ENHANCEMENT OF THE AN1 POPULATION BY BAFF: POTENTIAL ROLE IN AUTOIMMUNE DISEASE

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Dedication

To Shaheen and Meherin,

Amma, Abba, and Apajan

ENHANCEMENT OF THE AN1 POPULATION BY BAFF: POTENTIAL ROLE IN AUTOIMMUNE DISEASE

By

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DISSERTATION

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ENHANCEMENT OF THE AN1 POPULATION BY BAFF: POTENTIAL ROLE IN AUTOIMMUNE DISEASE

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Eva Lasmin Sadat

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Supervising Professor: Richard H. Scheuermann, Ph.D.

The cytokine BAFF is a TNF family member found to be essential for the homeostasis of B cells. Numerous studies have shown BAFF to be involved in the pathogenesis of autoimmune diseases, including Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis.

In an effort to understand the role of BAFF in the pathogenesis of autoimmune diseases, extensive *in vitro* studies on primary B cells have been performed. A recent study has described the existence of an anergic or silent autoreactive B cell population called An1, present within the wild-type repertoire of B cells in the periphery. Our differentiation studies using primary murine splenic B cells indicate an increase in this An1 population in response to BAFF stimulation. In order to ascertain whether this population is truly anergic, calcium flux assays have been performed. Interestingly, these assays show a decrease in response to

BCR signaling in BAFF-treated cultures compared to untreated controls. Additional studies with sorted populations of cells indicate that these An1 cells are emerging mainly from the mature and the T2 populations. Survival studies using Annexin V as an apoptosis marker show that BAFF increases the survival of B cells in each of their different stages affirming the role of BAFF in promoting survival of the An1 cells. Microarray gene expression studies done on splenic B cells treated with or without BAFF show higher expression of a set of genes that have been reported to be upregulated in anergic B cells. Purified B cells from mice injected with BAFF also showed an increase in AA4.1 hi cells, which include the An1 cells. B cells from these mice show a lowered calcium flux upon BCR stimulation indicating anergic properties.

These data suggest that BAFF stimulation results in the induction of B cell anergy, both *in vitro* and *in vivo*. The induction of anergy in B cells in response to stimulation through the antigen receptor may provide a mechanism by which autoreactive cells can evade deletion. Ultimately, the presence of these anergic B cells in the periphery poses a risk of activation and reversion to autoreactivity thus leading to automimmune disease.

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Prior publications and presentations

Publications:

Eva Sadat, Suwan Sinha, Jamie Lee, Shengxi Liu, Xin Bai, Richard Scheuermann. Manuscript in preparation. BAFF promotes enhancement of An1 cells: Potential role in autoimmune disease.

Jamie A Lee, Robert S Sinkovits, Dennis Mock, Eva L Rab, Jennifer Cai, Peng Yang, Brian Saunders, Robert C Hsueh, Sangdun Choi, Shankar Subramaniam and Richard H Scheuermann, and in collaboration with the Alliance for Cellular Signaling. **Components of the antigen processing and presentation pathway revealed by gene expression microarray analysis following B cell antigen receptor (BCR) stimulation.** *BMC Bioinformatics* 2006, **7**:237

Conference presentations

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Eva Rab, Jamie Lee, Xin Bai, Richard Scheuermann. Presentation of poster. A microarray analysis tool applied to BAFF stimulated B cell data. Annual Retreat, 2004, Department of Pathology, UT Southwestern Medical Center, Dallas, Texas

List of Tables and Figures

Abbreviations

BAD	BCL-2-antagonist of cell death
BAFF	B cell Activating Factor of the Tumor Necrosis Factor (TNF) family
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BIM	BCL-2 interacting mediator of cell death
BLNK	B cell linker protein
BrdU	bromodeoxyuridine
BTK	Bruton's tyrosine kinase
CFSE	carboxyfluorescein succinimidyl ester
CLASSIFI	Cluster assignment for biological inference
Crisp3	cysteine-rich secretory protein 3
Csk	C-terminal src tyrosine kinase
DAG	diacylglycerol
EAE	experimental autoimmune encephalomyelitis
Egr1	early growth response 1
Egr2	early growth response 2
EIF4E	eukaryotic initiation factor 4E
FBS	fetal bovine serum

FDR	false discovery rate
GO	Gene Ontology
IFNα	interferon alpha
IFNγ	interferon gamma
Igμ	Immunoglobulin μ heavy chain
IgM	Immunoglobulin M
IL-10	interleukin-10
IL-7	Interleukin 7
IP_3	inositol trisphosphate
ITAM	single immunoreceptor tyrosine-based activation motif
LPS	lipopolysaccharide
MACs	magnetic activated cell sorting
MCL1	myeloid cell leukaemia sequence 1
MZ	marginal zonal
Nab2	Ngfi-A binding protein 2
NF-AT	Nuclear factor of activated T cells
Nfatcc2	nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2
NF-κB	Nuclear factor kappa B
Nrgn	neurogranin
Pcp4	Purkinje cell protein 4)
PDK1	phosphoinositide-dependent protein kinase 1

PIP_2		phosphotidyl inositol bisphosphate
PLC- γ2		phospholipase C-γ2
pre-BCR		pre-B cell receptor
PS		phospholipid phosphotidylserine
PtdIns(3,4,5)P_3		phophatidylinositol-3,4,5-triphosphate
PTKs		protein tyrosine kinases
RA		Rheumatoid Arthritis
SAM		Significance analysis of Microarrays
sHEL		soluble for of HEL
SLE	-	Systemic Lupus Erythematosus
SS		Sjögren's syndrome
T1		transitional type 1
T2		transitional type 2
TACI		transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor
TNF		Tumor Necrosis Factor
TRAF		TNF receptor associated factor

Chapter 1

Background

B cell development

B lymphocytes play an essential role in the immune system as a primary line of defense by secreting antibodies against pathogens and foreign bodies. Early development of cells along the B lymphocyte lineage pathway occurs after successive gene rearrangement in the heavy (H) chain and then in the light (L) chain loci (Funk, Kincade et al. 1994). B cell development begins in the bone marrow with the pro-B-cell in which the heavy chain rearrangement occurs by VDJ recombination. The resulting intact μ heavy chain (Igμ) is the hallmark for the next stage in development, which is the pre-B cell stage. The pre-BCR (pre-B cell receptor) that is expressed on the surface of the pre-B cell is made up of an Igμ heavy chain and surrogate light chains. As the cells mature, the surrogate light chains are replaced with rearranged light chains. Once a complete IgM molecule is expressed on the cell surface, the cell is defined as an immature B cell (Rosenberg and Kincade 1994) expressing a mature BCR which can bind antigen. The cells then go through a negative selection process depending on whether the BCR can bind to any self antigen so that self-reactive cells can be eliminated. This occurs by three different processes, receptor editing, clonal deletion and anergy, the details of which are discussed in more detail in the "Negative Selection" section. Once the cells successfully complete this checkpoint, they leave the bone marrow as transitional type 1 or T1 cells. Once the T1 cells reach the spleen, they differentiate to the transitional 2 or T2 cells. These then either develop into the mature B cells or marginal zonal (MZ) cells. The mature B cells can further differentiate into plasma cells or memory B cells upon antigenic stimulation.

B cells can be differentiated from other cells in the body by antibodies against markers which are expressed on their cell surface that are unique for them. Such markers include CD45R (B220), a protein tyrosine phosphatase and CD19, which participates in B cell signaling. Once B cells have been identified, the next step is to identify the stage of differentiation of the cells. It has been seen that, at every differentiation stage, the cells express proteins other than immunoglobulin that are characteristic of each stage. CD43 is present in the pro B cell stage but is lost during maturation to the immature stage. IL-7 receptor is also present in the earlier B cell precursors since IL-7 is an essential growth factor for B and T cells. HSA or CD24 is expressed only in the early stages of B cell development (Hardy, Carmack et al. 1991; Osmond, Rolink et al. 1998). Another early B cell marker is AA4.1 which is expressed at lower levels beyond the T2 stage (Thomas, Srivastava et al. 2006). Other markers that are expressed at differential levels at each stage of B cell development include CD23, and IgD which are expressed at higher levels at the T2 and mature stage but at low levels or absent in the T1 and MZ populations; IgM expressed at higher levels on the T2 and MZ populations; and CD21 which is expressed at high levels on the T2 and MZ, at intermediate levels on the mature B cells and at low levels on the T1 cells (Loder, Mutschler et al. 1999). Thus the pattern of expression of a combination of the markers can help identify the maturation stage of the B cell

Proper development of B cells is essential for maintaining a healthy immune system. Any block in this pathway leads to immune deficiency due to lack of antibody production. An imbalance in the homeostasis of these cells leading to their inappropriate production can also create problems leading to malignancy or autoimmune disease.

Negative selection

After B cells proceed through several differentiation steps in the bone marrow, they develop a functional B cell receptor at the immature stage. In order to provide protection against pathogens, there is a great diversity of the antigen receptor generated by random rearrangement of the immunoglobulin genes. This inevitably results in the development of a great many of the receptors that recognize antigens that are expressed within the organism, also known as self-antigens. In fact about 50% to 75% of the newly produced immature B cells are reactive to self (Nemazee and Burki 1989; Wardemann, Yurasov et al. 2003). So how does the immune system know what is foreign and potentially harmful, and what is self? Being able to differentiate between self (own cells and tissues) versus non-self (microbial invaders) lies in the heart of the functional processes of the immune system. Each T and B cell carries surface receptors which specifically recognize a given antigen. This enables the immune system to recognize a wide range of foreign antigens. However, since microbes are made up of similar carbohydrates, proteins and lipids which constitute our own tissues, it is difficult to differentiate them from self-antigens. Nevertheless, in most cases, the immune cells are capable of being modified to ignore self and most of us are therefore tolerant of our own antigens.

There are two levels of tolerance, central and peripheral, which enables the cells to become tolerized and therefore prevent autoimmune disease. Central tolerance is the elimination of self-reactive cells during the development of the cells in the bone marrow. This includes receptor editing, which is a process by which renewed immunoglobulin gene rearrangement results in the elimination of the previous light chain and the generation of a

new light chain to pair with the existing heavy chain. This allows for about a third of these receptors to be reprogrammed to give a non-self reactive receptor (Gay, Saunders et al. 1993; Tiegs, Russell et al. 1993). Methods of detecting receptor editing include measurement of upregulation of RAG gene expression and (Nemazee and Burki 1989) and of new VJ recombinations (Fang, Weintraub et al. 1998). However, if receptor editing fails to give a receptor that is non self-reactive, then the cell will undergo clonal deletion. This results from the autoreactive B cells being deleted by apoptosis (Nemazee and Buerki 1989; Nemazee and Burki 1989; Nemazee, Russell et al. 1991). Upon failure of both of these processes of central tolerance, the self-reactive B cell may escape to the periphery where a third form of negative selection comes into play.

Peripheral tolerance results in autoreactive cells being rendered functionally unresponsive, a state that has been termed anergy. Tolerance can result when the B cells receive signal 1 without signal 2. Signal 1 is provided by antigen binding to the B cell receptor (BCR). In T dependent antibody responses, signal 2 is elicited by the binding of CD40L expressed on B cells to CD40 on T cells (Hodgkin and Basten 1995). Cells that receive only the first signal, become tolerant or anergic.

The hen egg lysozyme Ig Tg model has been useful in studying the different forms of negative selection (Cyster, Healy et al. 1996). In this model, Tg B cells that bear an Ig receptor specific for HEL die when they bind membrane bound HEL (mHel), showing clonal deletion (Hartley, Crosbie et al. 1991; Hartley, Cooke et al. 1993). On the other hand, expression of a soluble form of HEL (sHEL) results in insufficient cross-linking of the antigen receptors of the B cells, so that instead of deletion, the cells become unresponsive or

anergic (Goodnow, Crosbie et al. 1988; Goodnow, Crosbie et al. 1989). Although anergy has been described as a process that takes place in the periphery, it has recently also been observed in the bone marrow. Mice that were used for the study included a *p-azophenylarsonate* (Ars)-specific Ig-transgenic mice. The Ars specific transgenic BCR is cross-reactive with an undefined self antigen that induces an anergic phenotype in developing bone marrow cells. These cells exhibit high basal intracellular concentrations of Ca^{2+}, fail to induce normal tyrosine phosphorylation and calcium mobilization indicating their anergic nature (Benschop, Aviszus et al. 2001). This study signifies that tolerance mechanisms are much more complex than previously thought.

Anergic B cells

Anergy is a form of negative selection in which B cells that bind to self antigen with low affinity become unresponsive or silent. These cells have been studied using several transgenic models, including the MD4 anti-HEL model mentioned above as well as theArs/A1 transgenic model (Goodnow, Crosbie et al. 1989; Benschop, Aviszus et al. 2001). In the HEL transgenic model, all the B cells express a receptor that can recognize HEL. The MD4 mice with the transgenic B cells were bred with a transgenic mouse that expresses soluble HEL. Although cell development proceeds relatively normally in these mice, there is a strong skewing toward anergic B cells. Anergic B cells exhibit several features that differentiate them from normal cells. These include reduced lifespan (Fulcher and Basten 1994); high basal intracellular free calcium levels ($[Ca^{2+}]_i$) and no further increase in ($[Ca^{2+}]_i$) upon stimulation (Cooke, Heath et al. 1994; Benschop, Aviszus et al. 2001); high

phosphorylation of Erk; and altered migration and localization . In addition, anergic B cells do not proliferate or secrete antibodies upon stimulation (Cooke, Heath et al. 1994; Eris, Basten et al. 1994). Many of these features are shared by a recently described naturally occurring population of cells known as T3 or An1 B cells (Merrell, Benschop et al. 2006).

In order to identify anergic B cells, cell-surface-marker expression by B cells from two anergic immunoglobulin-transgenic mouse strains were compared to those expressed by non-autoreactive immunoglobulin transgenic mice and wild type mice (Merrell, Benschop et al. 2006). B cells from both ArsA1 (Benschop, Aviszus et al. 2001; Norsworthy, Fossati-Jimack et al. 2004) and α-HELtg/HELtg (Goodnow, Crosbie et al. 1988) mice expressed CD93. In addition, a majority of the B cells from the autoimmune models where CD23hi and IgM low. This phenotype is exactly similar to what has been previously described on transitional 3 or T3 cells (Allman, Lindsley et al. 2001). In contrast, the non-autoreactive immunoglobulin transgenic mice had virtually no T3 B cells. Since the anergic B cells in the autoimmune model show a T3 B cell phenotype, this was the first evidence that naturally occurring T3 B cells in wild type mice may constitute an anergic B cell population (Merrell, Benschop et al. 2006).

Some of the properties of the T3 B cells are very similar to anergic B cells. The T3 B cells are found in lymph nodes and peripheral blood which indicates that they are more mature in nature and not a developmental intermediary that are usually found in the spleen. T3 B cells, like anergic B cells, are impaired in signaling as measured by calcium mobilization and protein tyrosine phosphorylation (Cooke, Heath et al. 1994; Healy, Dolmetsch et al. 1997; Benschop, Aviszus et al. 2001). The *in vivo* fate and immunogen

responsiveness of T3 cells were measured by transferring the purified T3 population to *Igκ*$^{-/-}$ mice and immunizing the cells with goat anti-mouse *κ* antibodies. A polyclonal antibody response is induced that is aided by κ and goat-Ig-specific T cells. However the T3 cells do not respond to the stimulus. The developmental fate of these cells was also measured after adoptive transfer. The T3 cells remained CD93^{+} and did not contribute to the mature pool as readily as the T2 cells.

In addition, the gene expression profile of the anergic α-HELtg/HELtg cells were compared to antigen-naive α-HELtg B cells (Glynne, Ghandour et al. 2000). A very small set of genes were upregulated, which included Egr1 (early growth response 1), Egr2 (early growth response 2), Nab2 (Ngfi-A binding protein 2), Pcp4 (Purkinje cell protein 4) Cd72, Crisp3 (cysteine-rich secretory protein 3), and Nrgn (neurogranin). Very few genes were downregulated in anergic cells and they included Nfatcc2 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2) and Evi2a (ecotropic viral integration site 2a). T3 B cells were also seen to express increased amount of Egr2, Nab2 and Nrgn relative to follicular B cells, similar to α-HELtg/HELtg cells.

All these data provide evidence that T3 cells represent the anergic B cell population and were renamed An1 (Merrell, Benschop et al. 2006). This groundbreaking discovery of the existence of anergic cells within the wild type repertoire opens up new possibilities about how a break in tolerance could lead to autoimmunity through the reversal of anergy.

B cell receptor signaling

In order for B cells to develop normally, the B cell receptor signaling circuitry and components must be intact. The B cell receptor (BCR) is made up of an antigen binding component and a signaling component. The antigen-binding unit consists of a membrane immunoglobulin (mIg) heavy chain and light chain complex; the signaling component consists of a disulfide-linked heterodimer of the Igα and Igβ proteins. The immunoglobulin receptor is stably expressed at the plasma membrane in association with the Igα and Igβ invariant chain subunits (Reth 1992). The mIg chains have very short cytoplasmic ends, and therefore the much longer cytoplasmic tails of Igα and Igβ couple the BCR to the cytoplasmic signaling component. Both Igα and Igβ contain a single immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic tail and upon BCR crosslinking, signal transduction is initiated from these ITAMs (Flaswinkel and Reth 1994). The ITAMs consist of a conserved motif that are binding sites for SH2 domain-containing PTKs (protein tyrosine kinases) such as Blk, Fyn, or Lyn (Reth 1989; Cambier 1995). When the BCRs are crosslinked by antigen binding, they aggregate into glycosphingolipid-rich microdomains of the plasma membrane (Cheng, Brown et al. 2001). The receptor-associated kinases phosphorylate and activate each other, and then phosphorylate the ITAMs. These processes constitute the first steps in BCR-mediated signal transduction.

Regulatory mechanisms are present for this initial BCR signaling response. Src family kinases, such as Lyn, Blk and Fyn, consist of both an activatory domain and an inhibitory domain. Phosphorylation of the kinase domain results in activation while phosphorylation of the carboxy terminus results in inhibition (Pao, Famiglietti et al. 1998).

Lyn, a protein tyrosine kinase, plays a unique role in BCR signal initiation by having both positive and negative effects. Lyn phosphorylates Igα and Igβ ITAM tyrosines during development as well as during antigen-induced stimulation. Lyn-deficient mice have enhanced activation of downstream signaling components, elevated levels of intracellular calcium, proliferative responses, and B cell hyperactivity *in vivo* indicating that Lyn functions as a negative regulator of B cell signaling (Hibbs, Tarlinton et al. 1995; Nishizumi, Taniuchi et al. 1995).

