In contrast, the BCR of anti-Sm MZ B cells from these mice is uncoupled from its signalosme preventing the delivery of a signal, even Syk phosphorylation, a membrane proximal signaling event. This uncoupling is not evident in anti-Sm MZ B cells of 2-12H mice implying a role for affinity in uncoupling. Neither Fo, nor MZ B cells, from these mice show high basal ERK phosphorylation arguing against the involvement of the ERK signaling molecular switch in preventing TLR-mediated activation. Thus, we propose the existence of an alternative biochemical pathway for blocking TLR activation of autoreactive B cells that may be more physiologically relevant to anti-self B cells specific for antigens targeted in systemic lupus erythematosus and other autoimmune diseases.

Figure 1. Responses of 2-12H and 2-12H/Vκ4 B cells to *in vitro* anti-IgM stimulation.

A, Representative data of proliferative responses of CFSE-labeled B cells from wild-type and anti-Sm Tg mice incubated for 72 hr in the presence of 10 μg/ml $F(ab')_2$ anti-mouse IgM. Cell divisions as assessed by CFSE dye dilution. B, Cell survival as determined by flow cytometric analysis on isolated splenic B cells cultured in the presence of 10 μg/ml $F(ab')_2$ anti-mouse IgM for 72 hr. Cells negative for live/dead staining (y axis A) were scored as viable. Numbers show percentage of viable cells. Results represent the mean of three mice. Significant difference ** ($p<0.01$) and *** ($p<0.001$).



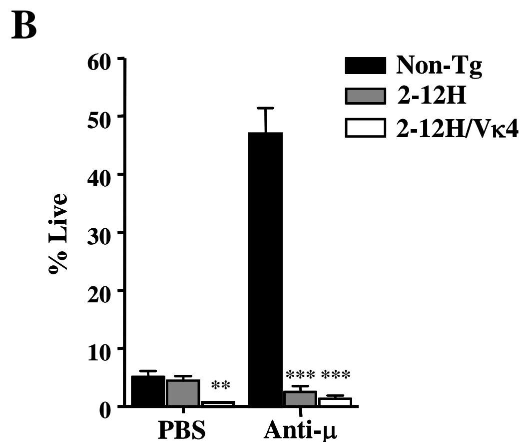
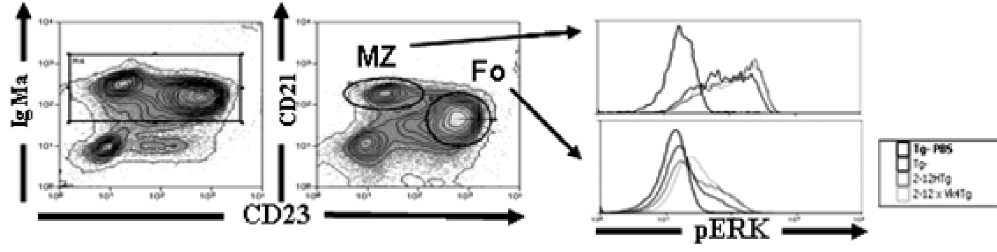
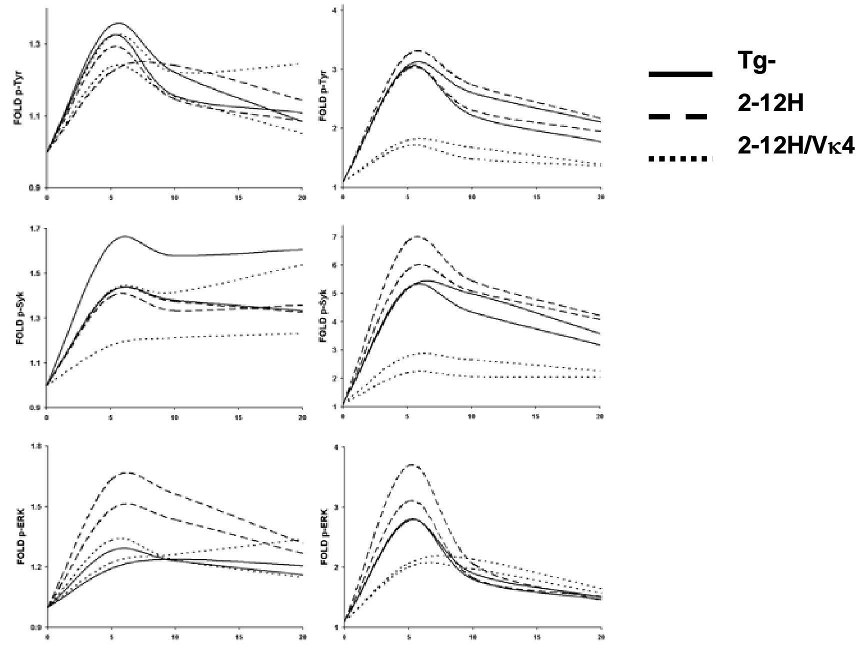


Figure 2. Subset-specific BCR-mediated signaling analysis of anti-Sm B cells by flow cytometry.

A, Purified B cells from each strain were stimulated as in Fig 1 for 5, 10, 15 and 20 minutes or unstimulated, fixed with formaldehyde, and permeabilized with methanol. Cells were then stained with the anti phospho-protein of interest plus IgM, CD21 and CD23 to distinguish subsets and analyzed by flow cytometry. *Top panel* shows example of gating scheme used to determine protein phosphorylaton in Fo and MZ B cells following stimulation (5min). *Bottom*, p-tyr (top), p-Syk (middle), p-ERK (bottom) phosphorylation kinetics was assessed. Fold increase over M.F.I. of unstimulated cells (0 min) was plotted. Data is representative of two independent experiments with two mice of each strain used per experiment. B, Basal phosphorylation levels of specific phospho-protein in unstimulated B cells. Data represents fold increase in MFI over unstimulated non-Tg B cells. Significant difference * ($p<0.05$) and ** ($p<0.01$).

A





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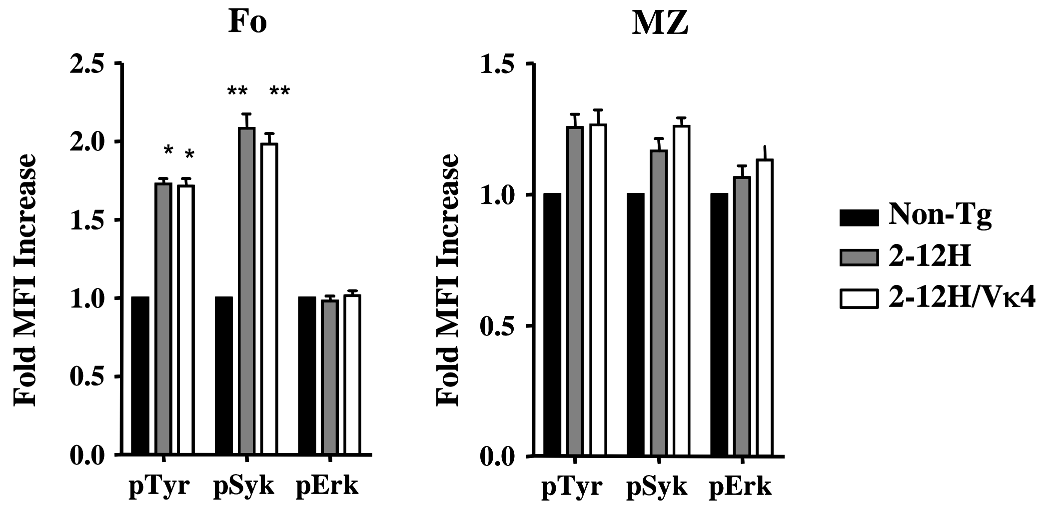


Figure 3. Flow cytometric anslysis of Bim, Bax and Bcl-X_L expression following anti-IgM stimulation.

A, Representative histogram showing changes in protein expression patterns in B cells from the indicated strains after 40hs of anti-IgM stimulation. B, Compiled data of protein expression of untreated (PBS) or anti-IgM (10 μg/ml F(ab')$_2$) treated B cells for the indicated times.

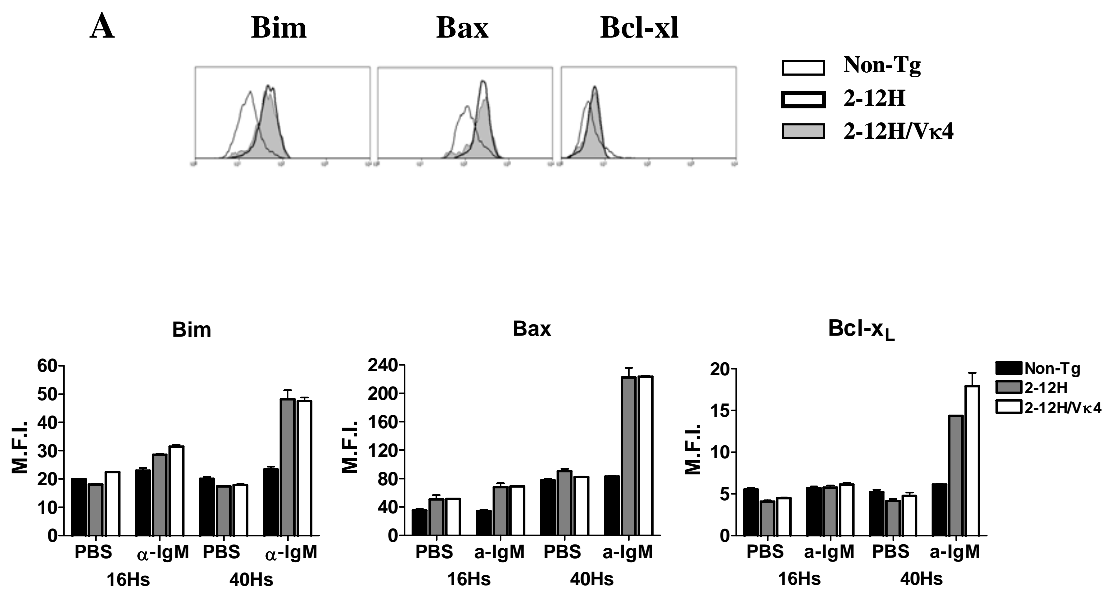
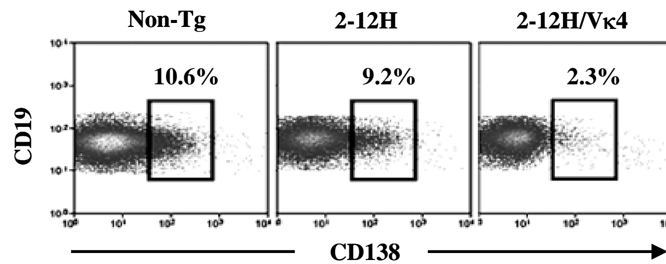


Figure 4. Analysis of CD138-expressing pre-PC B cells.