The initiation of BCR signaling is indirectly regulated by two other non-receptor associated molecules - CD45 and C-terminal src tyrosine kinase (Csk). CD45 is also known as B220 and it is a transmembrane tyrosine phosphatase. The absence of CD45 leads to several signaling abnormalities, which indicates its importance (Benatar, Carsetti et al. 1996). Csk acts in direct opposition to CD45 since its absence results in the hypophosphorylation of Lyn while the absence of CD45 leads to its hyperphosphorylation (Hata, Sabe et al. 1994). Csk can phosphorylate the carboxy terminus of Src family kinases and inactivate those kinases. CD45 counteracts this by dephosphorylating the negative regulatory sites, thereby allowing the activation of these kinases. Thus a fine balance of these two kinases determines the signaling activity of BCR

So, the first step in BCR signaling constitutes the phosphorylation of the ITAMS by the receptor-associated Src-family tyrosine kinases, such as Blk, Fyn, or Lyn (Pao, Famiglietti et al. 1998). The second step involves high affinity binding between the phosphorylated ITAM and the SH2 domain of a member of a second family of protein tyrosine kinases, known as Syk (Rowley, Burkhardt et al. 1995). This occurs only after both

of the ITAM motifs have been phosphorylated. Upon receptor clustering, the Syk kinases are brought to close proximity and can phosphorylate and activate each other. Syk is essential in coupling the BCR signaling to distal signal transduction and it does this by phosphorylating and interacting with the adaptor molecule BLNK (B cell linker protein), also known as BASH or SLP-65 (Fu, Turck et al. 1998; Goitsuka, Fujimura et al. 1998; Wienands, Schweikert et al. 1998). Interaction of the Syk kinase with BLNK leads to a cascade of events that ultimately lead to propagation of the signal from the cell membrane to the nucleus. BLNK helps recruit Tec kinases such as BTK (Bruton's tyrosine kinase), that in turn phosphorylate and activate the enzyme phospholipase C-γ2 (PLC- γ2). PLC- γ2 cleaves the membrane phosphotidyl inositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular Ca^{2+} concentration, which is required for the activation of transcription factors such as NF-κB and NF-AT (Nuclear factor of activated T cells) (Dolmetsch, Lewis et al. 1997).

Cell surface expression of the BCR is required for development of B cells. Thus cells that are unable to express a pre-BCR, are arrested at the pre-B cell stage (Kitamura, Kudo et al. 1992). Mature B cells also need a functional BCR in order to persist; loss of BCR results in death (Lam, Kuhn et al. 1997). Tonic signaling from the Igα and Igβ cytoplasmic tail is therefore required for development and survival of the B cell. The signaling strength can be altered by antigen binding which also influences developmental fate of the cell (Lam, Kuhn et al. 1997; Caldwell, Wilson et al. 1998; Qian, Santiago et al. 2001; Casola, Otipoby et al. 2004). Thus an immature bone marrow B cell is much more sensitive to tolerance induction when it binds to self antigen than a mature B cell. An immature B cell will undergo receptor

editing or cell death when it encounters self-antigen. On the other hand, when a mature B cell encounters self-antigen, it can undergo anergy or become unresponsive. However, upon binding to antigen with adequate T cell help, the cell becomes activated (Goodnow, Crosbie et al. 1989).

Overview of BAFF

In BAFF-deficient animals, B cell maturation was found to be impaired beyond the immature transitional type 1 (T1) stage (Mackay, Schneider et al. 2003). In addition, BCR ligation has been shown to up-regulate the expression of BAFF-R on B cells (Smith and Cancro 2003; Walmsley, Ooi et al. 2003). This showed that immature B cells need BAFF in order to mature. Mice that are transgenic for BAFF have an overabundance of B cells (Mackay, Woodcock et al. 1999; Gross, Johnston et al. 2000; Khare, Sarosi et al. 2000). These studies indicate the importance of BAFF in B cell differentiation.

BAFF stands for B cell Activating Factor of the Tumor Necrosis Factor (TNF) family, also referred to in the literature as BlyS, Tall-1, THANK, TNFsF13B and zTNF4 (Moore, Belvedere et al. 1999; Mukhopadhyay, Ni et al. 1999; Schneider, MacKay et al. 1999; Shu, Hu et al. 1999). It is important for the survival and homeostasis of B cells. It is a homotrimeric, type II transmembrane protein which is proteolytically processed at furin consensus sequence and is then secreted (Bodmer, Schneider et al. 2002). Binding of Ig complexes to the Fc receptors of myeloid cells results in an increased rate of processing of BAFF (Li, Su et al. 2008). BAFF can either exist in a membrane bound form or in a soluble form (Mackay, Schneider et al. 2003), as a homotrimer or as a capsid like structure consisting of at least twenty trimers.

BAFF is expressed by macrophages, monocytes and dendritic cells and at lower levels by T cells (Mackay, Schneider et al. 2003). In humans, follicular dendritic cells also express BAFF (Munoz-Fernandez, Blanco et al. 2006). Recent work has shown that there are other cell types that also produce BAFF. These include airway (Kato, Truong-Tran et al.

2006) and salivary gland epithelial cells (Ittah, Miceli-Richard et al. 2008), fibroblast-like synoviocytes (Ohata, Zvaifler et al. 2005), astrocytes (Krumbholz, Theil et al. 2005), some stromal bone marrow cells (Schaumann, Tuischer et al. 2007) and osteoclasts (Abe, Kido et al. 2006). There are several cytokines such as interferon alpha (IFNα), interferon gamma (IFNγ), interleukin-10 (IL-10), granulocyte colony-stimulating factor and CD40 ligand (CD40L), as well as lipopolysaccharide (LPS) which can activate the production of BAFF by macrophages, neutrophils and DCs (Mackay, Sierro et al. 2005).

The three receptors which bind BAFF include B cell maturation antigen (BCMA), transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor (TACI), and BAFF receptor (BAFF-R) or, BR3. The binding of BAFF to BAFF-R is essential for the BAFF specific response (Gross, Johnston et al. 2000; Marsters, Yan et al. 2000; Thompson, Schneider et al. 2000; Wu, Bressette et al. 2000; Thompson, Bixler et al. 2001). While all three of these receptors are expressed on B cells (Mackay, Schneider et al. 2003), BAFF-R is also expressed on activated T cells and regulatory T cells (Mackay and Leung 2006). BAFF binds specifically to BAFF-R whereas BCMA and TACI also bind to APRIL (a proliferation-inducing ligand), which is another TNF family member that has highest homology to BAFF (Mackay, Schneider et al. 2003; Schneider 2005). The absence of BAFF-R in mutant mice (A/WySnJ) results in a phenotype similar to the BAFF knockout mice which includes a profound lack of B cells. This indicates that this receptor is essential for the BAFF specific survival response (Gross, Dillon et al. 2001; Schiemann, Gommerman et al. 2001; Thompson, Bixler et al. 2001).

In contrast, TACI knockout mice have hyperresponsive B cells resulting in increased Ig levels and splenomegaly. This suggests that TACI has a negative regulatory effect on BAFF signaling (Yan, Wang et al. 2001; Seshasayee, Valdez et al. 2003). BCMA knockout mice do not have any obvious phenotype (Schiemann, Gommerman et al. 2001; Xu and Lam 2001) although the survival of plasma cells was seen to be BCMA dependent (Avery, Kalled et al. 2003). The receptors are expressed mainly by B cells (Rodig, Shahsafaei et al. 2005; Darce, Arendt et al. 2007).

In mice, the expression of the BAFF-R is low on newly formed immature B cells, but increases as the cells mature (Darce, Arendt et al. 2007). TACI is expressed by all the peripheral B cells and particularly by marginal zone B cells (Mackay and Schneider 2008). BCMA is expressed only by antibody producing cells (O'Connor, Raman et al. 2004). In humans, BAFF-R is expressed by all cells except the plasma cells in bone marrow. TACI is expressed by the CD27 memory B cells and by tonsillar and bone marrow plasma cells. It is also expressed by a subpopulation of activated CD27-non-germinal center cells and by a small subset of naïve B cells that are present in the blood and in the tonsils. BCMA is expressed by human plasma cells from tonsils, spleen and bone marrow (Rodig, Shahsafaei et al. 2005; Darce, Arendt et al. 2007; Groom, Fletcher et al. 2007).

BAFF mediated signaling

BAFF signaling is initiated by binding of the trimeric BAFF to three independent receptors (Liu, Hong et al. 2003). Although, both trimeric and oligomeric BAFF can initiate BAFF-R signaling, only oligomeric or membrane bound BAFF can signal through TACI

(Bossen, Cachero et al. 2008). Adapter molecules known as TNF receptor associated factors (TRAFs) bind linear TRAF-binding sequences present in BAFF-R, BCMA, and TACI. TRAFs are recruited with higher avidity to three receptors held in the correct geometry by the ligand (Ye and Wu 2000). In order for the binding of TACI to initiate NF-κB signaling, two TRAF2 or TRAF6 molecules must bind to TACI in close proximity, which requires the recruitment of at least 6 receptors (Ye and Wu 2000). On the other hand, BAFF-R signaling does not need such higher order receptor clustering. BAFF-R signals by recruiting TRAF3 and causing its degradation, thereby releasing the inhibition of the alternative NF-κB2 signaling pathway (Xu and Shu 2002; Xie, Stunz et al. 2007; Gardam, Sierro et al. 2008). Thus in the absence of BAFF, TRAF3 interacts with NF-κB inducing kinase (NIK) resulting in the degradation of NIK, which causes decreased NF-κB2 activation. Once TRAF3 is deleted, constitutive activation of NF-κB2 pathway ensues (Gardam, Sierro et al. 2008). Although TRAF2 does not interact with BAFF-R directly, the degradation of TRAF3 in the presence of BAFF-R is TRAF-2 dependent. Activation of the NF-κB2 pathway leads to increased B cell survival (Matsuzawa, Tseng et al. 2008; Vallabhapurapu, Matsuzawa et al. 2008).

Once signaling begins, a cascade of events takes place. Briefly, BAFF-induced signaling results in activation of phosphoinositide 3-kinase (PI3K). This leads to the accumulation of phophatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) as well as recruitment of the protein kinase, AKT1, and phoinositide-dependent protein kinase 1 (PDK1). AKT1 is then activated both by PDK1 and by mTORC2 or/and protein kinase Cß (PKCβ (Woodland, Fox et al. 2008). This results in the release of inhibition of mTORC1,

which in turn releases S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (EIF4E) binding protein 1 (4EBP1). Once S6K1 is phosphorylated, it activates ribosomes, while the phosphorylated 4EBP1 releases EIF4E and promotes mRNA translation (Patke, Mecklenbrauker et al. 2006).

Downstream transcription factors that are activated include myeloid cell leukaemia sequence 1 (MCL1) (Wang, Chao et al. 1999). MCL1 is a member of the B cell lymphoma 2 (BCL-2) family that is required for the survival of peripheral B and T cells. MCL-1 acts by inhibiting BCL-2 interacting mediator of cell death (BIM), which is an inducer of apoptosis. Another pro-apoptosis factor that is inhibited both by AKT1 and PIM2, is known as BCL-2-antagonist of cell death (BAD). BAFF mediated survival also involves prevention of PKCδ translocation to the nucleus (Ishikawa, Carrasco et al. 1997; Enzler, Bonizzi et al. 2006; Mills, Bonizzi et al. 2007; Otipoby, Sasaki et al. 2008).

BAFF-R signaling results in potent stimulation of the alternative NF-κB2 pathway and weak stimulation of the classical NF-κB1 pathway. Both NF-κB pathways are necessary to initiate B cell survival and BAFF-R signaling is enough to deliver that. Once the NF-κB pathways are signaled, several downstream components and events are activated. These include expression of anti-apoptotic proteins, localization of B cells in the marginal zone, as well as T-cell-independent antibody class switching. (Kayagaki, Yan et al. 2002; Enzler, Bonizzi et al. 2006; Sasaki, Derudder et al. 2006). Thus, signaling by BAFF is intricately involved in B cell homeostasis, function and development.

Role of BAFF in autoimmune disease

Systemic Lupus Erythematosus (SLE) is a relatively common autoimmune disease that predominantly affects women with a female to male ratio of 9/1 (Zouali 2002) characterized by inflammatory skin lesions and multiple organ damage. The symptoms of the disease are largely associated with autoantibody production against nuclear antigens, including antibodies to chromatin, DNA-associated proteins like histones and components of the DNA repair machinery. Thus, a hallmark of SLE is the presence of auto-antibodies, which results in immune complex-mediated glomerulonephritis and renal injury (Zouali 2002). Sjögren's syndrome (SS), another autoimmune disease, is characterized by infiltration of lymphocytes and damage to the exocrine glands. SS can develop alone or in association with other autoimmune diseases such as SLE or rheumatoid arthritis (RA) (Tabbara and Vera-Cristo 2000; Groom, Kalled et al. 2002). A lot of work has been focused on trying to understand the causes and agents involved in these complex autoimmune diseases, but effective treatments have yet to be found.

Mice that are transgenic for BAFF develop autoimmune disease-like symptoms within 5 months of age. They develop glomerular proteinaceous deposits in the kidney; and there is presence of increased humoral immunity in the transgenic mice. Elevated anti-nuclear antibodies in the serum, a hallmark of SLE, are present at high levels in the BAFF transgenic mice. At 8 months there is an obvious enlargement of glomeruli in the BAFF transgenic mice as compared with age-matched control littermates. The high serum autoantibodies and kidney lesions resemble the progressive renal disease seen in SLE patients and lupus prone mice (Mackay, Woodcock et al. 1999; Gross, Johnston et al. 2000;

Khare, Sarosi et al. 2000). Also, mouse models of SLE as well as human SLE patients have an overabundance of BAFF. Recently, high levels of BAFF have been detected in the circulating blood as well as in the saliva of patients with SS (Groom, Kalled et al. 2002). The level of BAFF has also been seen to correlate with the titer of autoantibodies in human SS (Mariette, Roux et al. 2003). In addition, elevated levels of BAFF have also been found in the serum of human patients with RA (Cheema, Roschke et al. 2001) and SLE (Zhang, Roschke et al. 2001) When the salivary glands of the BAFF transgenic mice are evaluated, they show infiltration of a pathogenic B cell population (Groom, Kalled et al. 2002). NZB/WF1 mice, which are genetically prone to autoimmune nephritis, have BAFF in their sera that correlates with disease progression. Gross et al has also shown that by injecting a TACI decoy receptor injected into the nephritis prone mice, the level of activated B cells are reduced leading to alleviation of nephritis (Gross, Johnston et al. 2000).

Other autoimmune diseases that may be related to an overabundance of BAFF include: autoimmune diabetes (Zekavat, Rostami et al. 2008); psoriasis (chronic autoimmune disease affecting skin and joints (Samoud-El Kissi, Galai et al. 2008); myasthenia gravis (autoimmune neuromuscular disease)(Kim, Yang et al. 2008; Ragheb, Lisak et al. 2008); childhood opsoclonus-myoclonus syndrome (OMS) (rare neurological disorder resulting from autoimmune process) (Fuhlhuber, Bick et al. 2009); experimental autoimmune encephalomyelitis (EAE) (animal model of multiple sclerosis) (Huntington, Tomioka et al. 2006) .

In addition to autoimmune diseases, BAFF has also been associated with neoplastic diseases such as chronic lymphocytic leukemia (CLL) (Bojarska-Junak, Hus et al. 2009;

Molica, Digiesi et al. 2009), non-Hodgkin's B lymphoma (Lwin, Crespo et al. 2009), multiple myeloma (MM) (Ju, Wang et al. 2009); (Tai, Li et al. 2006), and prostate cancer (Di Carlo, D'Antuono et al. 2009). It is currently being investigated whether these neoplastic diseases occur due to increased survival of the cells of lymphoid origin due to the presence of excess BAFF. Other diseases associated with BAFF include: idiopathic thrombocytopenia which is a low platelet count without any known cause (Molica, Digiesi et al. 2009; Zhou, Chen et al. 2009); chronic graft versus host disease (Sarantopoulos, Stevenson et al. 2009); common variable immunodeficiency (CVID) (Poodt, Driessen et al. 2009); CNS (central nervous system) inflammation (Kim, Park et al. 2009); inflammatory myopathies (group of diseases that involve chronic muscle inflammation) (Krystufkova, Vallerskog et al. 2009)

The association of BAFF with so many diseases underlines the importance of this TNF family member in maintaining B cell homeostasis and its dysregulation resulting in severe pathogenic outcome. This thesis focuses on the mechanism of the pathogenesis of autoimmune diseases by the presence of excess BAFF. Since autoimmune disease is the result of the action of autoreactive cells (cells that bind to self-antigens and are activated to produce antibodies against those antigens) one important mechanism of BAFF action would be by increasing the presence of these autoreactive B cells.

Chapter 2

Material and Methods

Primary B cell purification and culture

The studies that are described in this dissertation were mostly done with primary B cells. The primary B cells were isolated as described in the Alliance for Cellular Signaling (AFCS)/Nature signaling Gateway website (www.signaling-gateway.org), at http://www.signalinggateway.org/data/cgibin/ProtocolFile.cgi?pid=PP00000001.

This method was used to isolate splenic B cells from C57BL/6 mice at age 6 to 12 weeks. The mice were anesthetized with CO_2 and then sacrificed by cervical dislocation. Spleens were removed and placed in a 70 μm nylon mesh cell strainer (BD Biosciences, Flanklin Lakes, NJ). The strainer was placed in 5 mls of magnetic activated cell sorting (MACs) buffer (PBS / 2mM EDTA) with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in a Petri dish set on ice. 4 spleens were placed in each strainer and the splenocytes were extracted by mashing the spleen through the cell strainer using the rubber end of the plunger from a 3 cc syringe to create single cell suspension in MACs buffer. The MACs buffer from this first mashing was filtered through a second nylon mesh cell strainer placed on top of a 50 ml Falcon tube kept on ice. This was repeated 4 or more times until the splenic capsules looked white. Cells were pelleted at 400g for 8 minutes at 4^0C. The MACs buffer was then aspirated leaving a small volume in which the pellet was resuspended by flicking the tube. RBC (red blood cell) lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.2-7.4 with HCl) was added at 1 ml per spleen and incubated for 2 min at room temperature with gentle rolling. This was followed by immediately adding 40 mls of MACs buffer to stop the reaction. Cells were then collected by centrifugation at 400 g for 8 min at 4 ^{0}C. Supernatants were aspirated and

cell pellets were loosened by flicking. The cells were then resuspended in 1 ml MACs buffer and filtered through a fresh or clean nylon strainer. Clumps were also placed in the strainer. The tubes were washed with MACs buffer 3 times and passed through the nylon membrane. Live cells were counted by using Trypan blue and hemocytometer. The concentration of the cells was then adjusted to 1.1 x 10^5 /µl with MACS buffer. Magnetic microbeads that were coated with anti-CD43 and anti-Mac-1 antibodies (Miltenyi) were then added at a concentration of 1 ul per 10^6 and 0.5 ul per 10^6 cells respectively. The mixture was then incubated at 4 °C for 15 minutes, mixing once at around 7 min. After the incubation, 40 mls of MACs buffer was added to each tube to ensure that any free microbeads were washed away. The cells were then pelleted at 400xg for 8 minutes at 4 °C and then resuspended in 1 ml MACs buffer per spleen. Cells were then passed through a fresh cell strainer placed on a conical and the tube was washed 3x with 1 ml MACs buffer each time, so that a total of 3 mls was obtained. Cells were then separated on the AutoMACS (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. After separation, two fractions were obtained: The positive fraction contained cells that were CD43 +Mac-1 + which includes T cells, neutrophils, macrophages, monocytes. The negative fraction of cells consisted of the enriched B cell population with an average of 96% B220 + cells.