Top, representative histograms used to determine the presence of pre-PCs. Percentages are indicated. *Bottom*, Frequency of pre-PCs cells among total B cells (n ≥ 5). Significant difference * (p<0.05) and *** (p<0.001).



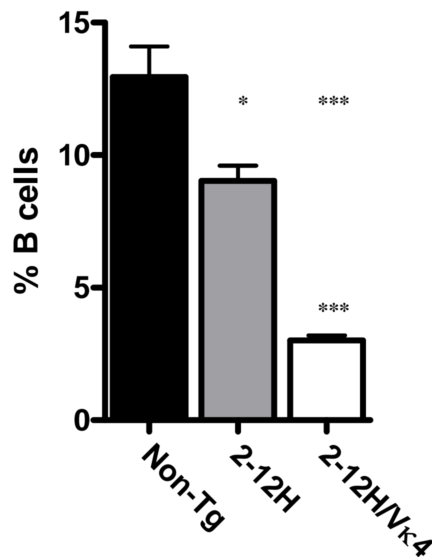
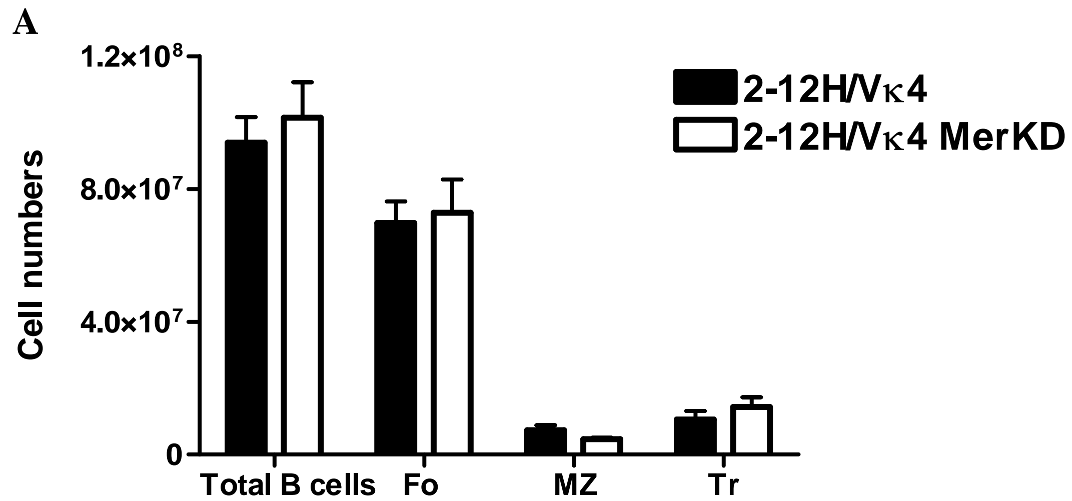


Figure 5. Analysis of 2-12H/Vκ4/*Mer*^{kd} mice.

A, Comparison of total, Fo, MZ and Tr B cell numbers in 2-12H/Vκ4 (n=11) and 2-12H/Vκ4/*Mer*^{kd} (n=8) mice. B, Serum total IgM^{a} and anti-Sm IgM^{a} levels were measured by ELISA.

A



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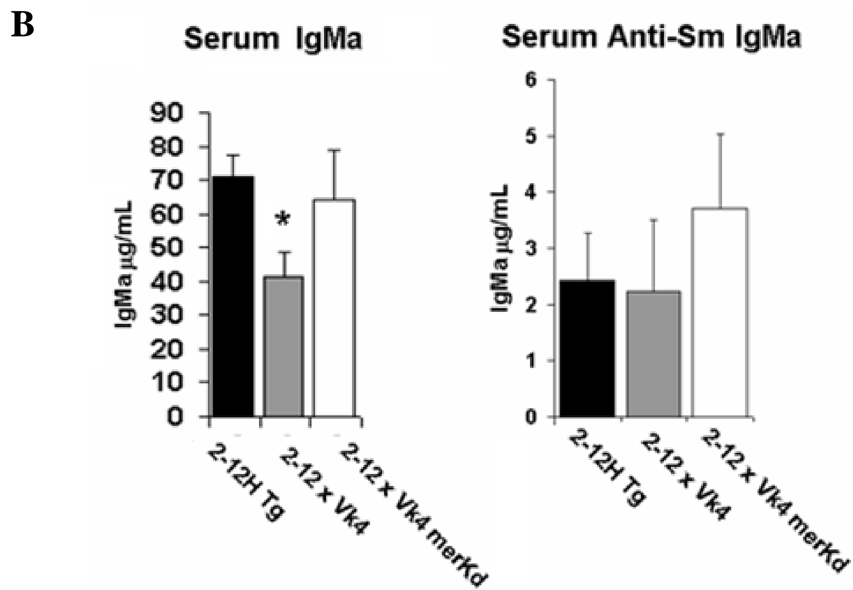

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CHAPTER 4

TLR induced antibody secretion by anergic anti-Sm B cells is regulated by cell death and inhibition to undergo plasma cell differentiation

ABSTRACT

Anergic B cells are unable to differentiate to antibody-secreting plasma cells (PCs) upon stimulation with toll-like receptor (TLR) agonists. How PC differentiation is blocked is not fully understood. In this report, I describe the regulation of PC differentiation by anergic anti-Sm B cells from mice transgenic for the 2-12H gene (2-12H) and the 2-12H gene and a Vκ4 gene (2-12H/Vκ4). Both mice generate anti-Sm B cells, but while 2-12H mice generate anergic anti-Sm follicular (Fo) B cells, but functional anti-Sm marginal zone (MZ) B cells, 2-12H/Vκ4 mice generate anergic anti-Sm Fo and MZ B cells. I hypothesize that anti-Sm B cells are block prior to PC differentiation following TLR stimulation. Anergic 2-12H and 2-12H/Vκ4 anti-Sm B cells proliferate in response to TLR stimulation. However, both 2-12H/Vκ4, and to a lesser degree, 2-12H B cells, are defective in antibody secretion. Two mechanisms regulate antibody secretion: activation-induced cell death and a block in PC differentiation, although the contributions of these mechanisms differ depending on B cell subset identity. These findings also indicate that, although activation induced cell death is functional at the transitional B cell stage, the block in PC differentiation does not become

fully functional until the Fo B cell stage. The regulation of PC differentiation of anergic anti-Sm B cells is independent of ERK activation contrasting with the block in PC differentiation by anergic anti-hen egg lysozyme (HEL) B cells, indicating the existence of an ERK independent mechanism for regulating PC differentiation by anergic B cells.

INTRODUCTION

The regulation of B cell differentiation to plasma cells (PCs) is critical to preventing the production of autoantibodies. Toll-like receptor (TLR) ligands are potent inducers of B cell activation, inducing proliferation and antibody secretion. Activation of B cells by TLR stimulation can bypass the requirements for other stimuli, such as cytokines or T cell help, in PC differentiation. As such, TLR activation has the potential danger of breaking tolerance and inducing autoimmune diseases. Autoreactive B cells can be regulated by multiple mechanisms [1, 2, 3, 4, 5, 6, 7]. Anergy, a state characterized by cell unresponsiveness, can offer a potential window for break in tolerance due to the retention of self-reactive cells in the periphery. Nonetheless, most anergic B cells remain unable to differentiate to PCs following TLR stimulation [8, 9, 10, 11]. The mechanisms involved in the regulation of TLR activation of anergic B cells remains unclear, but it is important to understand how tolerance is maintained and broken for the design of new therapies for autoantibody-mediated diseases such as systemic lupus erythematosus.

PC differentiation is regulated by multiple transcription factors. B lymphocyte-induced maturation protein-1 (Blimp-1) is considered the master regulator of PC differentiation [12, 13]. Blimp-1 directly represses c-Myc, a protein involved in B cell proliferation, and therefore induces cell cycle arrest required for terminal PC differentiation. Pax-5, which functions to maintain B cell identity, is another target of Blimp-1 mediate suppression. Pax-5 represses, amongst many genes, the transcription factor gene XBP-1 and IRF-4 that are required for PC differentiation [14, 15, 16, 17, 18].

Anergic anti-HEL B cells are unable to differentiate to PCs following TLR stimulation (CpG or LPS). Chronic Ag engagement by the anti-HEL BCR leads to higher

basal levels of activated ERK, resulting in suppression of Blimp-1 expression [19, 20]. Strong BCR signaling has been previously shown to also inhibit TLR induced PC differentiation of naïve B cells by an ERK-dependent mechanism [19, 21, 22]. Thus, ERK acts as a molecular switch that governs expression of Blimp-1 expression and subsequent PC differentiation.