BAFF purification

In order to do *in vivo* studies, a large quantity and a steady and reliable source of BAFF was required. BAFF was generated in the lab using the following procedures:

Construction of vector: Total RNA from human peripheral blood mononuclear cells (PBMCs) was used for cDNA synthesis. The following primers were used for amplification of hsBAFF:

Forward primer: GCCGTTCAGGGTCCA-Bam-U134-3

Reverse primer: TCACAGCAGTTTCAATGC-ter-Sal-L

The polymerase chain reaction (PCR) was done using Taq polymerase under the following conditions: first denaturation, 5 min at 94 ^{0}C; denaturation, 30 s at 94 ^{0}C; annealing, 30 s at 55 ^{0}C; extension, 1 min at 72 ^{0}C for 35 cycles; and last extension, 10 min at 72 ^{0}C. The amplified product and the pFastBac HT-C vector were separately cut with BamHI and Sal I and then ligated together to create the plasmid vector. PFastBac HT has a strong promoter for high level of protein expression. The N terminal 6xHis tag was used for purification of recombinant fusion proteins using metal-chelating resin.

Expression of recombinant protein in SF9 cells: The constructed recombinant plasmid pFastBac HT-C-BAFF was transformed into E.coli competent cells, DH10. The resulting Bacmid DNA was isolated from 6 mls of the transformed DH10 culture using midi-prep (Qiagen, Valencia, CA). The isolated bacmid DNA, rhBAFF/DH10, was then used to transfect the SF9 insect cell line using the Cellfectin reagent (Invitrogen, Carlsbad, CA). The viral supernatant consisting of the P_1 Baculovirus was collected 72hrs after transfection and stored at -80 ^{0}C. The viral supernatant was then used to infect a new batch of SF9 cells in order to amplify the virus. The P_2 virus was collected and another round of amplification was performed. The P_3 virus was then used to infect SF9 cells. Large scale BAFF expression in

SF9 insect cells was performed in a 1 liter flask seeded with cells at a density of 2 x 10^6 cells/ml in a total volume of 500ml. The cells were infected with high titer viral stock at an MOI of 10 pfu / cell. The time span of maximal expression was determined to be 72 hrs. At this time point, the cells were harvested in 50 ml aliquots. The cells were pelleted by centrifugation and stored at -80 ^{0}C until needed.

Purification of BAFF protein from SF9 cell lysate: The cell lysates were prepared using native conditions in order to keep the proper structure of the protein. The first step was to resuspend the pellet in 6 ml Native Purification Buffer (25 mM NaH_2PO_4, 2.5 mM NaCl, pH 8) containing 0.5 ug / ml Leupeptin (without EDTA) which is a protease inhibitor (Roche). The cells were then lysed by 4 freeze-thaw cycles using alternating liquid nitrogen and a 42 $^{\circ}$C water bath. The DNA was sheared by passing the preparation thru an 18 gauge needle about 10 times. The lysate was then centrifuged (JA-20 rotor, Beckmann Centrifuge) at 14,000 rpm (15,400 g) for 15 min. at 4 ^{0}C and then carefully transferred to a fresh tube without touching pellet. The lysate was centrifuged again at 4,500 rpm for 15 min. to remove any residual debris. 5 ul of the lysate was then used for SDS page analysis to ensure that the lysate contains the BAFF protein. BAFF was purified from the lysate using Ni-NTA agarose columns. The resin was washed with distilled water and then with binding buffer (native purification buffer + 10mM Imidazole), spinning down each time at 2,000 rpm for 1 min. The lysate was then added to the resin at a lysate to resin ratio of 25:1 and then allowed to bind to the resin at 4 ^{0}C overnight under constant rotation.

After spinning down the overnight bound resin with lysate, the supernatant consisting of the lysate was collected. The column was then packed with the resin. After allowing the resin to settle, the supernatant (lysate) was slowly added on top and then allowed to flow through and collected. These fractions were named as flow thru. Wash buffer (native purification buffer + 20 mM imidazole) was then added to the column. About 23 fractions of 1 ml each were collected. The O.D (optical density) of these fractions was measured to ensure that all the non-specific proteins that were bound to the column were washed off. Elution buffer (native purification buffer + 200 mM Imidazole) was then added to the column and about 25 fractions, ½ ml each were collected. The O.D of the eluents (elution fractions) was measured to ensure that the protein eluted properly. SDS page and Western Blot analysis was done to check the protein size and purity.

Dialysis of recombinant protein: The freshly eluted protein was dialyzed to remove imidazole using slide-a-lyzer cassettes (Thermo Scientific, Rockford, Illinois). The cassettes were first washed in water for 30 sec. The pooled eluted fractions were added to the cassette using a syringe. The cassette was then placed in a large beaker with about 1 liter of sodium phosphate buffer (250mM NaH_2PO_4 + 500mM NaCl) and stirred for 2 hrs at 4 ^{0}C. The buffer was then exchanged with about 1 liter of fresh buffer and the cassette was again stirred for another 2 hrs. This was repeated once more for 2 hrs. Finally, the buffer was exchanged with 1 liter of fresh buffer and the cassette was stirred overnight to ensure that all of the imidazole was removed. The cassette was then removed from the buffer and the protein

solution was taken out using a syringe. The buffer in which the protein was suspended was now replaced with the phosphate buffer using this dialysis procedure.

SDS-PAGE and Western blot (or Immunoblot) analysis: SF9 lysates that contained the BAFF protein were analyzed by SDS page (Polyacrylamide Gel Electrophoresis). . The samples were boiled for at least 3 minutes for denaturation and then separated using SDS-PAGE. Optical density of each of the samples was measured before loading onto the gel to ensure that roughly equal amounts of protein are loaded. A molecular weight reference was also loaded. Samples were separated by 10-12 % gel with Tris-glycine buffer at 50 mA constant current until dye front reached end of gel. Gels were blotted onto a nitrocellulose membrane by transferring overnight at 4 ^{0}C at 35 V. The membrane was then blocked to maximize signal:noise ratio. This was done by incubating with 5% non-fat dry milk powder in TBST buffer (50 mM Tris.HCL, ph 7.4, 150 mM NaCl, 0.1% Tween 20). The blot was then rinsed and the primary antibody, anti human recombinant BAFF antibody (R & D systems, Minneapolis, MN), suspended in the blocking buffer at a concentration of 1:500, was added. The blot was allowed to incubate with the primary antibody for 1 hr at room temperature. The blot was washed 2 times 5 minutes with TBST and then incubated with the secondary antibody, mouse anti-goat antibody conjugated with HRP (horse radish peroxidase enzyme) (Pierce Biotechnology, Rockford, IL), at a concentration of 1:10,000. The blot was next washed very carefully, about 4 times 10 minutes each with TBST to ensure that unspecific binding was removed. In order to detect the protein bands, enhanced chemiluminescence (Super Signal) (Pierce Biotechnology, Rockford, IL) was used. The film

was exposed to the blot, inititially for 30 s and developed. Depending upon clarity and thickness of the bands, the exposure time of the film was adjusted.

B cell survival assay: In order to test functionality of the lab-generated BAFF protein, primary splenic B cells were isolated, purified and cultured with or without different amounts of the lab-generated BAFF for 48hrs. The cells were then assessed for survival by staining with Annexin V-FITC (BD Pharmingen, San Jose, CA), which is an apoptosis marker that binds to membrane phosphotidylserine. Data were acquired with an LSRII (Becton Dickinson, San Diego, CA) and analyzed by FlowJo software (Tree Star)

In-vitro assays

Cell culture: All of the *in vitro* assays were performed on purified splenic B cells which were cultured in pre-warmed and CO_2 equilibrated media. The media consisted of high glucose DMEM medium containing 10% fetal bovine serum (FBS), 1 % penicillin / streptomycin solution (Invitrogen), 0.01 % ß-mercaptoethanol and 1% non-essential amino acids (all from Invitrogen, except FBS). Cells were cultured at 37°C in a 5% CO_2 incubator. These conditions were selected to maximize survival of primary B cells.

Annexin V staining: As cells undergo apoptosis, they translocate the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once PS is exposed to the surface, binding sites for Annexin V become available. Annexin V binds

with high affinity to PS in a Ca^{2+}-dependent manner (Reference-BD pharmingen). This assay allows the apoptotic cells to be detected.

The purified splenic B cells were cultured at 2 x 10^6/ml for desired timepoints. The cells were then harvested and spun down to remove media. They were then resuspended in 500ul cold binding buffer containing 0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$ (BD pharmingen) as per the manufacturer's protocol. The binding buffer comes as a 10x solution and must be diluted 1:10 with distilled water to get the 1x working solution. 5ul of Annexin V was added to each sample and the cells were incubated for about 10 minutes. The cells were then filtered through nylon membrane and taken for analysis. Data were acquired by FACscan and analyzed by FlowJo software (Tree Star).

Guava cell survival assay: Guava Viacount assay (Guava technologies, San Francisco, CA) was performed on the cells to measure the proportion of viable cells. Guava's patented viacount reagent consists of a vital nucleic acid dye LD751 (a membrane permeant dye that stains nucleic acid and mitochondria), and 7AAD that is a non-permeant dye which stains only dead cells that have lost their membrane integrity. By using this reagent it is possible to evaluate the exact number and percentage of viable cells present in each sample

Purified splenic B cells were cultured with or without BAFF for 24hrs, 48hrs and 72 hrs. About 50µl of the suspension was taken in a fresh tube and about 200 µl of the Guava Viacount reagent was added. The samples were mixed well and incubated for about 5 minutes at room temperature protected from light. They were then analyzed on the Guava PCA (personal cell acquisition) system, which is a bench top compact cytometer consisting

of a single green laser. It allows two-color detection and unlike other conventional cytometers, does not use sheath fluid. Instead, it takes up a fixed volume of sample so that the exact number of cells in the sample can be determined.

Guava MultiCaspase assay: The Guava multicaspase assay consists of a fluorochrome-conjugated caspase inhibitor, sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This is a non-cytotoxic, cell permeant dye that readily crosses cell membranes and covalently binds to multiple caspase family members that have been activated during apoptosis. This dye is again used with 7-AAD, which is a cell-impermeant dye. Thus, the double negative cells represent the live population, the double positives are the dead cells and the SR-VAD-FMK+ 7-AAD- cells are early apoptotic cells.

Purified splenic B cells were cultured for 48hrs and then harvested. Each sample was adjusted to a final concentration of about 5×10^5. 100µl of the cell suspension were transferred to a fresh tube and 5 ul of the 20X SR-VAD-FMK solution was added. Cells were incubated for 1 hour at 37^0C in the tissue culture incubator. After the cells were washed twice with wash buffer, they were resuspended in 100 µl of the wash buffer and about 5 µl of Caspase 7-AAD was added. The samples were incubated for 10 minutes at room temperature and then resuspended in 200 µl of wash buffer. The cells were then analyzed on the Guava PCA system.

BrdU proliferation assay: Bromodeoxyuridine (BrdU) is an analog of thymidine that gets incorporated into DNA during replication. BrdU uptake therefore gives a measure of cell division at the time of assay.

The purified B cells were cultured at 2 x 10^6/ml for desired timepoints and then pulsed with 25μM BrdU (Roche Diagnostics) 2 hrs before being harvested and stained. Cells were washed with FACs buffer without EDTA (PBS/ 0.5% FBS/ 0.1% NaN_3) and resuspended in 0.5ml PBS/ 1%PFA/ 0.01% Tween20 to fix and permeabilize the cells overnight. Cells were then washed 2x with the FACs buffer and then DNaseI treated by adding 10mg/ml DnaseI (Roche Diagnostics). Finally cells were stained with anti-BrdU-FITC at 10μg/ml (BD Pharmingen). Data were acquired by LSRII and analyzed by FlowJo software (Tree Star)

CFSE assay: CFSE or carboxyfluorescein succinimidyl ester, is a colorless and non-fluorescent reagent until the acetate groups in the compound are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein siccinimidyl ester. This group reacts with intracellular amines, forming fluorescent conjugates that are well retained in the cell as it undergoes cell division. The fluorescent label is inherited by daughter cells, but progressively diluted out due to the production of new cell material as the cells grow and divide during cell cycle progression. Thus daughter cells have decreased fluorescence intensity with each cell division cycle.

Briefly, cells were suspended at 1 x 10^6/ml in PBS and incubated at 37°C for 7 min with 0.25 μM CFSE. Following two serum/PBS washes to quench the uptake of CFSE, cells

were resuspended in media and cultured for 24hrs, 48hrs and 72hrs. Data was acquired using BD^{TM} LSRII and analyzed with FlowJo software (Tree Star)

Calcium flux assay: Calcium flux was used to evaluate whether the BAFF treated B cells obtain anergic properties. This is an effective method to measure B cell signaling since calcium flux is an early aspect of B cell signaling. Indo-1 functions by changing spectral properties such that it emits at 390 nm (violet) when bound to Ca++ and at 500 nm (green) when unbound. As the cells are activated and the calcium concentration in the cytoplasm increases, the dye binds more calcium. The color change from green to violet that Indo-1 undergoes can be plotted dynamically by programming the flow cytometer to display the two signals as a ratio of bound and free indo-1 against time. This allows measurement of changes in intracellular free calcium concentration [Ca (2+)].

Splenic B cells were cultured for 72hrs with and without BAFF. The cells were then incubated with 2 μM Indo-1AM for 40 minutes at 37°C followed by washing and resuspension in RPMI containing 10% FBS (Fetal bovine serum). Cells were washed again and resuspended in FACS buffer and were then stained with antibodies against AA4.1, CD23 and IgM. The cells were then kept in the dark on ice until calcium flux measurement. Right before measuring calcium flux, the cells were incubated in 37°C for 1 min and the baseline fluorescence was measured for unstimulated cells for 30 sec. The cells were then stimulated with anti-IgM Fab'2 (Jackson Immunoresearch) to induce BCR cross-linking and initiate calcium flux. Changes in intracellular-free calcium concentration was measured by the indo-

1 ratio (FL5/FL4 ratio) recorded over the next 41/2 min. Data were acquired with MoFLO (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

Differentiation marker staining: The splenic B cells were stained with several different markers to identify B cell differentiation stages.

Cells were suspended in FACS buffer, which consists of phosphate buffered saline (PBS) containing 1% FCS and 0.1% sodium azide. The following antibodies were used: CD23-PE, IgM-PerCP-Cy5.5, CD24-PE, CD21-FITC, CD19-PE-Cy7, B220-APC (BD Pharmingen) and AA4.1-APC (E-Biosciences). Cells were analyzed on FACS Calibur and on LSRII cytometers and analyzed using FlowJo software (Tree Star).

Cell sorting

The splenic B cells were sorted into the four differentiation stages, which include the transitional 1 (T1), transitional 2 (T2), marginal zone (MZ) and mature B cell populations. The T1 cells are CD23 low, AA4.1 high, T2 cells are CD23 high, AA4.1 high, mature are CD23 hi, AA4.1 low, and MZ cells are CD23 low, AA4.1 low.

The cells were stained for 20 minutes with anti-CD23 and anti-AA4.1 antibodies and then sorted into Mature, T1, T2 and MZ B cell populations using FACs CyAn (BD Pharmingen, Rockville, MD) and analyzed using FlowJo software (Tree Star).

In vivo injections

In order to study the effects of BAFF *in vivo*, 8 to 12 week old C57BL/6 male mice were given intraperitoneal injections of either 10μg BAFF (experimental group) or equivalent amount of PBS (control group) for 3 consecutive days. The mice were sacrificed the fourth day and the purified B cells were analyzed for any changes.

Microarray data generation

Microarray experiments were performed to analyze the biological response of B cells to various ligand stimulations.

Primary B cell culture and stimulation: Briefly, RNA samples were isolated from the cells cultured in media alone or with different ligand combinations for 6hrs, 24hrs and 48hrs. The treatments included BAFF (Cell Sciences, Canton, MA), anti-CD40 (BD Pharmingen, San Jose, CA), Anti-IgM (Jackson ImmunoResearch, West Grove, PA), BAFF + anti-IgM, anti-CD40 + anti-IgM, BAFF + anti-CD40, and LPS (Sigma Aldrich, St.Louis, MO), or media alone.

RNA extraction: After culturing the cells for desired timepoints, they were harvested and transferred from the wells to 15ml conical tubes. The cells were then centrifuged for 5min at 400xg to pellet cells and the supernatants were removed. 1 ml of cold TriPure reagent (Roche, Indianapolis, IN) was added to each sample and the cells were lysed by vortexing. The lysed cells were then stored at -80^0C until ready for RNA extraction.

The frozen cell lysates were thawed rapidly at 37^0C and then chloroform (Sigma, St Louis, MO) was added to 20% of TriPure volume. The tubes were then shaken for 30 sec and then incubated at room temperature for 5 minutes. Tubes were then centrifuged for 15min at 12,000 x g. The aqueous phase was then transferred to a fresh tube. Chloroform was added at equal volume as the aqueous phase. The tubes were shaken for 30s and then centrifuged at 4^0C 12,000 rpm for 15 min. Again the aqueous phase was transferred to a fresh tube and isopropanol (Sigma) was added at a volume equal to 50% of original TriPure volume. The tubes were inverted to mix and then incubated at room temperature for 5 min. Samples were centrifuged at 4°C 12,000xg for 10min. The supernatant was then carefully removed to avoid losing the pellet. 1ml of cold 75% ethanol was added to remove water and the tubes were than centrifuged briefly. The remaining supernatant was removed with a pipette and the pellet was allowed to air dry for 7 to 8 minutes until the pellets become clear. The pellets were resuspended in 10ul RNAse free water. RNA concentrations were then determined by diluting 1ul of sample into 79ul Tris-EDTA. The absorbance was read at 260nm. The RNA samples were stored at -80^0C.