Anti-Sm B cells are regulated by multiple mechanisms of tolerance, including anergy. In anti-Sm 2-12H Tg mice, anti-Sm B cells are also regulated at a pre-PC stage. These cells express CD138 (a pan PC marker), but have not upregulated the expression of Blimp-1. Thus, anti-Sm B cells are activated to become PCs, but are blocked at an early PC stage from completing PC differentiation in non-autoimmune mice [23, 24, 25]. High affinity anti-Sm from mice carrying both the 2-12H transgene and a Vκ4 transgene (2-12H/Vκ4 mice) have a more pronounced defect in antibody secretion following TLR stimulation than 2-12H mice (Chapter 2). In addition, pre-PCs are almost absent in 2-12H/Vκ4 mice, indicating a regulation of PC differentiation prior to pre-PC stage (Chapter 3). This would suggest a role for BCR affinity in the establishment of regulatory mechanisms maintaining tolerance. Unlike the low affinity anti-Sm B cells from 2-12H/Vκ8 mice, 2-12H/Vκ4 B cells are positively selected into the MZ B cell subset. The presence of this mature subpopulation in addition to the Fo B cell population allows us to assess the impact of tolerance on MZ B cells and gain insight into the regulation of anti-Sm B cells.

Herein, I show that, although anti-Sm B cells are able to proliferate to TLR stimulation, they have a defect in antibody secretion and are regulated by a combination of activation-induced cell death and a block in PC differentiation. This block occurs prior to CD138, XBP-1 and IRF-4 upregulation. Anti-Sm B cell death might be a consequence of increased Bim expression following TLR stimulation or a lag in the upregulation of Bcl-X_L.

The degree at which each of the two mechanisms act is dependent on the B cell subset. Moreover, the influential role of the BCR affinity was readily apparent as 2-12H/Vκ4 B cells exhibit the highest degree of regulation affecting even MZ B cells. The inhibition of ERK pathway did not restore anti-Sm antibody secretion as observed in anergic anti-HEL B cells. Thus, the mechanisms regulating TLR activation of anti-Sm B cell are influenced by BCR affinity and are independent of ERK activation, suggesting the existence of an ERK-independent pathway in the regulation of PC differentiation by anergic B cells.

MATERIALS AND METHODS

Mice

Anti-Sm 2-12H Tg mice and 2-12H/Vκ4 double Tg mice have been described previously (Chapter 2).

Flow Cytometry

Single-cell suspensions were prepared and analyzed as described in chapter 2. The Abs specific for IgM, B220, CD21, CD23 and CD138 were obtained from BD Biosciences.

For intracellular staining of pro and anti-apoptotic proteins, after the indicated times, cells were fixed with 2% PFA (Electron Microscopy Sciences) and permeabilized with BD Perm/Wash™ buffer (BD Biosciences) containing saponin. Anti-Bim (poly Rab) and anti-Bax (Ms IgG1 6A7) were obtained from BD Biosciences and Bcl-X_L PE (SC-8392) was purchased from Santa Cruz Biotechnology. Anti-Rabbit Alexa647 and anti-Ms IgG1 Alexa488 were obtained from Probes-Invitrogen. For intracellular staining of transcription factors involved in PC differentiation cells were fixed with 2% PFA and permeabilized with ice cold MeOH for 30 min. Cells were stained with B220, IgM and CD138 (BD Biosciences). Cells were also stained with mouse anti-Pax-5 (Ms IgG1 BD Biosciences), rabbit anti-XBP-1 and goat anti-IRF-4 (Santa Cruz Biotechnology). Goat anti-Rabbit Alexa647 and goat anti-Mouse IgG1Alexa488 (Probes-Invitrogen) and donkey anti-goat Cy5 (Jackson ImmunoResearch) were used as secondary Abs.

Cell purification

Splenic B cells were isolated by negative selection using IMag^{TM} Streptavidin Particles Plus (BD Biosciences) following manufacturers protocol for magnetic labeling and separation as described in Chapter 2. B cells were 92–98% pure as determined by flow cytometry.

Cell sorting

For cell sorting experiments, splenic cells were stained with anti-CD21, anti-CD23, anti-B220 and anti-CD138 Abs (BD Biosciences) and propidium iodide added (to discriminate dead cells) and sorted on a MoFlo high-speed sorter (DakoCytomation). Sorted populations were >95% pure as determined by reanalysis.

In vitro stimulation

Purified or sorted B cells were ($1x10^6$/ml) were cultured in 24 well plates in complete RPMI containing 10% FCS, L-glutamine, penicillin, streptomycin, and 2-mercaptoethanol. For proliferation analysis B cells were labeled with CFSE (Probes-Invitrogen) at a concentration of 1 μM for 10 min at room temperature.

Cells were stimulated with (0.1, 1 or 10 μg/ml) LPS or (0.1 or 1 μg/ml) CpG (InvivoGen) as indicated. For survival and proliferation analysis cells were stimulated for 72hs then washed and stained with LIVE/DEAD dead cell stain kit (Probes-Invitrogen). To analyze pro and anti- apoptotic protein expression B cells were cultured for the indicated times.

For analysis of XBP-1, IRF-4 and Pax-5, sorted B cells were stimulated for 72 hs and analyzed by flow cytometry as described above.

Cell signaling by Flow Cytometry

Cell signaling was determined by flow as previously described in Chapter 3. Cells were incubated for 30 minutes in the presence or absence of 10μM U0126 (Promega) to test the efficiency of the MEK inhibitor on ERK phosphorylation. Cells were stimulated with anti-CD40 (BD Biosciences) for 15 min to induce maximum phosphorylation.

ELISAs

Quantitation of total IgM in supernatants was done by ELISA as previously described in Chapter 2. Supernatants were collected 72hs following stimulation.

Statistical analysis

The Student *t* test was used to assess the significance of the differences between groups. A value of $p < 0.05$ was considered significant.

RESULTS

Anti-Sm B cells proliferate following TLR stimulation, but show enhanced cell death.

We previously showed that 2-12H and 2-12H/Vκ4 B cells secrete significantly less antibody than non-Tg B cells following LPS or CpG stimulation. Since proliferation and antibody secretion are independently regulated, we determined whether anti-Sm B cells were similarly deficient in ability to proliferate following LPS and CpG stimulation. Purified non-Tg, 2-12H and 2-12H/Vκ4 B cells were CFSE labeled and simulated with different concentrations of LPS or CpG. Proliferation and cell death were assessed by flow cytometry gating on live cells (Fig. 1A-B). 2-12H B cells proliferate similarly to non-Tg B cells, and underwent multiple cycles of division at all ligand concentrations tested (Fig 2A, top two rows). Thus, 2-12H and 2-12H/Vκ4 B cells are responsive to LPS and CpG-induced proliferation signals indicating that their deficiency in antibody secretion is due to a selective deficiency in TLR-induced differentiation signals.

Proliferation of 2-12H and 2-12H/Vκ4 B cells differed from non-Tg B cells in two ways. First, 2-12H and 2-12H/Vκ4 B cells consistently underwent fewer cycles of cell division than non-Tg B cells. Second, a higher proportion 2-12H/Vκ4 B cells proliferated in response to both ligands than non-Tg or 2-12H Tg B cells (Fig. 2A, bottom row). One possible explanation for this is that dividing and non-dividing anti-Sm B cells were more likely to undergo cell death than non-Tg B cells. To test this possibility we determined the frequency of divided and non-divided B cells that had undergone cell death after LPS or CpG stimulation. Compared to non-Tg B cells, a higher percentage of dividing 2-12H and 2-12H/Vκ4 B cells underwent cell death, which was most evident for the latter (Fig. 1C). Cells were lost at each cell cycle since a broad range of CFSE was detected, similar to the range

observed for live cells (data not shown). Thus, cell death is greater in 2-12H and 2-12H/Vκ4 B cells than non-Tg B cells accounting for the overall fewer divisions per cell in these cultures (Fig. 1A). In addition, non-dividing 2-12H/Vκ4 B cells underwent a higher frequency of cell death than 2-12H and non-Tg B cells, which can account for the presence of fewer undivided 2-12H/Vκ4 B cells in these cultures (data not shown). Undivided 2-12H and non-Tg B cells were not different in frequency of cell death, consistent the equal frequencies of undivided B cells in cultures of these cells.

Taken together, these results show that the anti-Sm B cells of 2-12H and 2-12H/Vκ4 mice proliferate in response to TLR stimulation indicating that these B cells are responsive to LPS and CpG. However, both TLR ligands are less effective at rescuing anti-Sm B cells from cell death than non-Tg B cells.

Anti-Sm B cells up-regulate Bim following LPS stimulation.

A balance between pro- and anti-apoptotic protein expression regulates activation induced B cell death [26, 27]. We therefore determined if a shift in this balance to favor pro-apoptotic protein expression could explain the deficiency in LPS or CpG-induced rescue of anti-Sm B cells. Splenic B cells were stimulated with LPS (10 μg/mL) and Bim, Bax and Bcl-X_L protein expression profiles determined at 16 and 40 hrs post-stimulation by intracellular flow cytometry. At 16 hrs, Bim levels were equal in 2-12H/Vκ4 and non-Tg B cells, but slightly lower in 2-12H B cells. In addition, Bax levels were somewhat higher in non-stimulated 2-12H and 2-12H/Vκ4 B cells compared to non-Tg B cells. Bim and Bax expression increased in LPS stimulated B cells from mice of all three strains, although the Bim increase was greatest in 2-12H and 2-12H/Vκ4 B cells (Fig. 3). Bim and Bax had

increased further in 2-12H B cells and especially 2-12H/Vκ4 B cells at 40 hrs, while Bim had decreased to non-stimulated levels in non-Tg B cells (Fig. 3).