DNAse treatment: The RNA was DNAse treated using a DNA-free kit (Ambion) according to manufacturer's instructions. 0.1 volume of 10X Dnase 1 buffer was added to the total reaction volume of RNA. The sample was mixed gently and incubated at 37^0C for 30 min. The Dnase inactivation reagent was resuspended by vortexing the tube and then 5ul of the reagent was added to the sample. The sample was mixed well by vortexing. The tube was

incubated for 2 min at room temp and then centrifuged at 10,000 x g for 1 min to pellet the Dnase inactivation reagent. The RNA was then removed to a fresh tube and stored at -80^{0}C.

Cleaning up RNA using the Rneasy MinElute Cleanup: The Dnase treated RNA was cleaned up using the Rneasy MinElute Cleanup kit (Qiagen) according to manufacturer's instructions. The volume of the thawed sample was adjusted to 100ul with RNase-free water. 350 ul buffer RLT was added to the sample and mixed thoroughly. 250ul of 96-100% ethanol was added to the RNA and then mixed thoroughly by pipetting. Immediately, 700ul of the mixture was added to the Rneasy MinElute Spin Column in a 2ml collection tube. The tubes were then centrifuged for 15s at greater then 8000xg and the flow through was discarded. The spin columns were then transferred to a new 2ml collection tube. 500ul buffer RPE was added onto the spin column. The tubes were centrifuged for 15s at 8000xg to wash the column and the flow through was discarded. 500ul of 80% ethanol was added to the Rneasy MinElute Spin Column. The tube was then centrifuged for 2min at >8000xg to dry the silica-gel membrane and the flow through was discarded. The Rneasy MinElute Spin Column was transferred to a fresh 2ml collection tube. The cap of the spin column was opened and the column was centrifuged at full speed for 5 min. The columns were transferred to a new 1.5ml collection tube and water was used to elute the RNA by dropping the water directly onto the center of the silica-gel membrane. The tube was centrifuged for 1 min at maximum speed to elute the RNA. The RNA was then stored at -80^{0}C.

Real-time reverse-transcription polymerase chain reaction (RT-PCR): B cells were purified and cultured in media alone or in the presence of BAFF for 16hrs and 24hrs. The cells were harvested and the RNA was extracted from them. 1 μg of total RNA from each sample was then treated with DnaseI (Invitrogen). Dnase treatment was done by adding buffer, 1ul Dnase1 and water to make up total reaction volume of 10ul, to each ug of RNA. The reaction mix was then incubated at 20 min. Finally the reaction was stopped by adding 1 ul EDTA and then incubating at 65^0C for 10 minutes.

The RNA was then reverse transcribed at 42 ^{0}C for one hour in a total reaction volume of 20μl. The reaction mixture consisted of 1μl MMLV reverse transcriptase (Invitrogen), 4ul of 5X buffer (Invitrogen), 0.5 mM dNTPs (Invitrogen), and 5 ng/ul pd $(N)_6$ (Amersham) The reaction was then inactivated at 70^0C for 20min. The PCR reactions were then set up using Sybr Green Master Maker (Bio Rad, Hercules, CA). Thermal cycling began with a denaturation step of 10 min at 95^0C, thermal cycling for 40 cycles of 95^0C for 15 sec and 60^oC for 1 min (denaturation, annealing and extension). The real time PCR reactions were performed in a thermocycler (Strategene, La Jolla, CA) and data analyzed with the Applied BioInformatics (ABI) software. Standard curves were generated using RNA isolated from RBC depleted splenocytes. Mouse 18S rRNA levels were used for normalization. Fold change in gene expression of treated samples relative to untreated samples was measured. PCR primers were designed using Primer Quest from IDT (Integrated DNA technologies). The following primers were used:

CD72 Forward Primer: ACAGATGAGCAAGAAGTGGAGGCA

CD72 Reverse Primer: TCCCATCAGGAAACCTGAAAGCCT

Egr1 Forward Primer: AGGTGGTTTCCAGGTTCCCATGAT

Egr1 Reverse Primer: TCAGGTCTCCCTGTTGTTGTGGAA

Nab2 Forward Primer: GGGCAGTGCTCGAAGCTTTA

Nab2 Reverse Primer: TGGCGACAACTTCTCTCCAA

Microarray experiment procedure and analysis: The RNA samples were used to generate Cy5 (for sample RNA) and Cy3 (for RBC-depleted total splenocyte RNA) labeled cDNA and were hybridized together. All samples were run in quadruplicates. The slides with the hybridized cDNA were then washed, dried and then scanned. Spot features on each array were filtered to remove values that were saturated, non-uniform or below background. The signal/noise ratio for each spot was calculated and only if the spot's background corrected intensity was greater than the background intensity, the spot was considered good. This ratio was calculated using TIGR MIDAS which is an open source software for Microarray data analysis.

Once background filtering was completed, the data was normalized using LOWESS. LOWESS normalization functions by normalizing channelA intensities or channelB intensities of all spots by applying LOWESS algorithm and adjusting the channel intensities of each spot by the LOWESS factor. The LOWESS factor for normalization was set to global mode so that all the spots were considered for normalizing each spot (Quackenbush 2002). After applying LOWESS, the data set is further scaled and centered by iterative log mean centering and standard deviation regularization. Iterative log-mean centering normalization function normalizes channelA or channelB intensities of all spots in the data set by adjusting the mean of $\log_2$ ratio of channelB versus channelA intensities to zero. The

data range for mean centering defines which spots should be selected and was set to ±3 standard deviation. Standard deviation regularization function scales the channelA or channelB intensities of each spot so that each spot has the same standard deviation distribution. The standard deviation regularization was done under the block mode so that the spots within each block will have the same standard deviation.

Once normalization, scaling and centering was completed, the data was ready for clustering. Hierarchical clustering was done using Pearson Correlation and average linkage clustering. Statistical filtering was done using SAM (Significance analysis of Microarrays) (Tusher, Tibshirani et al. 2001). A false discovery rate (FDR) of 15% was used to determine which of the genes were differentially expressed between samples and untreated controls. Very low numbers of differentially expressed genes were present in BAFF treated samples. 15% of the median of all significant genes of the strong stimulants was used as the FDR for BAFF. A significance cutoff or the critical p value for each of the stimulations was calculated using the Bonferonni correction. This was done by taking the *alpha* value of 0.05 and dividing by the number of genes in each treatment.

Classifi analysis

CLASSIFI (Cluster assignment for biological inference) is applied to give biological meaning to the microarray data. The input file for CLASSIFI consists of 3 columns: the first column has the probe IDs, the second column has the gene names and the third one has the cluster IDs. Once the input file is uploaded into CLASSIFI, and the query is submitted, the results appear shortly depending on how big the file is. A cumulative hypergeometric

distribution analysis is then applied to the uploaded file to determine whether the probability of the co-clustering of a particular ontology is significant. Calculating this hypergeometric distribution involves several steps. The first step is to upload the input file which consists of the probe list with gene cluster assignments. CLASSIFI then queries the database and captures the primary GO annotations for every probe in the dataset. CLASSIFI then further queries the database to capture complete GO ancestry for every primary GO annotation for each probe. Next CLASSIFI enumerates n, f, g, c for each gene ontology in each gene cluster, n = number of probes with associated GO ID in the gene cluster, f = number of probes with the associated GO ID in the data set, g = number of probes in the data set, c = number of probes in the cluster. Using these values, the solution (p-values) of the hypergeometric equation is calculated. Finally, CLASSIFI orders ontologies based on p-values. The function of CLASSIFI is to allow specific biological functions to be assigned to the different clusters depending on whether the probability of the co-clustering of genes with that specific biological function is below a threshold value.

Chapter 3

Characterization of the effects of BAFF on B cells *in vitro*

Introduction

BAFF has been associated with autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis and Sjogren's syndrome. There are increased levels of autoreactive antibodies that correlate to increased levels of BAFF found in the sera of these patients. However it is not fully understood how BAFF may be associated with the generation of autoreactive B cells and ultimately the development of disease. It is essential to characterize BAFF in order to find the missing link between BAFF and autoimmune disease. Several *in vitro* studies have been carried out to get an overall basic understanding of the effect of BAFF on B cells in order to elucidate how BAFF may be involved in autoimmune disease. We chose to first look at BAFF effects *in vitro* so that we could set up a controlled environment that would eliminate any indirect effects caused by the presence of other cytokines/chemokines or cell-cell interactions within the animal. The first step in doing this was to understand how BAFF could be affecting survival of B cells. We specifically wanted to test which cell type is targeted for survival and whether that cell type is prone to becoming autoreactive. These *in vitro* studies were also aimed at characterizing the expression of cell surface proteins in order to differentiate the stage of B cells at which BAFF shows a direct effect. We also wanted to study the effect of BAFF on other relevant biological processes such as cell proliferation and differentiation in purified B cells. Since autoimmunity could result from a disruption of a fine balance of B cell homeostasis, it is important to study the effect of BAFF on all of these different biological functions.

BAFF stimulates survival of resting B cells

The first step in our analysis of survival was to evaluate the forward/side scatter (FSC/SSC) dot plots of cells that have been stimulated with BAFF or anti-CD40. FSC/SSC is an effective measurement of cell size and complexity. A higher FSC indicates larger cell size while higher SSC indicates higher granularity.

Thus the cells with the lower FSC/SSC (smaller size and granularity) consist of the resting B cells and the cells with the higher FSC/SSC (larger size and higher granularity) represent the activated or the blasting/proliferating cells. Since no apoptosis marker was used in this experiment, it was not possible to gate out the apoptotic cell population.

CD40L is known to stimulate resting B cell proliferation and therefore anti-CD40 which mimics binding of CD40L was used as a positive control. Figure 3.1 A, B and C depict the FSC/SSC dot plots of untreated, BAFF- and anti-CD40-treated cells, respectively. Within each plot, the oval shapes were drawn to separate the resting cell population (lower left) and the blasting population (upper right). In the untreated time 0 plot it was seen that most of the cells are in the resting zone (data not shown). The results show that the BAFF stimulated cells (Fig 3.1 B) have higher numbers of resting cells than the untreated control (Fig 3.1 A) while the anti-CD40 treated cells show a much higher proportion of blasting cells (Fig 3.1 C). The summary of the data from 3 replicates of each treatment is shown as bar graphs for 24hr plots (Fig 3.1 D) and 48hr plots (Fig 3.1 E). The sum of the resting and the activated populations gives the total live cell population. In both the 24hr and the

48hr plots, there is a significantly greater proportion of live cells in both BAFF and anti-CD40 treated samples (Fig 3.1 D and E). However, in the 24 hr plot, only 18% of the BAFF treated cells are seen to be activated compared to 36% of the anti-CD40 treated cells (Fig 3.1D). In contrast, 12 % of the BAFF treated cells are resting while only 3% of the anti-CD40 treated cells are resting in the 24hr plot (Fig 3.1 D). The same trends can be seen in the 48hr plot with an equivalent proportion of resting and blasting population in the BAFF treated cells (Fig 3.1 E). At both timepoints, BAFF shows a significantly greater number of resting B cells while anti-CD40 shows a greater number of activated B cells compared to untreated controls (Fig 3.1 D and E). These data suggest that BAFF causes an increase in the recovery of cells from culture primarily by promoting the survival of resting cells, while anti-CD40 contributes to cell recovery mainly by increasing activation of the cells.

The purity of the B cells that were separated from the splenic cells using a negative selection procedure is shown in Fig. 3.0. The cells were separated by adding anti-CD43 and anti-MAC-1 antibodies which are conjugated to magnetic beads. The mixture is then passed through a magnetic column allowing the B cells to pass through with minimal activation. The purified B cells are on average 96% pure. CD43 + cells include immature B cells, plasma cells, some mature B1 cells, granulocytes, monocytes, macrophages, platelets, natural killer cells, thymocytes and CD8 positive and CD4 positive T cells. CD43 is not expressed on resting peripheral B cells. This method of resting B cell enrichment has been used by other groups (Hein, Lorenz et al. 1998). CD11b or Mac-1 is expressed on monocytes/macrophages

and microglia, and to a lower extent on granulocytes, NK cells, CD5+ B1 cells and a subset of dendritic cells. The other cell populations in the negative fraction may consist of contaminating CD43+, MAC-1+ non B cells which may include natural killer cells, thymocytes, CD8+ and CD4+ T cells due to leakage in the staining.

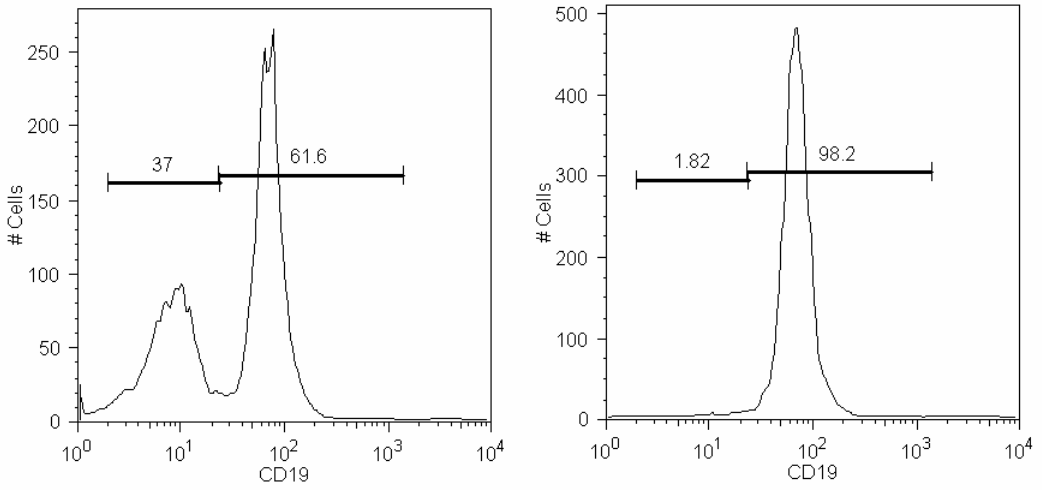


Fig 3.0 B cell purity

Flow cytometric analysis of B cell purity. Cells were separated using a negative selection procedure using anti-CD43 and anti-Mac-1 antibodies, and then stained with anti-CD19 antibodies.
(A) Splenic cells before separation
(B) Purified B cells post separation

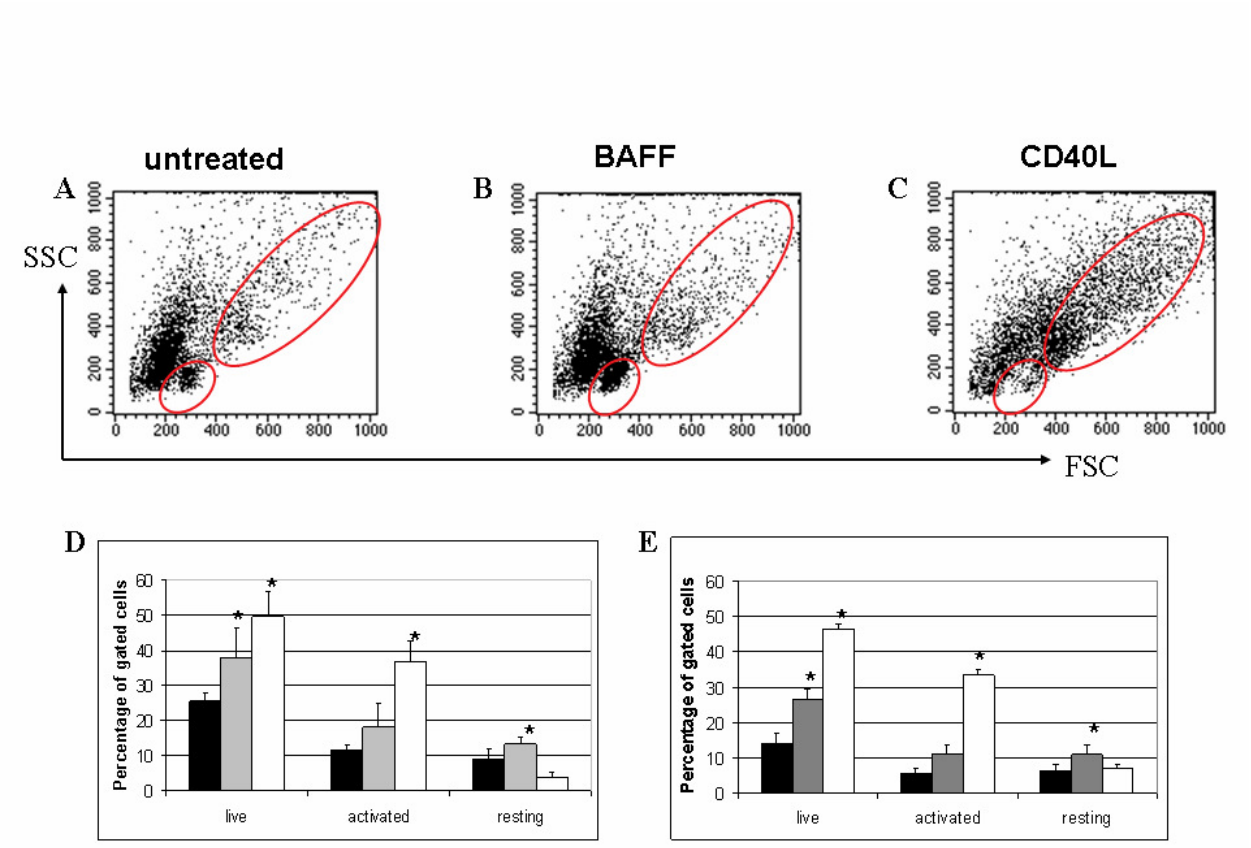


Fig 3.1 BAFF stimulates survival of resting B cells

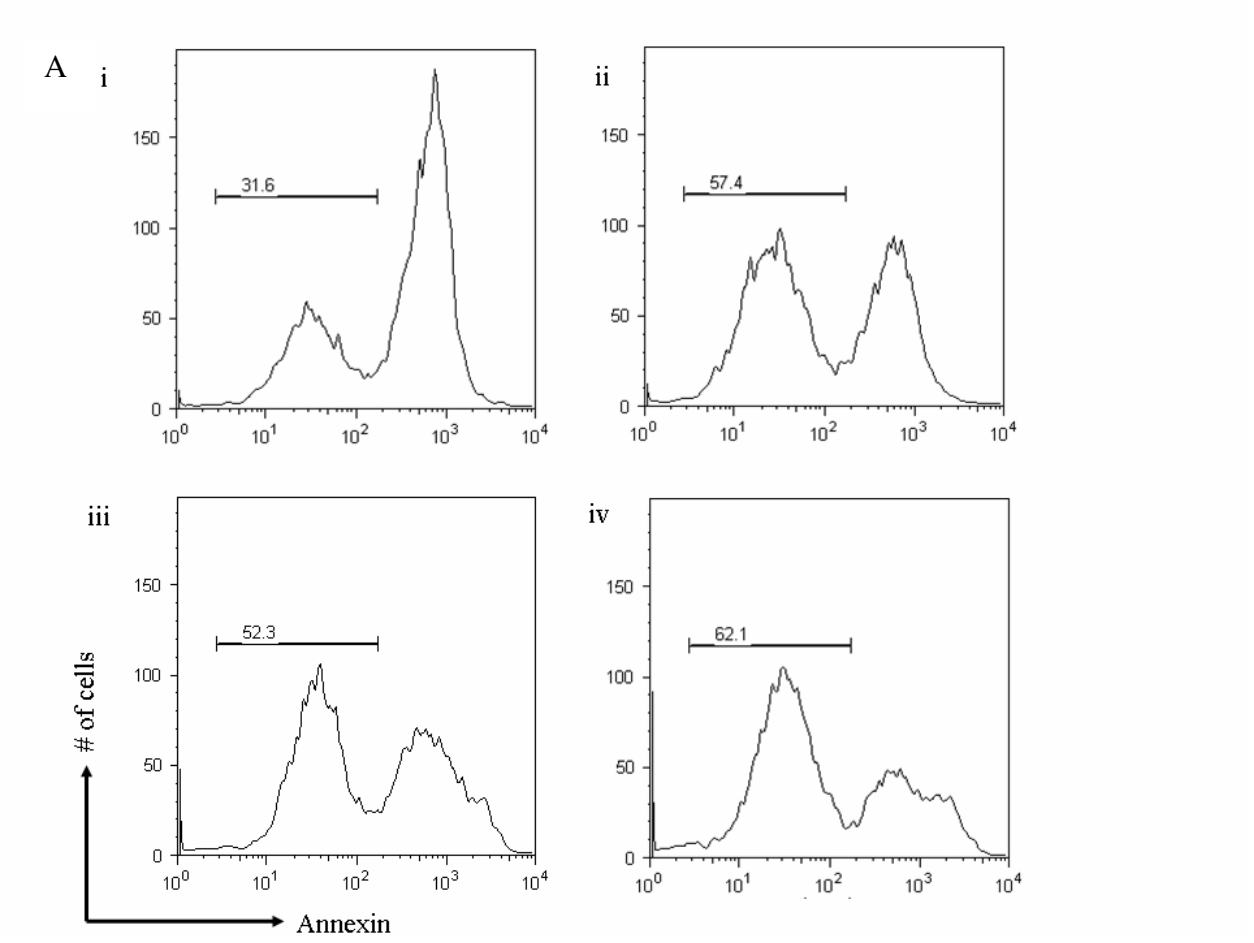
Purified splenic B cells were cultured either in media alone (black) or with BAFF (grey) or with anti-CD40 (white) for 24hrs and 48hrs and the FSC/SSC dot plots were examined.
(A-C) Representative dot plots of untreated, BAFF and anti-CD40 treated purified splenic B cells at 24 hrs. Lower encircled area represents the resting cells and the upper encircled area represents the blasting cells.
(D) 24hr bar graph summary of total live, activated and resting B cell populations
(E) 48hr bar graph summary of total live, activated and resting B cell populations
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.
* Represents p value of less than 0.05
** Represents p value of less than 0.01

BAFF enhances survival of BCR stimulated cells

B cells undergo death by neglect when cultured in vitro without stimulation. Several studies have shown that BAFF prevents apoptosis and increases the survival of B cells in culture. In addition to testing the survival effect of BAFF by itself, we also wanted to examine whether the BAFF signaling pathway is interconnected to B cell receptor (BCR) signaling. The strength of the BCR signal is a determining factor for the developmental fate of the cells. Strong binding of the BCR to membrane bound self antigen could result in apoptosis, since negative selection comes into play. On the other hand, weak binding of the BCR to soluble self-antigen results in inactivation of the B cells resulting in anergy. Thus any disruption or modulation of the BCR signaling could lead to a disruption in negative selection and homeostasis that could potentially lead to an increase in autoimmune cells. The effects of BAFF on BCR signaling can be studied by stimulating B cells with anti-IgM alone and in the presence of BAFF. Polyclonal anti-IgM effectively cross links BCR and is therefore a good substitute to use for *in vitro* studies to mimic antigen-induced cross linking. We used three different methods to examine the survival effects of BAFF on BCR signaling.

The first method involved the use of Annexin V staining as a marker of apoptotic cell death and analysis by traditional flow cytometry. As cells undergo apoptosis, they translocate the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once PS is exposed to the surface, binding sites for Annexin V become available. Annexin V binds with high affinity to PS in a Ca^{2+}-dependent manner (van Engeland, Ramaekers et al. 1996). This assay allows the apoptotic cells to be detected as shown by the annexin V positive population in the histogram plot. The live cells are annexin V negative (Fig 3.2A). The bar graphs were drawn using the proportion of the Annexin V negative (live) cells (Fig 3.2 B, D and E). The data shows that the treatment of the isolated splenic B cells with BAFF in culture promotes their survival, both in the presence and absence of stimulation through the B cell receptor. This can be seen in all three timepoints, 24hrs (Fig 3.2 B), 48 hrs (Fig 3.2 C) and 72 hrs (Fig 3.2 D) but the most dramatic effect is seen at 72 hrs, between the untreated and BAFF treated samples. For example it can be seen that at 72hrs only 45% of the untreated B cells were Annexin V negative (viable) whereas 90% of BAFF treated were Annexin V negative (Fig 3.2 D). In the presence of anti-IgM, which stimulates B cells through their antigen receptor, BAFF exhibited a similar effect, increasing the proportion of Annexin V-negative cells (Figure 3.2 B-D). These results suggest that BAFF treatment substantially reduces the level of spontaneous apoptosis of isolated splenic B cells when they are placed in tissue culture, both in the presence and absence of BCR stimulation. In the BAFF cultures most of the cells are resting B cells according to the FSC/SSC. In the anti-IgM

cultures, most of the cells are activated. In the BAFF + anti-IgM cultures, most of the cells are activated since anti-IgM is a strong activator. The cells that are rescued from spontaneous apoptosis in the BAFF + anti-IgM cultures are therefore mostly activated.


A
i
ii
iii
iv
31.6
57.4
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62.1
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Annexin

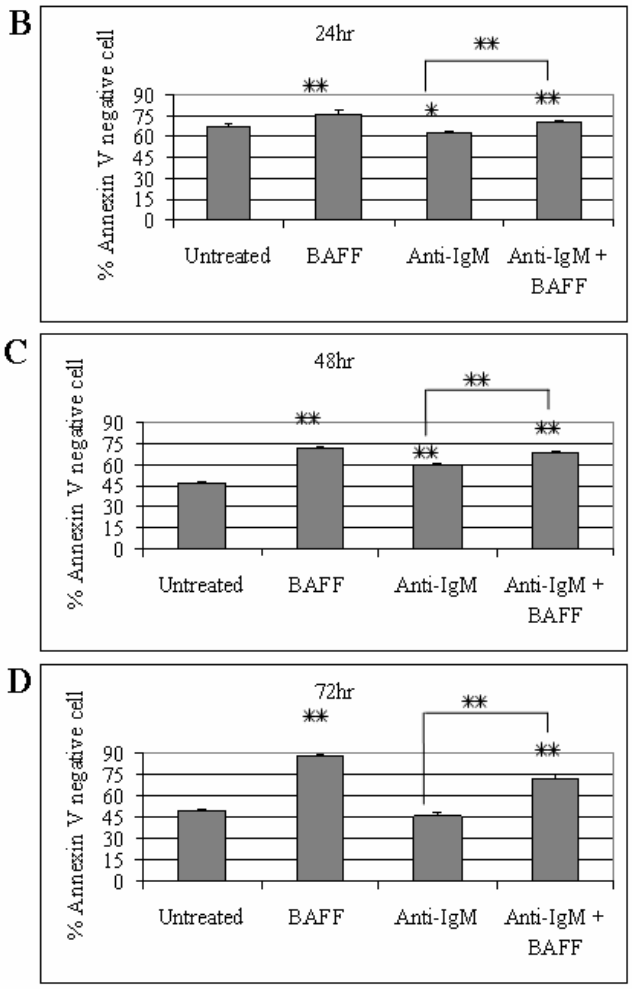


Fig 3.2 BAFF shows an anti-apoptotic effect on B cells *in vitro*

Flow cytometric analysis of Annexin V staining on purified splenic B cells.
(A) Representative histogram plots showing percentage of Annexin negative cells that were either left untreated (i) or cultured in the presence of BAFF (ii), anti-IgM (iii), or both (iv), for 48hrs.
(B-D) Bar graphs showing cells that were cultured in the presence or absence of BAFF, anti-IgM, or both, for 24hrs (B), 48hrs (C) and 72hrs (D). The percentage of Annexin V negative (non-apoptotic) cells are represented.
Shown are the average of triplicate samples. Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test. (n is greater than 3)
* Represents p value of less than 0.05
** Represents p value of less than 0.01

One of the drawbacks with the Annexin V assay using the FACs Calibur is that it captures data about cell proportions in culture, but does not take into account any cell loss that may have occurred as a result of cell death. And so we employed a second method that included flow analysis with the Guava PCA (Personal Cell Analysis system). The unique feature of the Guava personal cell analysis system (Guava PCA) is that it takes up a fixed volume of sample, unlike the traditional flow cytometry machines which mix variable amounts of sheath fluid to the sample. This enables the software to calculate the exact number of cells that are present in a given sample. Guava's patented viacount reagent consists of a vital nucleic acid dye LD751 (a membrane permeant dye that stains nucleic acid and mitochondria), and 7AAD that is a non-permeant dye which stains only dead cells (Fig 3.3 A-D). By using this reagent it is possible to evaluate the exact number and percentage of viable cells present in each sample. The data shows a large difference in viable cell counts between untreated versus BAFF-treated samples at all three timepoints (Fig 3.3 B, C and D). Additionally, it can be seen that there is also a higher number of viable cells in the anti-IgM + BAFF treated samples compared to the anti-IgM alone samples (Fig 3.3 B,C and D). This confirms the Annexin data in the protective function of BAFF against apoptotic death of B cells both in the presence and absence of BCR stimulation. Interestingly, while the traditional flow data shows no change in the proportion of anti-IgM treated B cells at 72hr (Fig 3.2 D) compared to untreated control, the Guava data shows that there is a much greater number of anti-IgM treated B cells compared to untreated control (Fig 3.3 D). This suggests that BCR stimulation may be inducing proliferation without directly affecting cell survival.

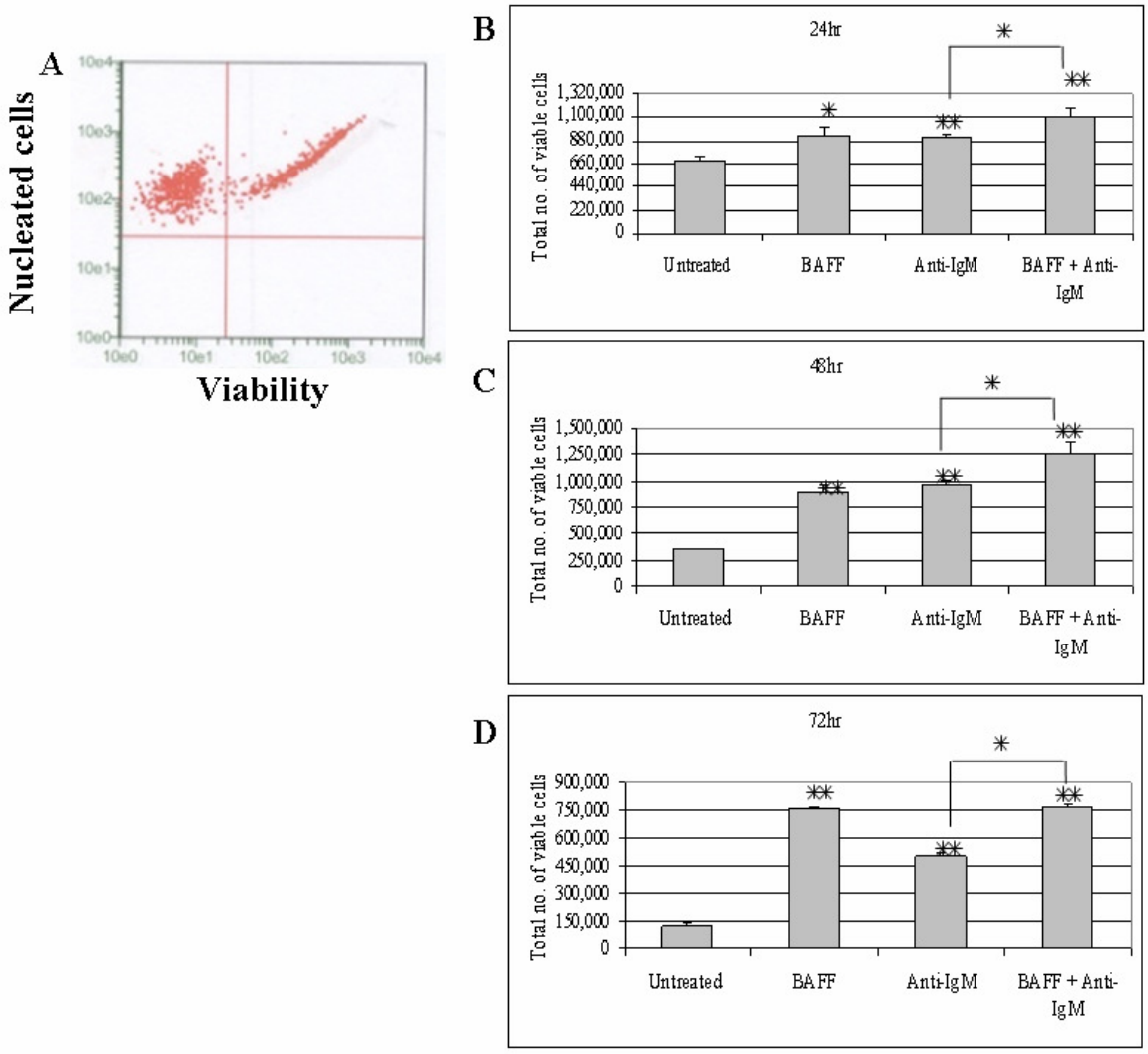


Fig 3.3 BAFF shows an anti-apoptotic effect on B cells *in vitro*

(A-D) Flow cytometric analysis of Guava Viacount staining on purified splenic B cells. . Cells were cultured in the presence or absence of BAFF, anti-IgM, or both, for 24hr (B), 48hr (C) and 72hr (D). The total numbers of viable cells are represented.

Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.

* Represents p value of less than 0.05

** Represents p value of less than 0.01

The third method involved using a fluorochrome-conjugated caspase inhibitor, sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This is a non-cytotoxic, cell permeant dye that readily crosses cell membranes and covalently binds to multiple caspase family members that have been activated during apoptosis. This dye is again used with 7-AAD, which is a cell-impermeant dye. In live and healthy cells, 7 AAD is excluded; however it can permeate late apoptotic and dead cells. Thus the double negative cells represent the live population, the double positives are the dead cells and the SR-VAD-FMK+ 7-AAD- cells are early apoptotic cells. This assay separates the early apoptotic cells, the live and the dead cells in each of the samples (Fig 3.4). The data shows that about 65% of the cells are live at 24 hrs in the control samples and the anti-IgM samples (AIG) while the BAFF and BAFF +AIG have about 10% more live cells (Fig 3.4A). This indicates again that BAFF protects the cells against spontaneous apoptosis both in the absence and presence of BCR stimulation. These results are reflected in the dead cell populations, which shows about 10% less dead cells in the BAFF treated samples compared to the untreated and AIG treated samples (Fig 3.4A). In the early apoptotic populations, there is no difference between the untreated control vs. BAFF and the AIG vs. BAFF + AIG samples (Fig 3.4 A). At the later time point of 48hrs, about twice as many live cells are present in the BAFF treated culture compared to the control in the live cell populations (Fig 3.4 B). There was also a 10% increase in the live cell population in the BAFF + AIG compared to AIG alone treated samples. This indicates that prevention of apoptosis by BAFF occurs mainly at the later stages rather than the very early stages of apoptosis.

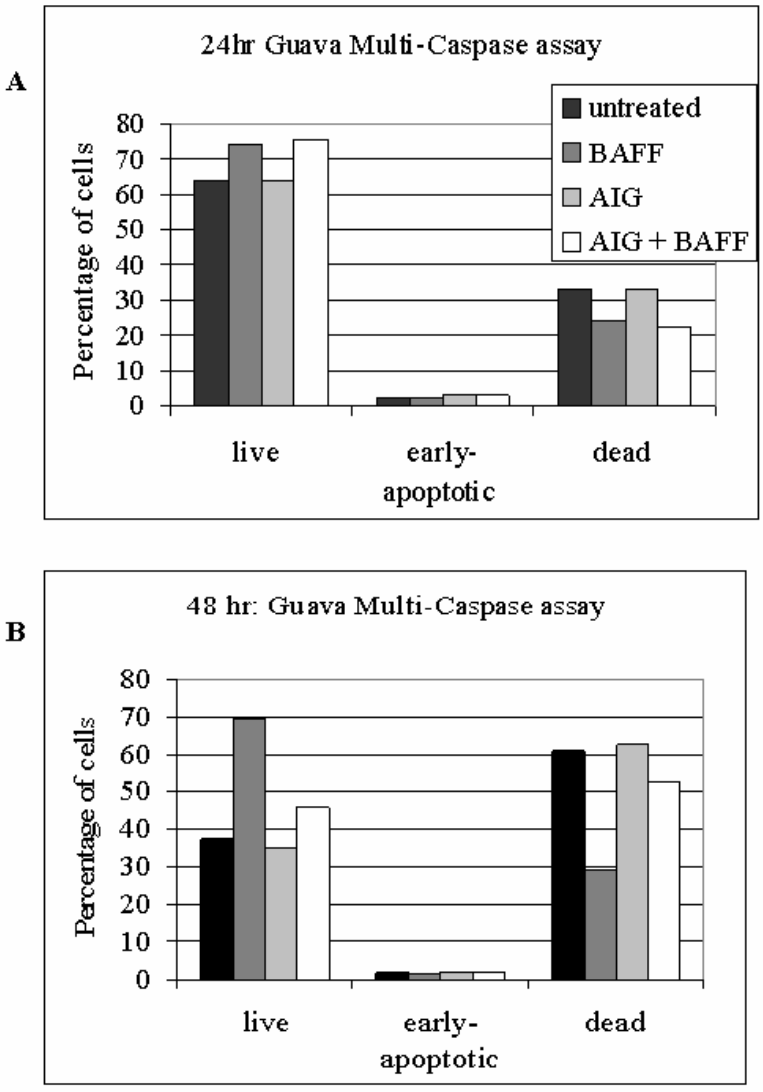


Figure 3.4 BAFF shows an anti-apoptotic effect on B cells in vitro

Flow cytometric analysis of Guava multicaspase assay on purified splenic B cell stimulated as indicated in (Fig 3.1) for (A)24hr and (B)48hrs. The percentage of live, early-apoptotic and dead cells are shown.