In contrast, the levels of the anti-apoptotic protein Bcl-X_L at 16hrs was lower in non-stimulated 2-12H and 2-12H/Vκ4 B cells compared to non-Tg B cells. Bcl-X_L expression levels, following LPS stimulaton, had increased substantially in non-Tg B cells at 16hrs, but little increased was observed in 2-12H and 2-12H/Vκ4 B cells. Bcl-X_L levels were further increased in non-Tg B cells at 40 hrs, while 2-12H and 2-12H/Vκ4 B cells had up-regulated Bcl-X_L expression to levels similar to the level in non-Tg B cells following LPS stimulation (Fig. 3). However, at this time Bim levels were much higher in these cells compared to non-Tg B cells. Thus, LPS stimulation induces a shift in the balance in of pro- and anti-apoptotic proteins to favor survival in non-Tg mice, but to favor apoptosis in 2-12H and 2-12H/Vκ4 B cells, which may provide at least a partial explanation for the deficiency in survival of anti-Sm B cells after LPS stimulation.

Fo, MZ and transitional anti-Sm B cells are defective in Ab secretion following TLR stimulation.

We have previously shown that 2-12H and 2-12H/Vκ4 Fo and MZ B cells are defective in BCR-mediated activation through different biochemical mechanisms. To determine whether Fo and MZ B cells are equally defective in TLR-mediated antibody secretion, we sorted transitional, Fo, and MZ cells for *in vitr*o LPS or CpG stimulation. Splenic B cells were stained for B220, CD21, CD23, and CD138 expression and transitional ($B220^+$, $CD21^-$, $CD23^-$), Fo ($B220^+$, $CD21^+$, $CD23^+$), and MZ ($B220^+$, $CD21^{high}$, $CD23^{low}$) were sorted (Fig. 4A). Pre-PCs were excluded by CD138 expression. Sorted cells were

cultured and stimulated with TLR ligands and supernatants collected for measurement of secreted antibody levels by ELISA after 4 days. As shown in Figs. 4B-D, 2-12H and 2-12H/Vκ4 B cells of all three subsets secreted less antibody in response to LPS and CpG than B cells of the corresponding population from non-Tg mice, with 2-12H/Vκ4 B cells consistently showing the lowest levels. R-848 (a TLR7 agonist) and poly I:C (a TLR3 agonist) stimulation generated a similar pattern of antibody secretion by Fo B cells (data not shown). Thus, we conclude that B cells of all three subsets from 2-12H and 2-12H/Vκ4 mice are profoundly deficient in antibody secretion following TLR activation, consistent with their deficient responses to BCR activation (Chapter 3). This inability to secrete antibody extended to transitional B cells 2-12H/Vκ4 B cells indicating that the defect in TLR induced antibody secretion occurs at or before the transitional B cell stage (Fig 4D).

Regulation of anti-Sm B cell secretion is independent of ERK phosphorylation.

ERK activation plays a central role in blocking TLR activation of anergic anti-HEL B cells by serving as a molecular switch that integrates signals from multiple receptors thereby controlling ASC differentiation. ERK is constitutively phosphorylated in anti-HEL B cells due to chronic BCR stimulation and although the mechanism for blocking ASC differentiation has not been elucidated, inhibition of ERK phosphorylation in these cells restores normal secretion by LPS and CpG activation [19, 20]. We have previously shown that basal phospho-ERK levels are not elevated in Fo and MZ B cells of 2-12H and 2-12H/Vκ4 mice (Chapter 3) suggesting that ERK signaling does not regulate TLR activation of these cells. To test this possibility further, we stimulated non-Tg and 2-12H/Vκ4 B cells with LPS or CpG in the presence or absence of the MEK inhibitor U0126 that blocks ERK

phosphorylation [28]. 2-12H/Vκ4 B cells displayed diminished antibody secretion following either LPS or CpG stimulation compared to non-Tg B cells, and antibody secretion was not restored by the addition of U0126 (Fig 3A and 4B). U0126 inhibited ERK phosphorylation, since U0126 abrogated anti-CD40-induced ERK phosphorylation in these cells (Fig 4C). These results demonstrate that the inhibition of TLR-induced ASC differentiation by 2-12H/Vκ4 B cells is independent ERK activation, suggesting the existence of an alternative regulatory mechanism for blocking ASC differentiation of anergic B cells.

Anti-Sm B cells are regulated by cell death and inhibition of plasma cell (PC) differentiation.

The higher frequency of cell death by 2-12H and 2-12H/Vκ4 B cells after TLR activation could account for the poor antibody secretion by 2-12H and 2-12H/Vκ4 B cells. Alternatively, a block in the upregulation of the PC transcriptional program could account for the poor antibody secretion observed. To discriminate between these possibilities, we determined the phenotype of TLR-activated B cells and whether the ASC transcriptional program had been activated. PC differentiation results in a decrease in B220 and an increase in CD138 and intracellular IgM. In addition, expression of the transcription factors XBP-1 and IRF-4 increases, while Pax-5 decreases. As shown in Fig. 5, we readily detected PC differentiating B cells among LPS activated non-Tg B cells by flow cytometry. These cells were intracellular IgM^{hi}, $B220^{low}$, $XBP\text{-}1^{hi}$, $IRF\text{-}4^{hi}$ and $CD138^{hi}$. Since all of these changes occurred together in mice of all three strains, we used the increase in intracellular IgM and XBP-1 to identify PC differentiating cells in all subsequent experiments. We were unable develop a flow cytometry assay for Blimp-1, the master regulator of PC differentiation, and were therefore unable to examine the expression of this transcription factor. However, cells

with increased intracellular IgM and XBP-1 likely express Blimp-1 since the both increases are Blimp-1 dependent [12, 13, 14, 16, 17].

To examine the PC differentiation by anergic anti-Sm B cells in response to TLR activation, sorted Fo, MZ, and transitional B cells were assayed individually, since these cells differ in their ability to secrete antibody. Non-Tg, 2-12H, and 2-12H/Vκ4 B cells were cultured with either LPS or CpG and 4 days later cells harvested for flow cytometry analysis. These were the same cultures used to measure antibody levels in Fig. 4. By day 4, ~50% of non-Tg Fo B cells were alive, but only ~40% and ~30% of 2-12H and 2-12H/Vκ4 Fo B cells, respectively (Fig. 6A). These populations also differed significantly in PC frequency. Whereas ~30% of live non-Tg Fo B cells were PCs, only ~20% and ~5% of 2-12H and 2-12H/Vκ4 B cells, respectively, were PCs (Fig. 6A). Thus, poor antibody secretion by 2-12H and 2-12H/Vκ4 Fo B cells is the result of defects at two levels; a failure to efficiently rescue these cells from cell death and a deficiency in the activation of the PC transcriptional program. Consistent with this, the relative ability to rescue from cell death and to activate the PC transcriptional program parallels the amount of antibody secreted (Figs. 4B and 6A).

LPS was equally able to rescue from cell death and activate the PC transcriptional program of non-Tg and 2-12H MZ B cells (Fig. 6B), agreeing with the similar levels of secreted antibody (Fig. 3C). This suggests that 2-12H MZ B cells are not anergic, as suggested by our previous analysis (Chapter 3) [29, 30]. By contrast, LPS was less able to rescue and activate the PC transcriptional program of 2-12H/Vκ4 MZ B cells (Fig. 6B), paralleling their poor ability to secrete antibody (Fig. 3C). We conclude from these findings that 2-12H MZ B cells are functional, but that 2-12H/Vκ4 MZ B cells are at least partially anergic. This

is in agreement with our finding with these cells that 2-12H/Vκ4 BCRs, but not 2-12H BCRs, are uncoupled from the signalsome (Chapter 3).

Relative to non-Tg transitional B cells, LPS was less able to rescue 2-12H and 2-12H/Vκ4 transitional B cells them from cell death. This inability to rescue 2-12H/Vκ4 transitional B cells was more pronounced than the inability to rescue anti-Sm Fo B cells. LPS was able to partially activate 2-12H and 2-12H/Vκ4 PC transcriptional program (Fig. 6C). Non-Tg and 2-12H transitional B cells were not statistically different in activation of the PC transcriptional program, but this was likely due to the considerable variability evident with 2-12H transitional B cells. Thus, the intrinsic mechanisms that block activation are in place at the transitional B cell stage, but the ability to block the activation of the PC transcriptional program is not as functional as it is in Fo B cells.

Since there are differences between TLRs in the signaling pathways they activate [7, 31], we compared the ability of LPS (TLR4) and CpG (TLR9) to rescue cells from cell death and to activate the PC transcriptional program. In contrast to LPS, CpG rescued Fo B cells from mice of all three strains equally well. However, 2-12H and particularly 2-12H/Vκ4 B cells activated the PC transcriptional program less well in response to CpG than non-Tg B cells (Fig. 6D). Non-Tg and 2-12H Fo B cells were not statistically different in the activation of the PC transcriptional program, but this likely reflects the considerable variation between mice. This difference parallels the difference in antibody secretion by these cells (Fig. 3B). Thus, whereas LPS is unable to efficiently rescue 2-12H and 2-12H/Vκ4 Fo B cells from cell death and to efficiently activate the PC transcriptional program, CpG exhibits a deficiency in only the latter.

DISCUSSION

TLR-induced activation of autoreactive B cells can lead to autoantibody production and disease. Activation *via* TLRs bypasses the mechanisms that block BCR-induced activation of some autoreactive B cells [1, 2, 4, 5, 32, 33, 34, 35]. TLR stimulation can also bypass T cell tolerance mechanisms leading to autoantibody production [35, 36, 37, 38, 39]. Blocking TLR activation may be particularly important to MZ B cells, since their unique anatomical location may provide them with a greater exposure to LPS and CpG motifs associated with bacterial and viral infections [40]. In addition, TLR signals in conjunction with BCR signals are required for autoreactive B cell activation *in vivo*. Thus, prevention of TLR induced activation is important to maintaining tolerance to self-antigens. In this study, we investigated the mechanisms regulating TLR activation of B cells specific for Sm, a self-antigen commonly targeted in mouse and human SLE [41]. We demonstrate that, while anergic anti-Sm B cells respond to LPS and CpG activation by robust proliferation, they are less able to rescue anergic B cells from cell death and to activate the PC transcriptional program, thereby preventing antibody secretion.