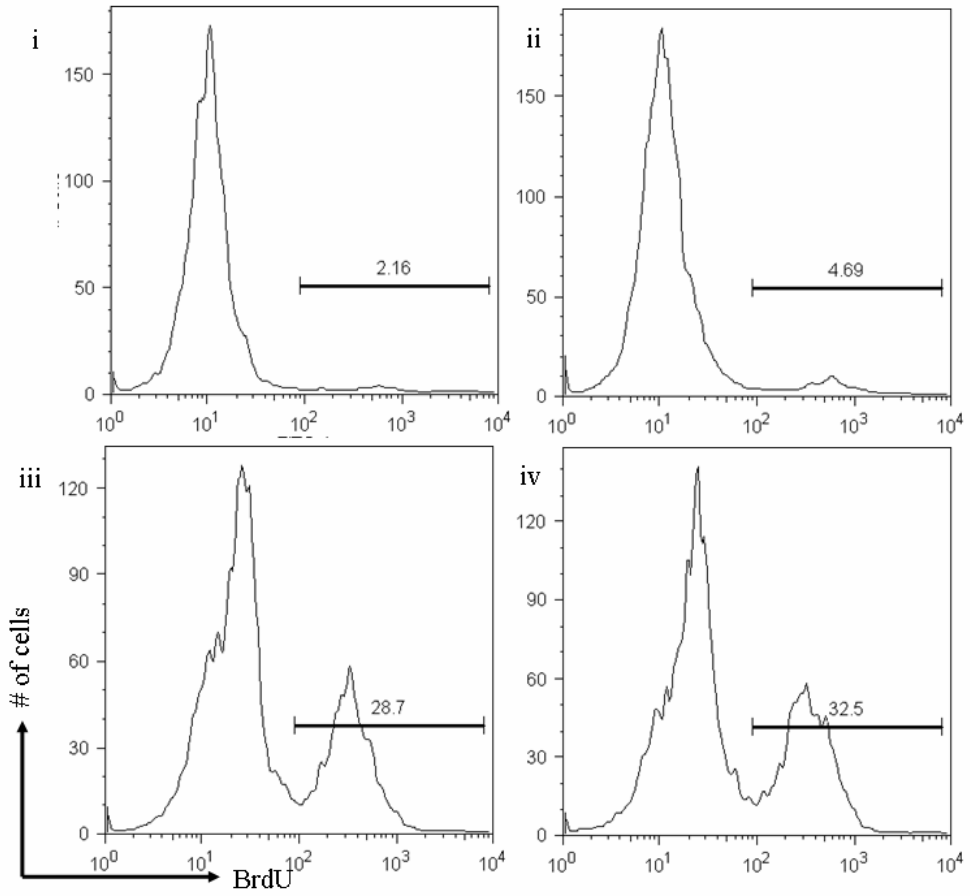
BAFF increases proportion of proliferating cells stimulated by BCRs

Previous literature has shown conflicting data on whether BAFF can cause proliferation of B cells. Although some studies have shown a proliferative effect of BAFF on B cells in the presence of B cell signaling (Schneider, MacKay et al. 1999), others have shown BAFF to have just a survival effect (Batten, Groom et al. 2000; Rauch, Tussiwand et al. 2009). In order to understand whether the survival effect by BAFF is solely responsible for increasing B cell numbers or whether proliferation is also involved, it was necessary for us to study the effect of BAFF on proliferation of B cells *in vitro*. To determine whether BAFF can stimulate growth, two different proliferation assays, BrdU uptake and CFSE dilution, were performed. Bromodeoxyuridine (BrdU) is an analog of thymidine that gets incorporated into DNA during replication. BrdU uptake therefore gives a measure of DNA synthesis at the time of assay and is represented by the BrdU positive cells (Fig 3.5A). CFSE or carboxyfluorescein diacetate succinimidyl ester is a colorless and non-fluorescent reagent. The acetate groups in the compound are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein siccinimidyl ester. This group reacts with intracellular amines and forms fluorescent conjugates that are retained in the cell as it undergoes cell division. The fluorescent label is inherited by daughter cells, but progressively diluted out due to the production of new cell material as the cells grow and divide during cell cycle progression. Thus daughter cells have decreased fluorescent intensity with each cell division cycle. Therefore, cells that remain undivided have higher fluorescence intensity than those that have undergone cell divisions (Fig 3.5 E and F). CFSE, therefore, measures the overall cumulative proliferation that has taken place upto the time of assay (Bronner-Fraser 1985).

There are very few BrdU positive cells at 24hrs in BAFF-treated cultures (Fig 3.5 B) and none at all at 48 or 72hrs (Fig. 3.5 C and D). In contrast, there is a significant increase in the proportion of BrdU positive cells in cultures treated with BAFF together with anti-IgM compared to anti-IgM alone treated cells (Fig 3.5 B-D). This indicates that BAFF alone has little to no effect on proliferation when compared to unstimulated controls. However, BAFF together with BCR signaling show significantly greater numbers of proliferating cells. Another interesting general observation that can be made from these data is that there seems to be the highest amount of DNA replication (BrdU incorporation) taking place at 48hrs. This indicates that at around 48hrs, cell division is at its peak in cultured purified B cells (Fig 3.5 B-D).

The CFSE assay shows no change in proportion of CFSE low cells in BAFF-treated compared to untreated cultured B cells indicating that there has been no accumulation of dividing cells by 24hrs (Fig 3.5 G). At 48hr (Fig 3.5 H) there is maximum peak accumulation of cells that have undergone division, and this does not go up much by 72 hrs (Fig 3.5 I). Again we see no change in CFSE low cells by BAFF treatment, but a decrease at 48hrs (Fig 3.5 G-I). BCR crosslinking results in stimulation of B cells in culture as seen by the significantly higher proportion of CFSE low cells compared to control at 48 and 72hrs (Fig 3.5 H, I). Also, the significantly higher proportion of cells in BAFF + anti-IgM compared to anti-IgM alone treated cells indicates that BAFF may be costimulating the proliferation induced by BCR. However, given that this increase is modest and that BAFF by itself has no effect on proliferation, the increase in the proportion of BCR stimulated pool of proliferating cells could be attributed to survival effects by BAFF.

These data indicate that BAFF can fine tune B cell signaling resulting in enhanced survival. This could have a profound effect on increasing the total number of autoreactive cells which would otherwise not survive in the absence of BAFF.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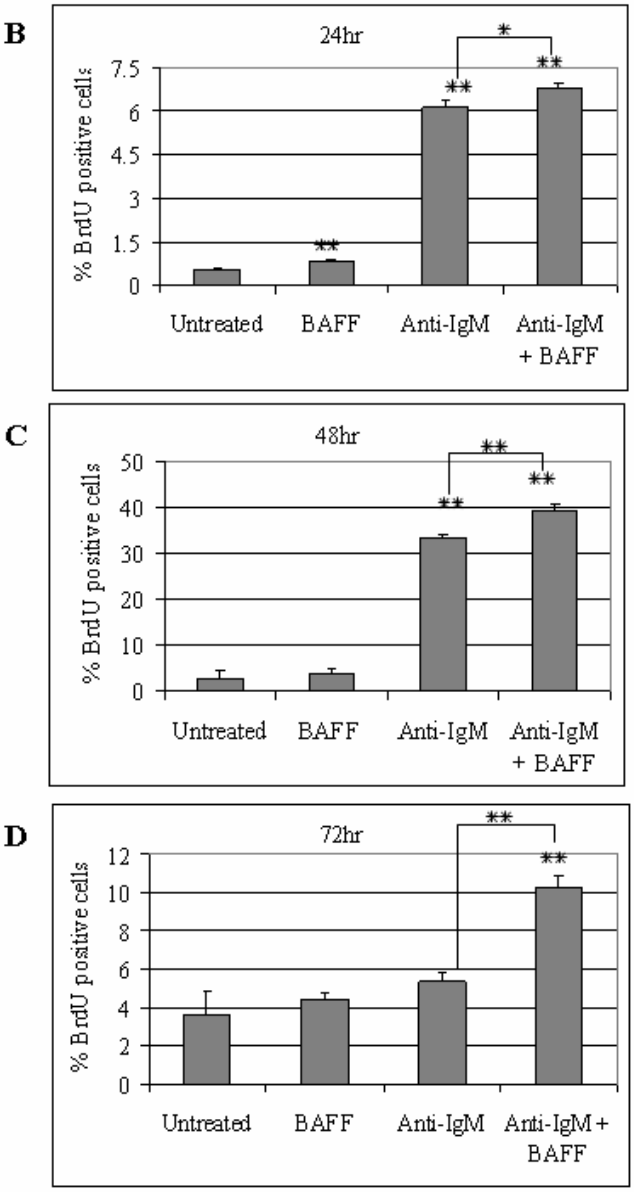
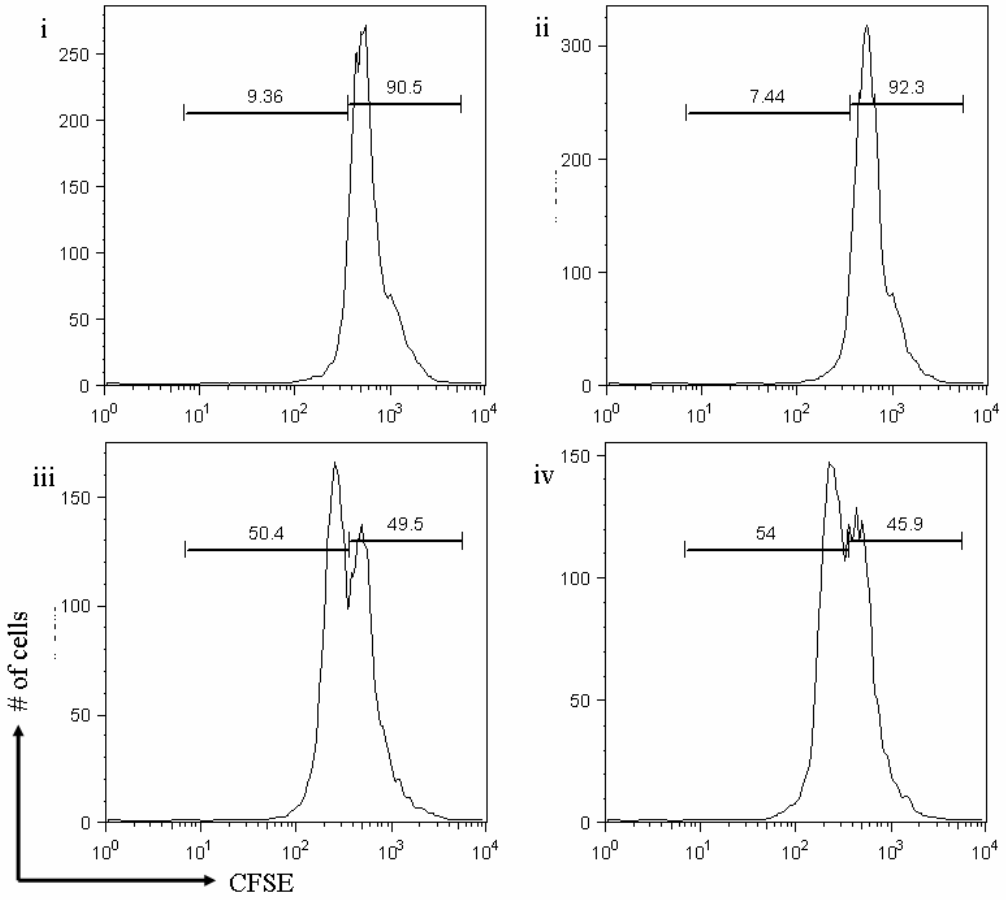
ii
iii
iv
2.16
4.69
28.7
32.5
# of cells
BrdU


B
24hr
% BrdU positive cells
7.5
6
4.5
3
1.5
0
*
**
**
**
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF
C
48hr
% BrdU positive cells
50
40
30
20
10
0
**
**
**
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF
D
72hr
% BrdU positive cells
12
10
8
6
4
2
0
**
**
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF


i
9.36
90.5
ii
7.44
92.3
iii
50.4
49.5
iv
54
45.9
# of cells
CFSE

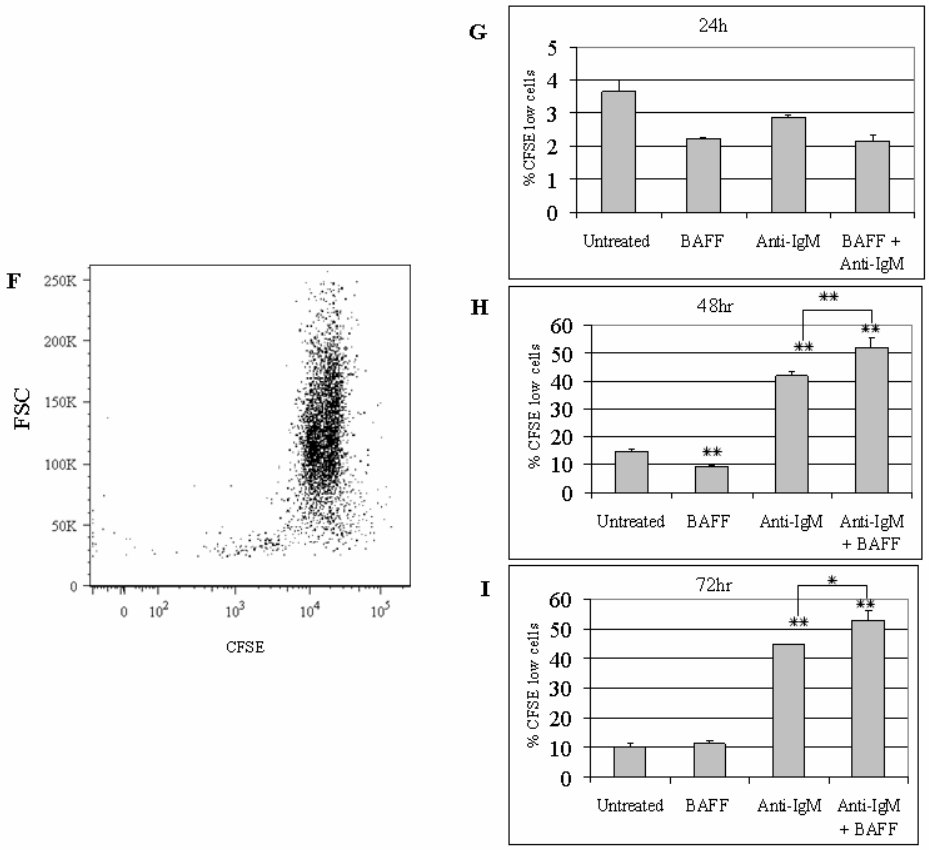


Fig 3.5 BAFF increases proportion of proliferating cells induced by BCR stimulation

Proliferative responses of purified splenic B cells after 24, 48 and 72hrs of stimulation in media alone or w/ BAFF, Anti-IgM alone or with BAFF and Anti-IgM together.
(A) Representative histogram plots showing percentage of CFSE lo and hi cells that were either left untreated (i) or cultured in the presence of BAFF (ii), anti-IgM (iii), or both (iv) for 48hrs.
(B-D) BrdU uptake by B cells cultured for 24hr (B), 48hr (C) and 72hr (D). The percentage of BrdU positive cells are shown.
(E) Representative histogram plots showing percentage of CFSE lo and hi cells that were either left untreated (i) or cultured in the presence of BAFF (ii), anti-IgM (iii), or both (iv) for 48hrs.
(F) Dot plot of CFSE dilution in splenic B cells
(G-I) CFSE dilution of B cells cultured as indicated in (A-D), for 24hr (G), 48hr (H) and 72hr (I). The percentage of CFSE low cells are represented.
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.
* Represents p value of less than 0.05; ** Represents p value of less than 0.1

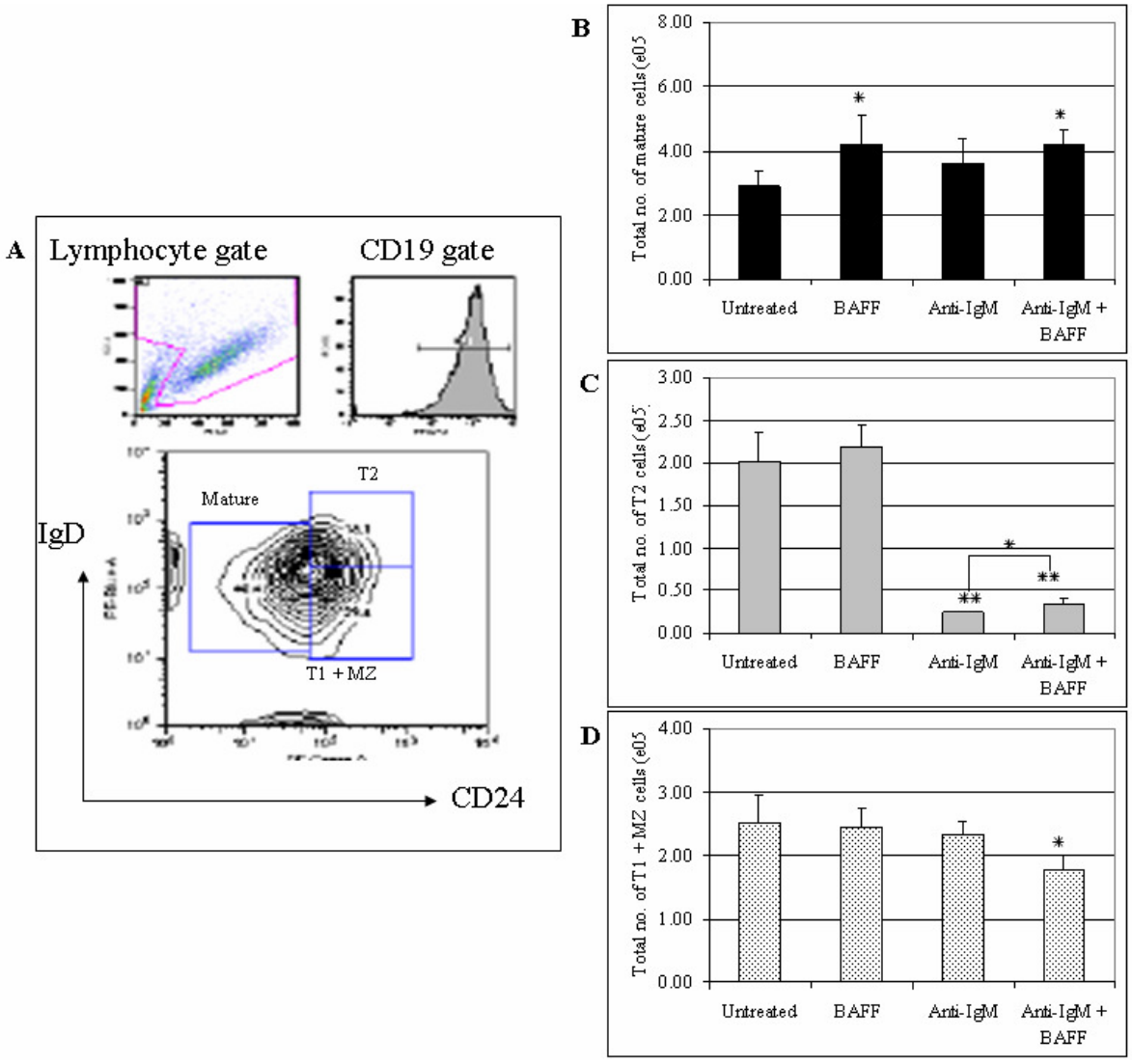
BAFF increases the total number of all B cell populations

We next wanted to determine which cell populations are specifically targeted by BAFF. It is necessary to evaluate which cells might be responding to BAFF in order to decipher how this could be leading to autoimmune disease. One hypothesis is that BAFF could be affecting the T2 cells that are prone to harbor autoreactive cells and are susceptible to negative selection. By examining the SSC vs FSC, it can be established that the majority of the cells in the anti-IgM and BAFF + anti-IgM cultures are activated, while the majority of the cells in the untreated and the BAFF treated cultures are resting.