The robust proliferation of 2-12H and 2-12H/Vκ4 B cells induced by LPS and CpG indicates that their deficiency in antibody secretion is not due to an inability to signal in response to TLR stimulation. In contrast to non-Tg B cells in which a large fraction (35-40%) do not divide at high concentrations of LPS (10 μg/ml), nearly all 2-12H/Vκ4 B cells undergo one or more cell divisions at this concentration. This could be due to a greater sensitivity to LPS, but our data showing increased cell death of non-dividing 2-12H/Vκ4 B cells compared to non-dividing non-Tg B cells suggests that this difference is likely to be due to an increased rate of cell death of non-dividing 2-12H/Vκ4 B cells. Consistent with this,

non-dividing 2-12H B cells, which do not differ from non-dividing B cells of non-Tg mice, do not differ in the frequency of cell death. Dividing 2-12H and 2-12H/Vκ4 B cells undergo increased cell death during clonal expansion compared to non-Tg B cells, likely accounting for the fewer overall number of cell divisions that these cells are able to undergo. 2-12H and 2-12H/Vκ4 B cells respond similarly to CpG stimulation through TLR9 suggesting that a higher rate of cell death before and during clonal expansion are a more general response to TLR signaling. Thus, the anti-Sm B cells of 2-12H and 2-12H/Vκ4 B cells proliferate in response to TLR stimulation, but undergo a higher rate of cell death than non-Tg B cells. The ability to proliferate, but not secrete, in response to LPS and CpG is not a property of all anergic B cells. Anergic anti-HEL B cells are similar to these anti-Sm B cells, but anti-dsDNA and anti-insulin B cells are unable to either proliferate or secrete in response to LPS [42, 43].

All B cells undergo cell death *in vitro* unless rescued by a survival signal. Compared to non-Tg B cells, anergic anti-Sm B cells, particularly 2-12H/Vκ4 B cells, are more prone to cell death *in vitro* in the absence of stimuli and in response to BCR and BAFF-R stimulation (Chapters 2 and 3). This higher rate of cell death by anergic anti-Sm B cells in the presence of these stimuli could be passive, and due to a deficiency in the ability of survival stimuli to block an ongoing cell death program. However, BCR and TLR stimulation may actively promote cell death, since both upregulate the pro-apoptotic proteins Bim and Bax. LPS stimulation increases the level of the anti-apoptotic protein Bcl-X_L, but this does not occur until late in the culture period after a period in which the Bcl-X_L level was low and Bim and Bax levels were high, which likely promotes cell death. Thus, induction of cell death

following TLR stimulation likely plays a role in the regulation of anti-Sm B cells as we discuss below.

Since anti-Sm B cells of the Fo and MZ subsets differ in their ability to signal through their BCR, we compared individual subsets to respond to LPS and CpG, and observed significant differences in both proliferation and secretion. In general, anti-Sm Fo B cells exhibited the greatest deficiency in antibody secretion and PC differentiation (Figs. 3 and 6). In addition, 2-12H/Vκ4 B cells of all subsets were more deficient in secretion and PC differentiation than 2-12H B cells or non-Tg B cell, likely due to their higher affinity for Sm (Chapter 2). Thus, 2-12H/Vκ4 Fo B cells were the most severely deficient population, secreting less than 10% of the antibody secreted by non-Tg B cells. This deficiency of 2-12H/Vκ4 B cells to secrete in response to LPS is evident at the transitional B cell stage. Interestingly, while 2-12H/Vκ4 MZ B cells were severely deficient in antibody secretion, 2-12H MZ B cells were near normal indicating that the anti-Sm MZ B cells of 2-12H mice are functional and fundamentally different from those of 2-12H/Vκ4 mice. This parallels the defect in stimulation through their BCRs; 2-12H/Vκ4, but not 2-12H, MZ B cells exhibit an uncoupling of the BCR signalsome. Why 2-12H and 2-12H/Vκ4 MZ B cells differ functionally is unclear. Although L chain use by the former is diverse (S. K. McCray and S. H. C., unpublished observation), they stain with Sm similarly suggesting that they have a similar affinity. Thus, another quality of the BCR, such as the ability to bind endogenous Sm, must be responsible for determining regulation.

Two mechanisms appear to contribute to the poor secretion by anergic anti-Sm B cells. First, increased cell death would likely account for some of this. In response to LPS, there are fewer live B cells of all three subsets in 4-day cultures of 2-12H/Vκ4 and 2-12H B

cells than non-Tg B cells (Fig. 6). Thus, there are fewer anti-Sm B cells than non-Tg B cells available for secretion. Second, anergic anti-Sm B cells of all subsets are deficient in PC differentiation. A smaller percentage of the live 2-12H and 2-12H/Vκ4 B cells have undergone PC differentiation than non-Tg B cells (Fig. 6). The defects in survival and PC differentiation are more severe for 2-12H/Vκ4 B cells than 2-12H B cells consistent with the lower levels of secreted antibody by B cells of all subsets (Fig. 3). The relative contributions of these two mechanisms vary between subsets. For example, 2-12H/Vκ4 Fo B cells are primarily regulated by a block in PC differentiation, while 2-12H/Vκ4 transitional B cells are regulated primarily through cell death. Since the majority of maturing transitional B cells are likely to become Fo B cells, and thus, the greater reliance on cell death to regulate 2-12H/Vκ4 transitional B cells suggests that the mechanism for blocking PC differentiation is not fully active until after the transitional B cell stage. 2-12H/Vκ4 MZ B cells are as likely to survive activation by LPS, but more likely to activate the PC differentiation program (Fig. 6), accounting for the greater ability of these cells to secrete antibody (Fig. 3). Consistent with the antibody secretion data indicating that 2-12H MZ B cells are not anergic, we observed no difference in cell death or PC differentiation between these cells and non-Tg MZ B cells. Thus, Fo and MZ B cells are regulated independently; in 2-12H mice anti-Sm Fo B cells are regulated, while the anti-Sm MZ B cells are not, whereas both are regulated in 2-12H/Vκ4 mice, albeit to different degrees.

Our comparison of LPS and CpG stimulation of Fo B cells indicates that there are differences between TLR ligands in regulation of antibody secretion. 2-12H/Vκ4 and 2-12H B cells are deficient in secretion in response to CpG (Chapter 2) and this defect is evident among Fo and MZ B cells (Fig. 3). However, in contrast to LPS, there is not a difference in

cell death between Fo B cells of non-Tg, 2-12H, and 2-12H/Vκ4 mice, but 2-12H/Vκ4 Fo B cells exhibit a block in PC differentiation relative to the others. LPS signals via a MyD88 dependent and a MyD88 independent, whereas CpG signals through only a MyD88 dependent pathway [31]. How the activation of one versus two of these pathways results in the observed differences is unclear, but it should be noted that there are differences between TLR9 and TLR7 stimulation and autoantibody production even though they are not know to be different in the activated pathways [38, 44, 45]. Thus, differences in activated pathways among TLRs results in different regulatory mechanisms.

The mechanism for the block in PC differentiation is not known. Our findings indicate that the block is prior to the upregulation of Blimp-1, the master regulator of PC differentiation that is necessary and sufficient for PC differentiation. Although we were unable to measure Blimp-1 expression, we infer from the absence of XBP-1 and IRF4 expression, B220 downregulation, and the increase in intracellular Ig, that most LPS stimulated anti-Sm B cells do not express Blimp-1, since all of these changes are Blimp-1 dependent [12, 13, 14, 46]. Goodnow and colleagues have demonstrated that ERK acts as a molecular switch that integrates multiple signals to regulate PC differentiation. Anti-HEL B cells have high basal phospho-ERK levels that result in a block in PC differentiation through prevention of Blimp-1 upregulation [19, 20]. The block in PC differentiation by anergic anti-Sm B cells does not appear to be regulated by this ERK molecular switch. The basal phospho-ERK levels are not elevated in these cells relative to non-Tg B cells (Chapter 3) and LPS activation in the presence of U0126, a powerful inhibitor of ERK phosphorylation, shows no difference in antibody secretion. This difference in dependence on ERK may be a function of a difference in BCR affinity for antigen, the form of antigen (soluble vs. membrane bound),

or additional signals through other receptors. Regardless, these data indicate that anergic anti-Sm B cells the block in PC differentiation is independent of ERK phosphorylation and therefore that there are at least two pathways for inhibiting PC differentiation by anergic B cells.

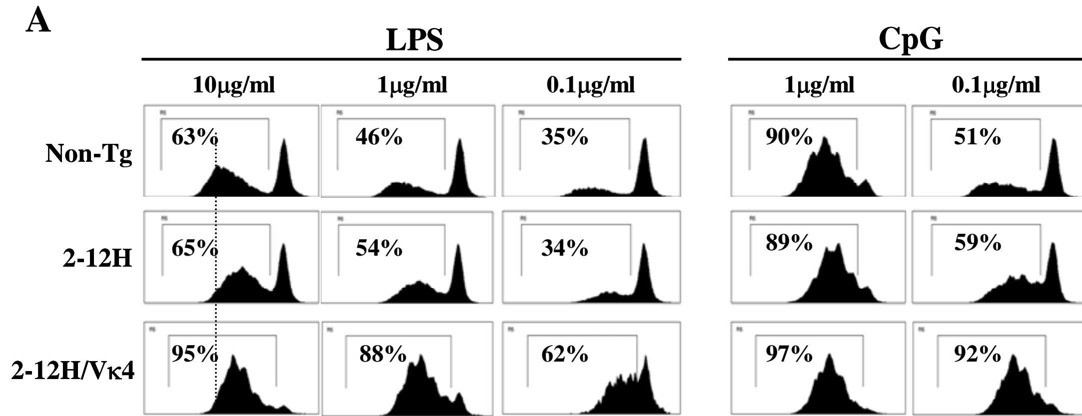
An important consideration for how TLR activation is regulated in anergic anti-Sm B cells is the potential effect of antigen in the cultures. Sm is displayed on the surface of apoptotic cells and therefore Sm may engage the BCRs of cultured B cells. The presence of antigen in the culture could cause an increase in cell death, since we have shown that BCR stimulation of anti-Sm B cells induces increased expression of pro-apoptotic genes and cell death (Chapter 3). Second, signaling via the BCR could maintain high levels of Pax-5 or BCL-6 that inhibit Blimp-1 expression and therefore inhibit PC differentiation [12, 16, 47, 48]. Strong BCR signaling (anti-IgM) blocks LPS-induced PC differentiation of non-Tg B cells as well as HEL antigen blocks induction of PC differentiation of naïve anti-HEL B cells [19]. Thus, the inability to be able to eliminate self-antigen from these cultures could have important consequences to activation and antibody secretion.

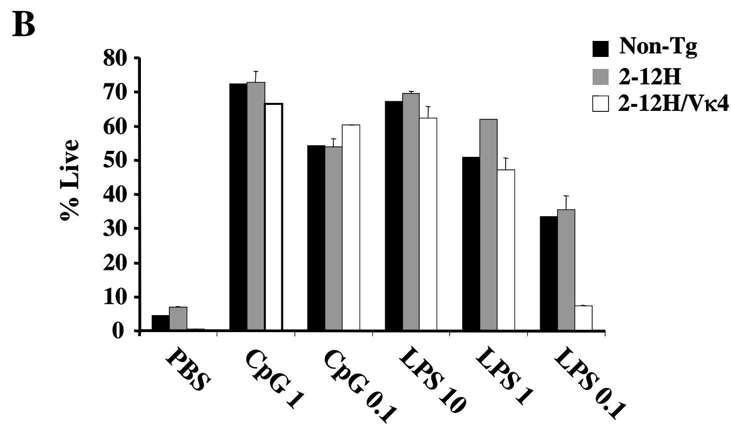
In summary, this study shows that 2-12H/Vκ4, and to a lesser extent 2-12H B cells, are defective in antibody secretion following TLR stimulation, even though proliferation is minimally affected. Activation-induced cell death and a block in PC differentiation are two mechanisms that act, in different degrees depending on subset identity, to regulate antibody secretion by anergic anti-Sm B cells. While cell death seems to be more important in regulating transitional B cells, a block in PC differentiation is more important to Fo B cell regulation. In addition, high affinity 2-12H/Vκ4 B cells display the highest degree of regulation, consistent with a role for BCR affinity in determining the mechanism of

regulation. ERK inhibition does not restore anti-Sm secretion indicating that the ERK mediated regulation of PC differentiation identified in anergic anti-HEL B cells is not responsible for the block in PC differentiation of anti-Sm B cells. Thus, these data indicate the existence of an alternative ERK independent pathway for blocking PC differentiation in anergic B cells.

Figure 1. Responses of 2-12H and 2-12H/Vκ4 B cells to *in vitro* TLR stimulation.

A, Representative data of proliferative responses of CFSE-labeled B cells from wild-type and anti-Sm Tg mice incubated for 72 hr in the presence of indicated amounts of LPS and CpG. Cell divisions as assessed by CFSE dye dilution. B, Cell survival as determined by flow cytometric analysis on isolated splenic B cells cultured in the presence of LPS and CpG for 72 hr. Cells negative for live/dead staining, as shown in C *left*, were scored as viable. C, Percent of cells that have undergone proliferation and subsequently death. *Left*, gating logic utilized. *Right*, numbers show percentage of cells in gate 3.


A
LPS
CpG
10μg/ml
1μg/ml
0.1μg/ml
1μg/ml
0.1μg/ml
Non-Tg
63%
46%
35%
90%
51%
2-12H
65%
54%
34%
89%
59%
2-12H/Vκ4
95%
88%
62%
97%
92%


B
Non-Tg
2-12H
2-12H/Vκ4
% Live
80
70
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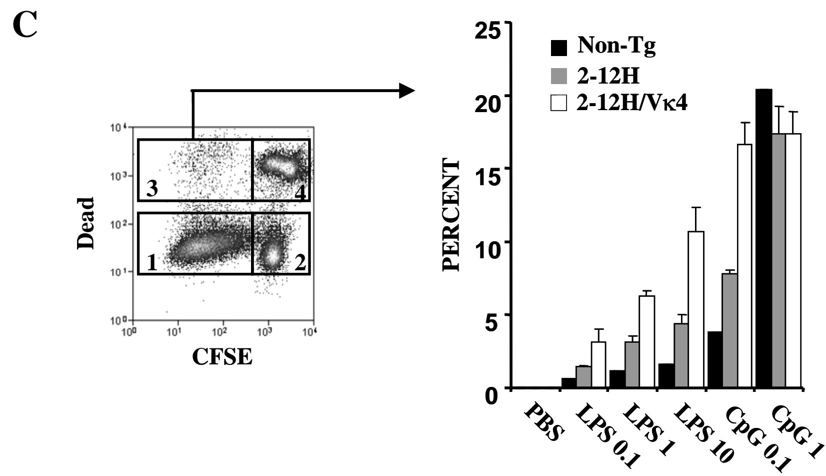
PBS
CpG 1
CpG 0.1
LPS 10
LPS 1
LPS 0.1


C
Dead
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1
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Non-Tg
2-12H
2-12H/Vκ4
PERCENT
25
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0
PBS
LPS 0.1
LPS 1
LPS 10
CpG 0.1
CpG 1

Figure 2. Flow cytometric anslysis of Bim, Bax and Bcl-X_L expression following LPS stimulation. Compiled data of protein expression of untreated (PBS) or LPS (10 μg/ml) treated B cells for the indicated times.

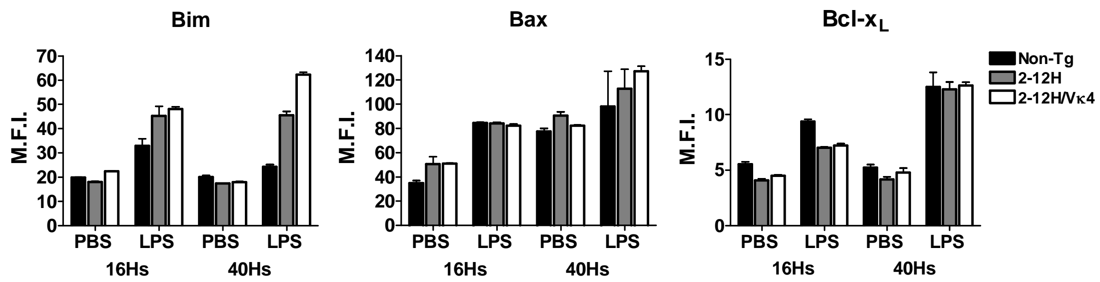


Figure 3. TLR induced Ab secretion by anti-Sm B cells subsets.

A, Gating scheme used to sort the FO, MZ, and Tr B cells is indicated using 2-12H spleen cells. Propidium iodide (PI)+ as well as CD138+ cell were excluded. B, Ab secretion by LPS (10 μg/ml) or CpG (1 μg/ml) stimulated sorted Fo B cells. Cells were stimulated for 4 days and total IgM Ab in the supernatants were measured by ELISA. C, Ab secretion by sorted MZ B cells stimulated assessed as described in B. D, Ab secretion by sorted Tr B cells stimulated with LPS as described in B. Secretion was normalized to secretion by non-Tg control. Data obtained from several independent experiments (n≥6) Significant difference * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