In order to test the effect of BAFF on the different populations of B cells, we used differentiation markers to separate out each of the four stages of B cells. The cells were first gated on the lymphocyte gate, then on CD19+, a pan B cell marker, and were then separated using CD24 and IgD (Fig 3.6A). The transitional type 2 (T2) cells are CD24hi IgDhi, the mature are the CD24 lo IgD int and the transitional (T1) and marginal zone (MZ) cells are CD24hi IgD lo. The proportion of cells in each of the populations was determined from the FACs plots. Additionally, the total number of cells in each sample was determined using the Guava viability assay. The total number of cells in each stage was then calculated and shown as bar graphs (Fig 3.6 B-G). At 0hrs, the mature B cells are present at a proportion of 76%, the MZ and T1 at 14% and the T2 at 6%. Since 2 x 10^6 cells were plated, we started off with a total number of 1520,000 mature cells; 280,000 T1 and MZ and 120,000 of T2 cells. The results show that by 48hrs, there is a total of 900,000 mature B cells under BAFF treatment compared to only 420,000 in control sample (Fig 3.6E). Similarly, there are 220,000 T2 cells

and about 200,000 T1 and MZ cells under BAFF treatment but only 140,000 T2 and 110,000 T1 and MZ cells in control sample (Fig 3.6 F and G) indicating that under BAFF treatment there is a higher number of cells compared to control cultures. This could either be due to a protective effect of BAFF which increases the survival of the cells or it could be due to a proliferative effect of BAFF. At this point the data does not show which functional process of BAFF results in such high number of cells compared to controls.(Fig.3.6 E-G). When comparing 0hr untreated to BAFF treated, it can be seen that there is an increase in the total number of T2 cells by BAFF (220,000 in BAFF treated compared to 120,000 at 0h) and a corresponding decrease in the total number of T1 + MZ (200,000 in BAFF treated culture compared to 280,000 at 0hr). This could indicate differentiation of the T1cells to the T2 stage resulting in an overall increase in the T2 pool. Data from sorted T1 cells also show an increase in the proportion of AA4.1 hi, CD23 + cells which include the T2 cells (Data not shown). In addition, at 24hrs, there is a significantly lower number of T2 cells by anti-IgM treatment compared to the untreated control samples (Fig 3.6 C). This indicates that anti-IgM could be inducing apoptosis of the T2 cells by 24hrs. BAFF seems to be blocking this apoptotic effect of anti-IgM as seen by the significantly higher number of T2 cells under BAFF + anti-IgM treatment compared to anti-IgM alone (Fig 3.6C). At 48hrs, both M and T1 + MZ cells have a higher number of cells under BAFF + anti-IgM treatment compared to anti-IgM alone (Fig 3.6 E and G). This indicates that BAFF is capable of modulating B cell signaling resulting in targeted enhancement of survival or proliferation in the mature and T1 + MZ populations. In addition, BAFF by itself is expanding all the populations, which include the immature autoimmune prone T1 and T2 B cells (Fig 3.6 E-G). In the presence of

B cell signaling, BAFF is promoting the T1+MZ and the mature B cell population. Since, autoreactive cells that have escaped into the periphery could have mature B cell properties, an expansion of these cells could also have significance in relation to autoimmune disease.


A
Lymphocyte gate
CD19 gate
IgD
Mature
T2
T1 + MZ
CD24
B
Total no. of mature cells (e05
8.00
6.00
4.00
2.00
0.00
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF
C
Total no. of T2 cells (e05
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF
D
Total no. of T1 + MZ cells (e05
4.00
3.00
2.00
1.00
0.00
Untreated
BAFF
Anti-IgM
Anti-IgM + BAFF

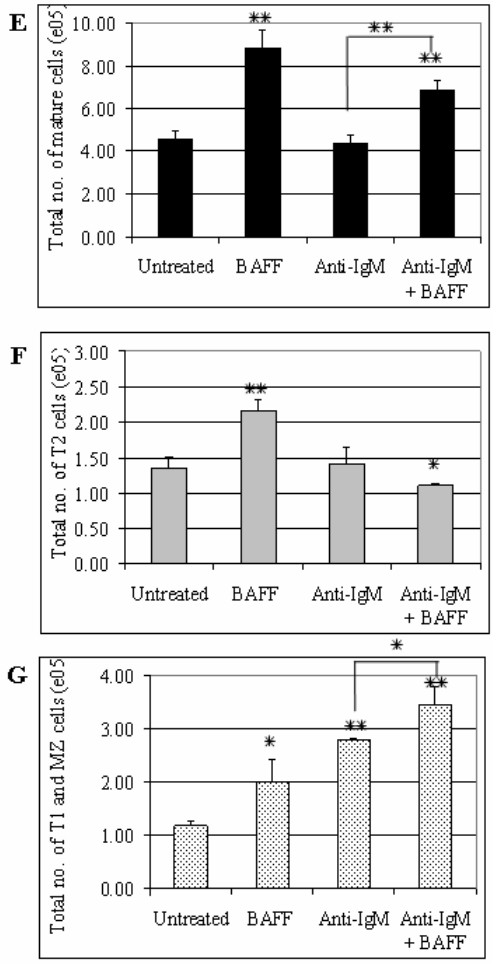


Figure 3.6 Higher number of cells in all the different stages of B cells in BAFF treated cultures compared to untreated control by 48hrs. Purified splenic B cells were stained with differentiation markers and gated on lymphocyte gate, CD19+ cells and then separated based on staining with CD24 and IgD (A) after 24 hrs (B – D) and 48hrs (E-G) of culture in media alone, BAFF alone, Anti-IgM alone or with BAFF and anti-IgM in combination. 2 million cells were initially plated.
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.
* Represents p value of less than 0.05
** Represents p value of less than 0.01

BAFF promotes survival of all the cell populations

Although we see a change in the B cell populations by the effect of BAFF, it cannot be deciphered which biological response leads to this difference. In order to evaluate whether it is survival or proliferation that plays a role in a higher proportion of these cell populations compared to the time-matched control, further detailed experiments were needed.

In order to test which B cell population is protected from apoptosis by the presence of BAFF, cells cultured with or without BAFF, anti-IgM or BAFF + anti-IgM for 48hrs, and were then stained with differentiation markers as well as with Annexin V to assay for survival. The purified splenic B cells were first gated on the lymphocyte gate (Fig 3.7 i), then on Annexin negative cells (Fig 3.7 ii), which were further gated on AA4.1 hi and AA4.1 lo cell populations (Fig 3.7 iii). Finally the cells were separated based on CD24 and CD21 expression (Fig 3.7 iv and v). AA4.1hi cells represent the immature B cell populations, which can be further separated into CD24hi CD21hi T2 and CD24 lo CD21hi T1 cells (Fig 3.7 v). AA4.1 lo cells represent the mature and the MZ B cell populations, which can be further separated into CD21hi MZ and the CD21 int mature cells (Fig 3.7 iv). At 0hrs, splenic B cells constitute the mature B cells at 76%, the MZ at 11%, the T2 at 6% and the T1 at 3%. Since 2 x 10^6 cells were plated, we started off with a total number of 1520,000 mature cells, 220000 MZ, 60,000 T1and 120,000 T2 cells

The results show that under BAFF treatment, there is 20% more of the T2 cells when compared to untreated control (Fig 3.8 ii). There is a slight dip in the percentage of the T1 population (Fig 3.8 i) and no change in the MZ or mature cells (Fig 3.8 iii and iv). This indicates that BAFF may be enhancing survival of just the T2 cells at 48hrs (Fig 3.8 ii). MZ

cells show a slightly lower percentage of cells under anti-IgM stimulation, and an even lower number of cells by anti-IgM + BAFF (Fig 3.8 iii) treatment. This could indicate differentiation of the MZ cells occurring in the presence of BAFF and BCR stimulation. Since MZ cells have properties of memory B cells, they could be differentiating into plasma cells and therefore losing or changing their marker expression. The mature B cells also had a higher percentage of cells under anti-IgM treatment and an even greater number of cells with anti-IgM + BAFF treatment (Fig 3.8 iv). This could indicate that BCR stimulation is inducing proliferation of the mature B cell population resulting in a higher proportion of the cells and that BAFF further stimulates this proliferation.

When we now look at total concentration of cells, we see that BAFF is actually capable of enhancing survival of all four of the different populations (Fig 3.9 i-iv). In addition, by comparing BAFF + anti-IgM to anti-IgM alone, it can be seen that there is a lower number of the MZ cells (Fig 3.9 iii), which could indicate maturation of the MZ cells. There is also enhanced survival in the mature (Fig 3.9 iv), T1 (Fig 3.9 i) and T2 (Fig 3.9 ii) cell populations by BAFF treatment in the presence of anti-IgM. This suggests that BAFF together with BCR signaling is capable of further promoting the survival of these cell populations. Since the immature B cells harbor more autoreactive cells that may have escaped negative selection, this could provide an important link connecting BAFF and autoimmunity.

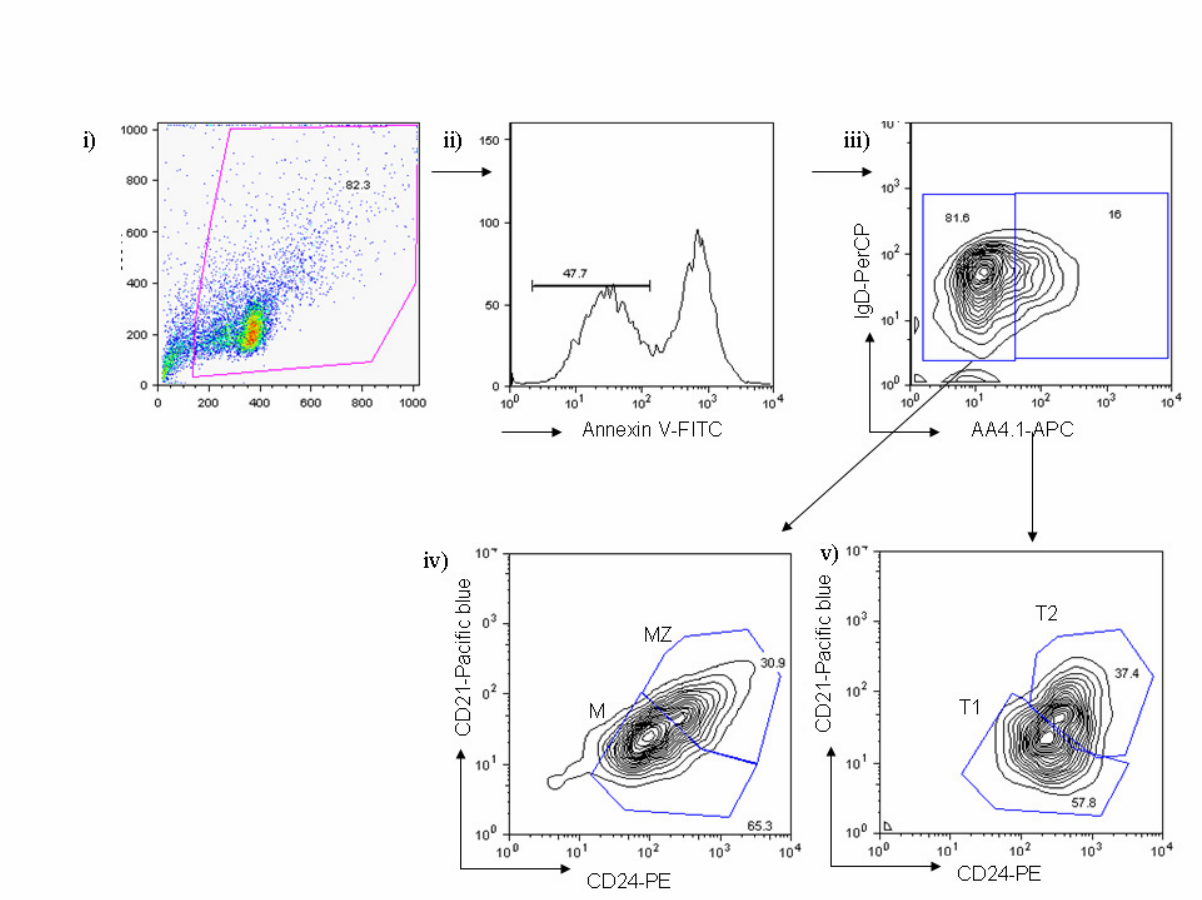


Figure 3.7 Separation of B cells based on AA4.1, CD21 and CD24 expression

Separation of purified splenic B cells that are annexin negative into the different populations based on the following gating strategy:
Cells are first gated on lymphocytes (i), then on Annexin negative (ii), then on AA4.1 high and AA4.1 low (iii) and then finally separated based on CD21 and CD24 expression (iv and v)

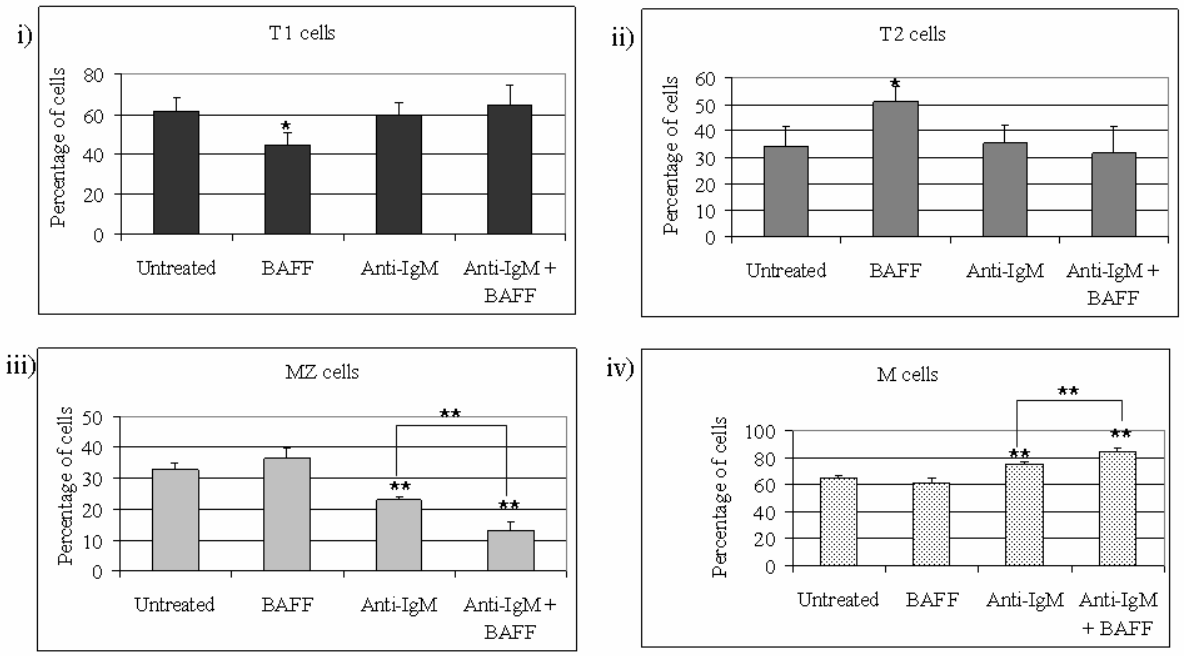


Figure 3.8 Increased survival of T2 cells with BAFF treatment at 48hrs

Flow cytometric analysis of splenic purified B cells cultured in media, with BAFF or anti-IgM alone or in combination for 48hrs and then stained with antibodies against AA4.1, CD23 and CD21 to identify the different populations. The cells were also stained with Annexin V to measure apoptosis.
Percentage of annexin V negative cells of (i) T1 cells (ii) T2 (iii) MZ and (iv) M B cells are shown.
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.
T1: transitional type 1; T2: transitional type 2; MZ: marginal zone; M: mature
* Represents p value of less than 0.05
** Represents p value of less than 0.01 using the Student's t-test

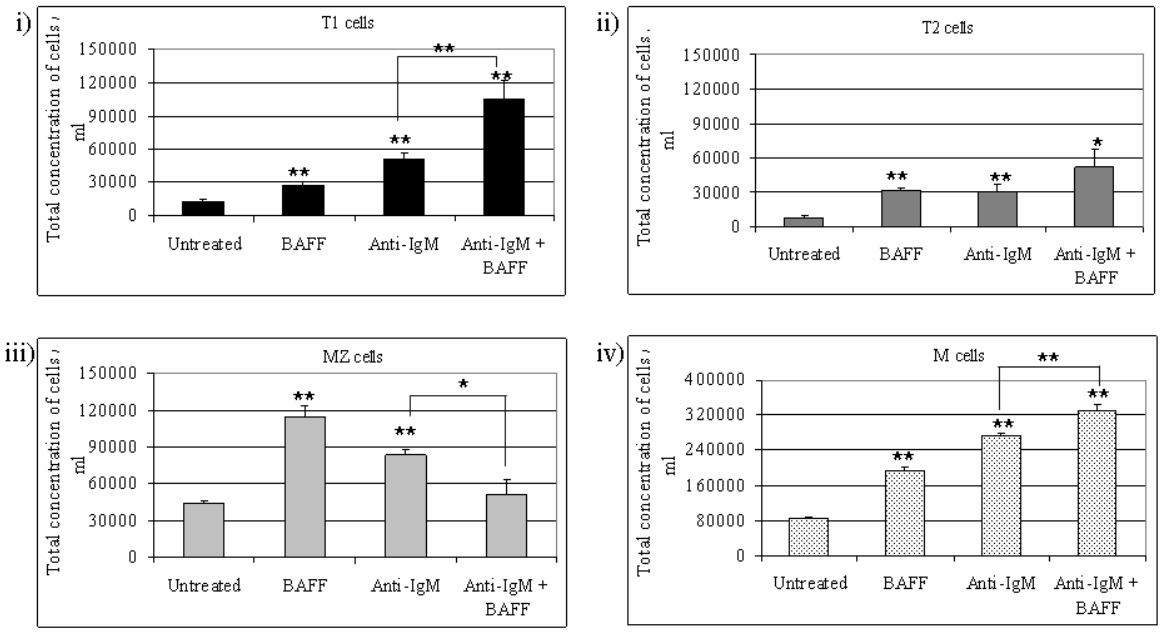


Figure 3.9 BAFF protects B cells from spontaneous apoptosis at all different stages of development

Flow cytometric analysis of splenic purified B cells cultured in media, with BAFF or anti-IgM alone or in combination fir 48hrs and then stained with antibodies against AA4.1, CD23 and CD21. The cells were further stained with Annexin V. Total cell concentration of (i) T1, (ii) T2, (iii) MZ and (iv) M
T1: transitional type 1; T2: transitional type 2; MZ: marginal zone; M: mature
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.