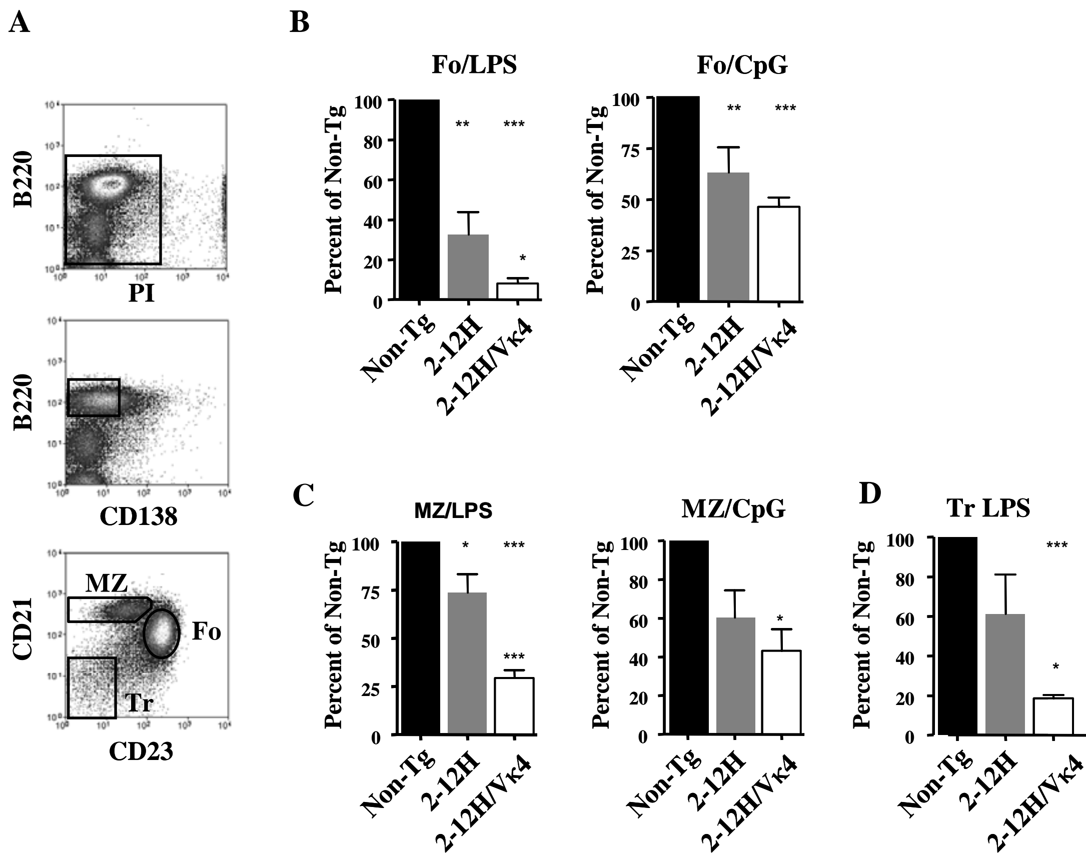


Figure 4. Effect of ERK inhibition on Ab secretion of 2-12H/Vk4 B cell to TLR stimulation. A, Purified B cells were pre-incubated or untreated for 30 min with10μM MEK Inhibitor U0126 and then cultured for 5 days with LPS (10 μg/ml). B, same as in A but cell were stimulated with CpG (1 μg/ml). C, U0126 effectively inhibits ERK activation. Purified B cells were pre-incubated as described in A and stimulated with anti-CD40 (10 μg/ml) for 15 min. ERK phosphorylation was assessed by flow cytometric analysis as described in chapter 3. (n=3)

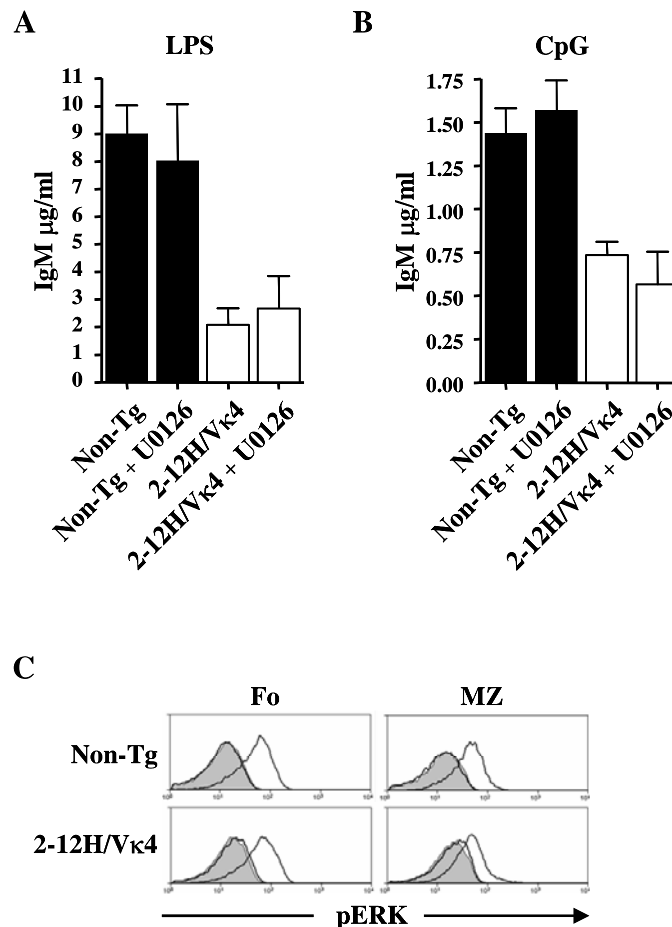


Figure 5. PC differentiation assessed by Flow Cytometry analysis.

Purified B cells were stimulated *in vitro* with TLR ligands for 4 days. Cells were processed as described in materials and methods and stained with antibodies against B220, IgM, XBP-1, IRF-4 and CD138. Example shows non-Tg B cells stimulated with 10 μg/ml LPS (*left column*) or freshly isolated (*right column*). Dead cells were excluded by Live/Dead stain. B cells that have differentiated to PCs are boxed.

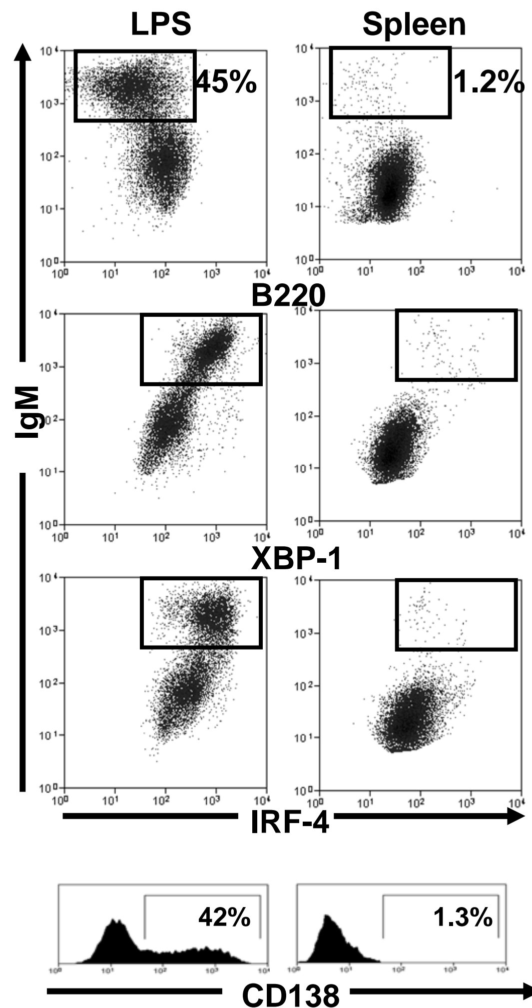
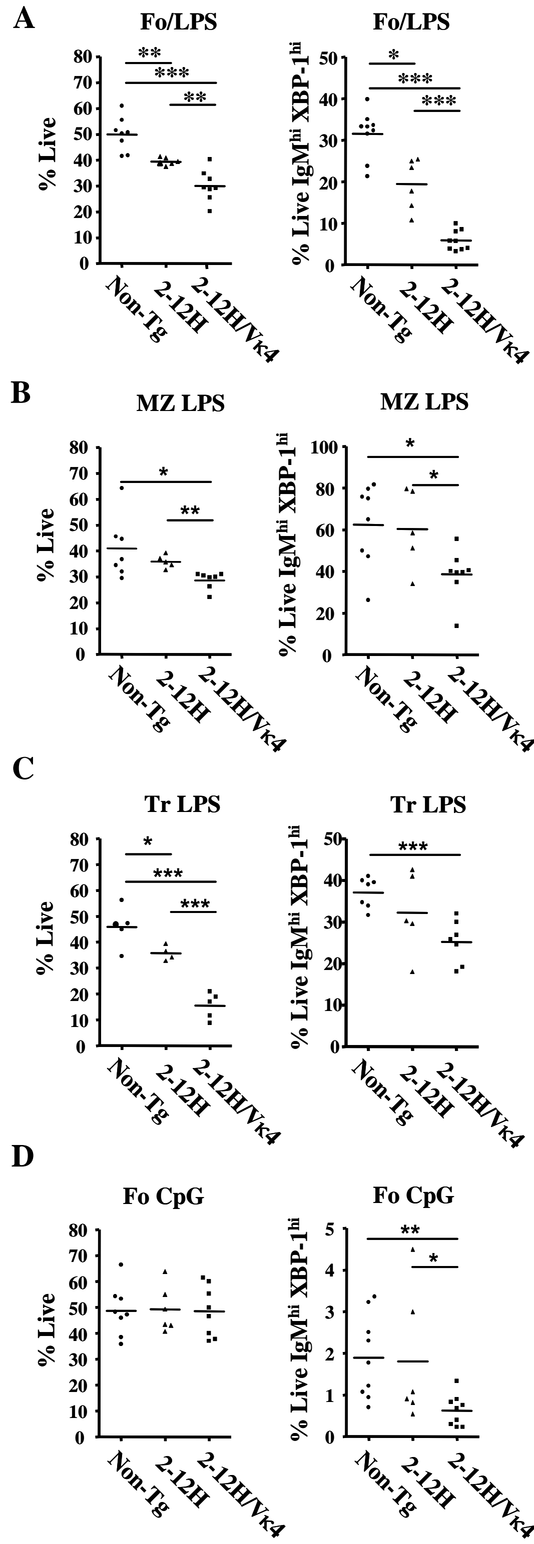


Figure 6. Induction of Cell Death and PC differentiation in anti-Sm B cells subsets induced by TLR stimulation.

Cells were sorted and stimulated with TLR ligands as described in Fig 3. Cell death (*left column*) was determined by assessing staining with LIVE/DEAD stain kit. PC differentiation (*right column*) was determined by gating as described in Fig 5. A, Sorted Fo B cells stimulated with 10 μg/ml LPS. B, Sorted MZ B cells stimulated with 10 μg/ml LPS. C, Sorted Tr B cells stimulated with 10 μg/ml LPS. D, Sorted Fo B cells stimulated with 1 μg/ml CpG. Each symbol represents cells from individual sorts. Significant difference * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).



A
Fo/LPS
% Live
Fo/LPS
% Live IgMhi XBP-1hi
Non-Tg
2-12H
2-12H/Vκ4
B
MZ LPS
% Live
MZ LPS
% Live IgMhi XBP-1hi
C
Tr LPS
% Live
Tr LPS
% Live IgMhi XBP-1hi
D
Fo CpG
% Live
Fo CpG
% Live IgMhi XBP-1hi

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CHAPTER 5

Summary and future directions

The immune system has developed mechanisms to silence B cells specific for self antigens through mechanisms of tolerance. Tolerance encompasses many processes and mechanisms that regulate and suppress the activation of self-reactive cells [1, 2, 3, 4, 5, 6, 7]. When these mechanisms fail autoimmune disease ensues. Immunoglobulin Tg mice have been very effective tool in the elucidation of the mechanisms of B cell tolerance. These mechanisms include central and peripheral deletion, receptor editing, developmental arrest, anergy and follicular exclusion [7, 8, 9]. Anergy is of great interest because it offers a window for a break in tolerance due to the retention of self-reactive cells in the periphery [6, 7, 10]. Early analyses of B cell anergy using Ig-Tg mouse models has generally considered B cells as a single homogeneous population whish shared common characteristics amongst the different models. These characteristics included low BCR levels, a short life span, follicular exclusion and defective or uncoupled BCR signaling cascade [5, 11, 12, 13]. Nonetheless, other recent models do not share these same characteristics [12, 14, 15, 16, 17]. The differences observed between these models indicated that anergy encompasses a broad spectrum of complex and multi-factorial states of cell unresponsiveness. Thus, better understanding of B cell anergy is fundamental for the design of new therapies for autoantibody-mediated diseases.

Sm, a component of a ribonucleoprotein, is a target of the immune system in the autoimmune disease systemic lupus erythematosus (SLE) [18, 19, 20]. For the work presented

here I utilized Ig-Tg mouse models specific for Sm. I utilized mice transgenic for the 2-12H gene (2-12H) and the 2-12H gene and a Vκ4 gene (2-12H/Vκ4). 2-12H Tg mice present a polyclonal B cell population of anti-Sm B cells with varying degrees of BCR affinity [14, 21]. In contrast to the 2-12H/Vκ4 B cells are basically monoclonal. Using mice expressing one BCR clonotype is useful to tease apart the multiple mechanisms involved in regulation of autoreactive B cells, which in real life will involve numerous clonotypes of different affinities for antigen. Furthermore, the 2-12H/Vκ4 mice offer the first ever opportunity to assess regulation of self-reactive MZ B cell. The goal of this work was to gain insight into the regulation of anti-Sm B cells and to advance the current understanding of B cell anergy.

The studies presented in chapter 2 revealed that anti-Sm B cells are regulated by anergy but that the BCR affinity for Sm is a determining factor. It has previously been shown that low affinity 2-12H/Vκ8 anti-Sm B cells are limited to differentiation to the Fo B cell stage and, although anergic, they are able to survive in the presences of competitor B cells [14]. In contrast, high affinity 2-12H/Vκ4 B cells differentiate to Fo, MZ and B-1 B cells. Nonetheless, they are unable to survive in a competitive environment. Suggesting that high BCR affinity induces a mechanism of regulation not observed in low affinity anergic B cells. This defect correlates with a minimal responsiveness to BAFF, which is unable to rescue these cells from cell death. Because BAFF is required for B cell maturation and maintenance [22, 23, 24, 25] we hypothesize that the observe defect must be induced after maturation of anti-Sm B cells. However, it is not clear why 2-12H/Vκ4 B cells have a normal life span in a monoclonal environment. A likely explanation is that there is some BAFF signaling and that this low level is sufficient to allow B cell survival when B cell numbers are low.

The minimal BAFF responsiveness by 2-12H/Vκ4 B cells does not seem to be due to BAFF receptor expression defects or anomalies. The most likely explanation is that the signaling ability of these receptors is compromised. Because these cells are still able to mature, I predict that the defect in BAFF responsiveness is with only the survival pathway, such as the Akt and Pim2 pathways leading to upregulation of the anti-apoptotic protein Mcl-1 [22, 26] and the alternative NF-κB activation pathway which is involved in Bcl-X_L upregulation [27, 28, 29]. Although, recent studies undermine the role of Bcl-X_L in BAFF mediated survival [30, 31, 32, 33]. It will be interesting in the future to determine to define precisely the defect in BAFF signaling that prevents survival in a competitive environment. Preliminary data indicates a defect in the Akt survival pathway (R. Bussick, personal communication).

In chapter 3, I assessed the ability of the BCR on cells of each subset to signal in response to BCR ligation to determine whether anergic anti-Sm Fo and MZ B cells are regulated similarly. Although anti-Sm Fo B cells from both 2-12H and 2-12H/Vκ4 mice are signaling competent, they are induced to undergo rapid cell death, which can be explained by an imbalance in pro- and anti-apoptotic proteins that favors apoptosis. 2-12H MZ B cells are able to induce signaling following BCR ligation and are not anergic, as previously demonstrated [34, 35]. In contrast, 2-12H/Vκ4 MZ B cells exhibit a weak protein tyrosine phosphorylation response and little increase in Syk and ERK phosphorylation. Furthermore, they are unable to respond to endogenous self antigens, since in contrast to 2-12H MZ B cells; they do not constitutively generate anti-Sm pre-plasma cells and are not activated by an increase in apoptotic cell load. These data demonstrate that 2-12H/Vκ4 MZ B cells are

regulated by uncoupling of the BCR from the signalosome and are anergic. Anergy of self-reactive MZ B cells has not been previously described.

The uncoupling of the BCR from signalosome in 2-12H/Vκ4 could be due to negative regulators of BCR signaling. CD5, a negative regulator of signaling expressed mainly by B-1a cells, is a potential target of future research [36, 37, 38, 39]. 2-12H/Vκ4 MZ B cells express high levels of CD5 (data not shown) and could be responsible for the uncoupling observed. Alternatively, the Igα/Igβ signaling components of the BCR may be dissociated from the Ig component due to chronic engagement with self-antigen [40, 41].

In Chapter 4, I assess the mechanisms regulating TLR induced activation and differentiation to PCs of anergic anti-Sm B cells. Anergic 2-12H and 2-12H/Vκ4 anti-Sm B cells proliferate in response to TLR stimulation. However, both 2-12H/Vκ4, and to a lesser degree, 2-12H B cells, are defective in antibody secretion. Two mechanisms regulate antibody secretion: activation-induced cell death and a block in PC differentiation, although the contributions of these mechanisms differ depending on B cell subset identity and is influenced by BCR affinity. The block in PC differentiation is not fully established until B cells reach the Fo B cell stage suggesting that this mechanism is established after maturation. Cell death of anti-Sm B cells might be a consequence of increased Bim expression following TLR stimulation or a lag in the upregulation of Bcl-X_L. Because anti-Sm B cells can proliferate to TLRs, it suggests that a particular signaling pathway involved in survival is defective. NF-κB and Akt pathways are to potential candidates due to their role in Bcl-X_L and A1 expression, two key anti-apoptotic proteins and therefore in future experiments we will want to determine whether signaling through these pathways is altered in anergic anti-Sm B cells [29, 42, 43].

The block in PC differentiation seems to occur prior to the upregulation of a plasma cell-specific transcription program. Although we were unable to measure Blimp-1 expression, we infer from the absence of XBP-1 and IRF4 expression, B220 downregulation, and the increase in intracellular Ig, that most LPS stimulated anti-Sm B cells do not express Blimp-1, since all of these changes are Blimp-1 dependent [44, 45, 46, 47, 48]. Nonetheless, future studies will try to address the expression of Blimp-1 as well as BCL-6 in TLR stimulated anti-Sm B cells.

Sm is displayed on the surface of apoptotic cells [35] and therefore Sm may engage the BCRs of cultured B cells. The presence of antigen in the culture could cause an increase in cell death, since we have shown that BCR stimulation of anti-Sm B cells induces increased expression of pro-apoptotic genes and cell death (Chapter 3). Second, signaling via the BCR could maintain high levels of Pax-5 or BCL-6 that inhibit Blimp-1 expression and therefore inhibit PC differentiation. Blocking Sm in culture might result potentially problematic. An alternative possibility is to inhibit pharmacologically BCR signaling in order to test this possibility.

Anergic anti-HEL B cells blocked from PC differentiation by a mechanism dependent on ERK activation [49, 50]. In this chapter, I have demonstrated that anti-Sm PC block is independent of ERK activation, suggesting the existence of an ERK-independent pathway in the regulation of PC differentiation of anergic B cells. The identification of this pathway is of great interest and would be a priority in future experiments as well as to determine if these mechanisms are maintained *in vivo*.

Overall, this work has demonstrated multiple mechanisms for the regulation of anti-Sm B cell activation by BCR and TLR signals. These mechanisms are unique compared to

anergic B cells specific to other self-antigens, which could be due to the form and distribution of the antigen [6, 7, 10]. Furthermore, I have shown how the BCR affinity can influence the degree to which a mechanism acts on anergic anti-Sm B cells. Since both BCR and TLR signaling can activate B cells, multiple mechanisms are required to prevent antigen specific activation through the BCR and antigen non-specific, bystander activation through a TLR [51]. The regulation of TLR signaling is of added importance due to the recent studies showing that sequential signaling through the BCR and a TLR. can activate autoreactive B cells [52, 53, 54, 55]. For Sm and other ribonucleoprotein antigens, the involvement of TLR7, which binds ssRNA is paramount [55, 56, 57, 58]. Thus, an understanding of the biochemical pathways involved in blocking anti-Sm B cell activation by BCR and TLR signals will be important to the identification of new therapeutic targets to prevent the production of pathogenic autoantibodies characteristic of human SLE. The present work has laid the groundwork for the identification of relevant signaling pathways.

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