* Represents p value of less than 0.05
** Represents p value of less than 0.01

One caveat in analyzing these samples is to keep in mind that the gating strategy may result in over- or under-estimating the size of the populations due to overlap or unclear separation of the populations. Also, it is difficult to evaluate whether any differentiation is taking place in a mixed population since a change in the proportion of the population could also result from increased survival or proliferation. In order to understand more deeply how each of these populations is behaving, the cells were sorted into the four individual populations and were cultured in the presence or absence of BAFF for 48hrs. In addition, the mature and the T2 cells were also cultured with anti-IgM alone or with BAFF + anti-IgM for 48hrs (Fig 3.10). The cells were plated at 2 million cells/ml. Although the total number of cells after 48hrs of culture was not determined, the percentage of annexin negative cells is shown (Fig. 3.10). The results show a general trend in a higher percentage of live cells in all of these populations by BAFF treatment compared to untreated controls (Fig 3.10 i-iv). Although this enhanced survival effect is modest for MZ and T2 cells (Fig 3.10 ii and iii), the same trends in increase in survival in the different populations were obtained when the experiment was repeated several times. This further confirms the protective effect of BAFF on all stages of B cell differentiation. When BAFF + anti-IgM is compared to anti-IgM alone treatment in the M sorted populations (Fig 3.10 iv), it is seen that there is significantly enhanced survival in the mature cells, indicating that in combination with BCR signaling, BAFF has a further protective effect on mature B cells.

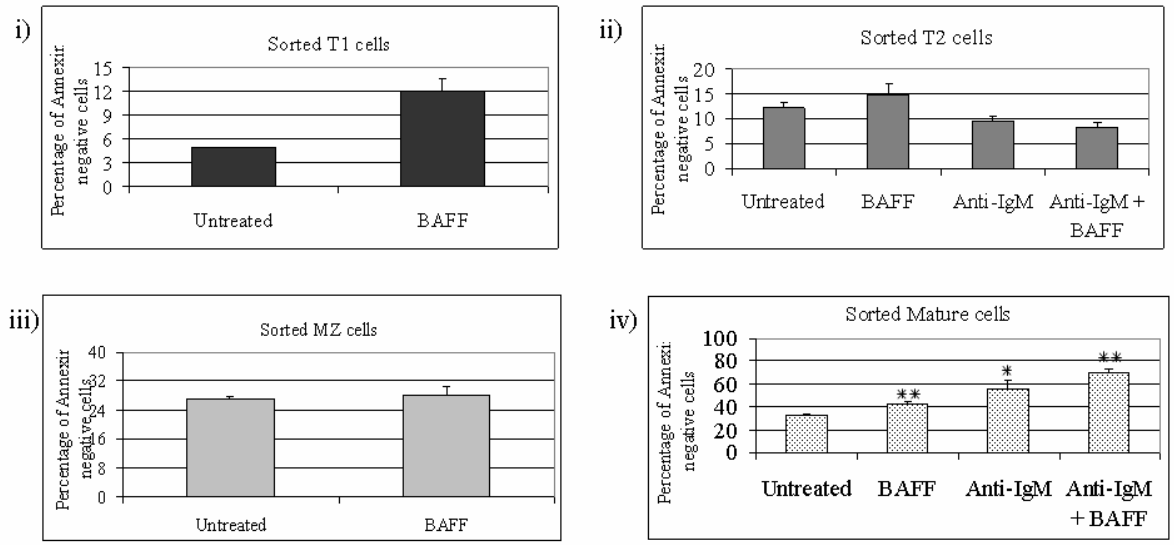


Figure 3.10 BAFF protects B cells from spontaneous apoptosis at all different stages of development

Purified splenic B cells were sorted into the four different populations based on differentiation marker expression and then left untreated or cultured with BAFF for 48hrs (i and iii). The T2 and Mature cells were either left untreated or cultured with BAFF alone, anti-IgM alone or with BAFF and anti-IgM in combination for 48hrs (ii and iv). The cells were then stained with differentiation markers in addition to Annexin V to measure apoptosis and analyzed by flow cytometry.

T1: transitional type 1; T2: transitional type 2; MZ: marginal zone; M: mature

Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test. The experiment was repeated 3 times for the T1, T2 and MZ populations and 4 times for the mature B cell population.

* Represents p value of less than 0.05; ** Represents p value of less than 0.01

BAFF increases proportion of proliferating cells at all stages of B cell development

We were interested to see which B cell populations are stimulated to proliferate by the action of BAFF. We hypothesized that BAFF stimulates the T2 cells, which have a propensity to have autoreactive properties, to proliferate. In order to evaluate this, cells were not only stained with the differentiation markers but were also assayed for proliferation using BrdU incorporation assay.

The results show that BAFF increases proportion of DNA replicating B cells as seen by the higher percentage of BrdU positive cells by BAFF treatment compared to time-matched untreated control (Fig 3.11 A-C). Since this change is modest, this could be explained by an overall enhancement of survival of these cells rather than proliferation. Anti-IgM as well as BAFF + anti-IgM treatment show a significantly larger percentage of proliferating cells compared to control (Fig. 3.11 A-C). However, only the mature B cells show a significant difference between BAFF and anti-IgM + BAFF. This indicates that anti-IgM induces proliferation in the cells and that BAFF treatment, in the presence of BCR signaling, does not have any effect on proliferation except in the mature B cells. The significantly higher number of BrdU positive mature cells in the BAFF + anti-IgM treated culture indicate that BAFF may be interacting with B cell signaling only in the mature B cell population to enhance proliferation (Fig 3.11 A).

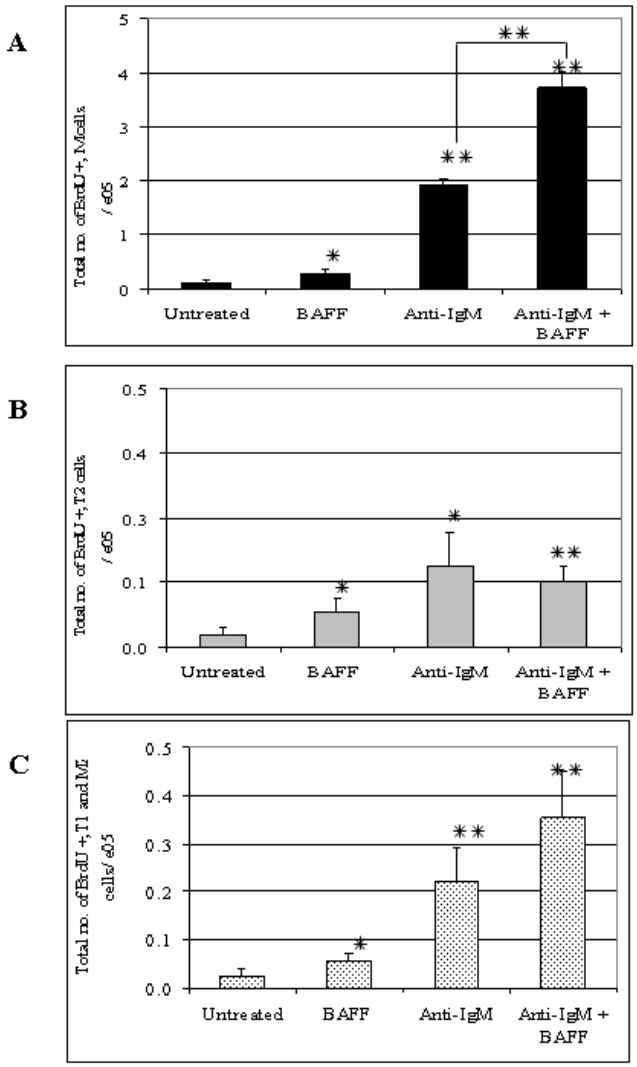


Figure 3.11 BAFF increases proliferating cells at all different stages of development
Flow cytometric analysis of splenic purified B cells cultured in media, with BAFF or anti-IgM alone or with BAFF and anti-IgM together for 48hrs and then stained with antibodies against CD19, CD24 and IgD. The cells were further assayed for BrdU incorporation and the total number of BrDU positive cells are shown.
A. Total number of BrdU + mature B cells;
B. Total number of BrdU + T2 cells
C. Total number of BrdU + T1 and MZ cells
T1: transitional type 1; T2: transitional type 2; MZ: marginal zone; M: mature
Statistical test was performed by comparing average of triplicates of treated samples with average of untreated controls using the Student's *t* test.
Represents p value of less than 0.05; ** Represents p value of less than 0.01

BAFF induces modest activation in purified B cells

In order to evaluate the effect of BAFF on activation of B cells, we used two different activation markers, CD25 and CD69. Again this would be an important aspect of BAFF stimulation that could result in activation of autoreactive cells. The data show that BAFF treated culture has a slight increase in CD25 as well as CD69 expression when compared to untreated controls (Fig.3.12). Anti-CD40 treatment shows a much greater increase in both activation marker expression. The anti-IgM treated cultures have an even greater increase in the activation markers, which is expected from BCR cross linking. However, when anti-CD40 or BAFF are added in combination with anti-IgM, there does not seem to be any significant change in activation. This indicates that although BAFF by itself has a slight effect on activation of the B cells, there is no effect when combined with BCR stimulation.

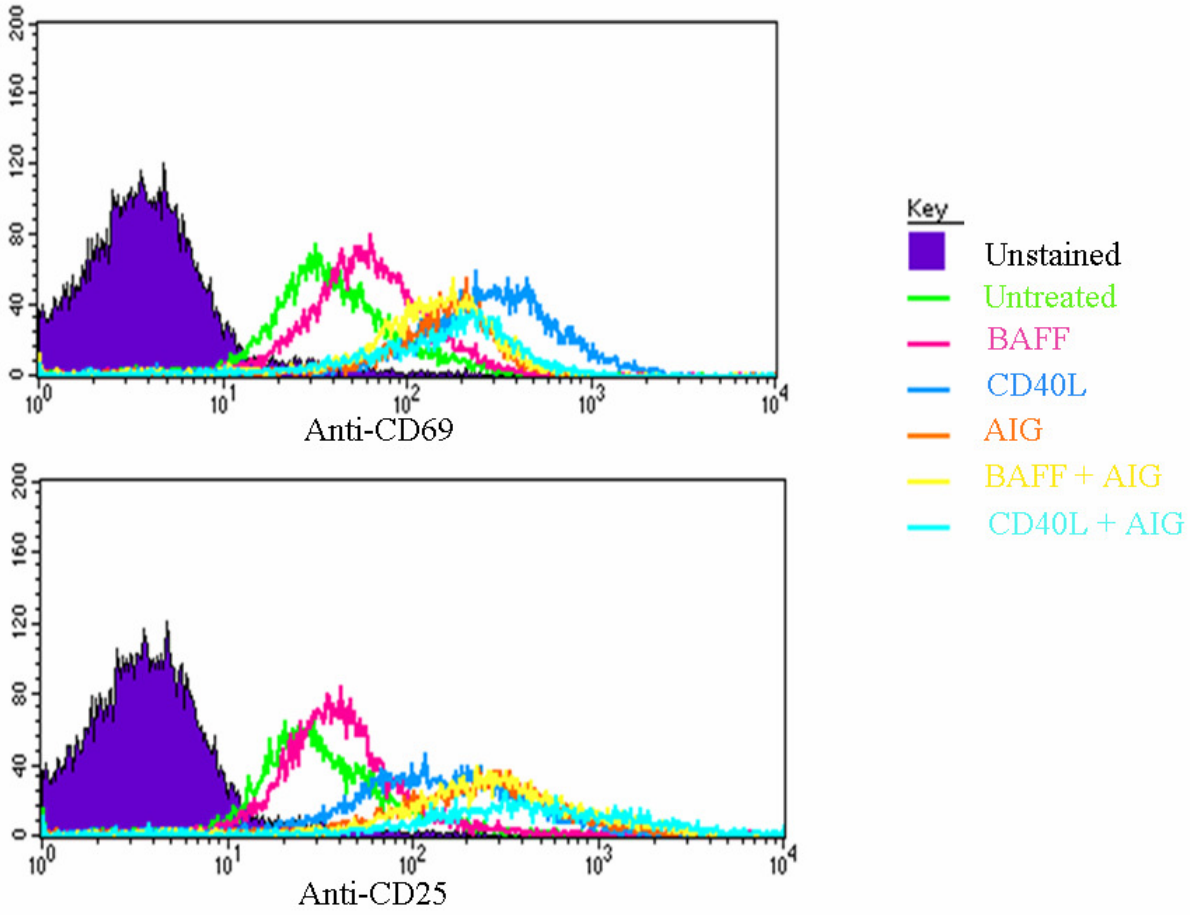


Figure 3.12 BAFF shows modest increase in activation of purified B cells

Flow cytometric analysis of splenic purified B cells cultured in media alone, or with BAFF, anti-CD40 or, anti-IgM alone or in combination and then stained with antibodies against CD69 and CD25. The histogram curves for anti-CD69 (top) and anti_CD25 (bottom) for each of the treatments are shown.

Effects of BAFF survival does not involve BTK signaling

Bruton's tyrosine kinase (BTK) is essential in B cell development and homeostasis. BCR crosslinking results in several BTK-mediated functional effects in B cells including calcium influx, cytoskeletal remodeling, apoptosis/survival, proliferation and gene expression (Desiderio 1997; Satterthwaite, Li et al. 1998). We wanted to test whether BTK is in any way linked to BAFF-mediated survival of B cells. BAFF has been reported to be involved in NF-kappa B signaling, both classical and non-classical (Kayagaki, Yan et al. 2002; Sasaki, Derudder et al. 2006). We wanted to investigate the possibility of BAFF being involved with BTK signaling, which would provide a novel signaling pathway for BAFF stimulation thereby opening up new possible mechanisms for BAFF-mediated autoimmunity. In order to test this, B cells were purified from BTK -/-, BTK lo and WT control mice. The cells were then cultured in media alone or with BAFF, anti-IgM alone or in combination. Media with and without serum was used to ensure that the presence or absence of serum does not have any effect on the experimental outcome (Fig. 3.13). The data show that BAFF has the same level of survival when compared to untreated cultures in B cells from the BTK -/-, BTK lo and WT mice. Although the total number of B cells are reduced in the BTK -/- and BTK lo mice, our data show that this is not due to lack of survival effects of BAFF.

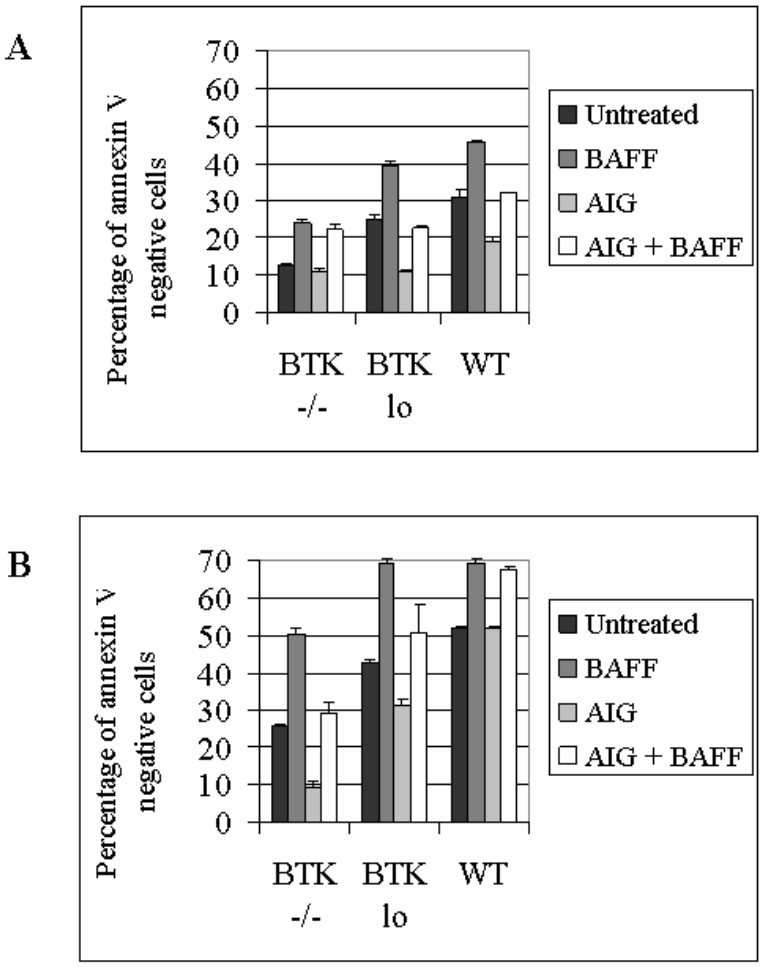


Figure 3.13 No effect on BAFF mediated survival by loss of BTK

Flow cytometric analysis of splenic purified B cells from BTK -/-, BTK lo and WT mice cultured for 40 hrs in media alone, with BAFF or anti-IgM alone or in combination, and then stained with Annexin V-FITC to measure apoptosis. Panel A shows media without serum and panel B shows media with serum.

Summary

Our experiments provide a comprehensive study of the *in vitro* effects of BAFF on purified splenic B cells. We were able to establish the survival effects of BAFF on B cells. Our studies confirmed and extended results from other studies (Batten, Groom et al. 2000; Rolink, Tschopp et al. 2002; Rauch, Tussiwand et al. 2009) showing that BAFF treatment promotes the survival of cells as judged both by the decrease in the proportion of cells expressing markers of apoptosis (PS and activated caspases) and by the increase in the absolute number of viable cells. In order to examine whether interaction of BAFF with B cell signaling affects survival, B cells were cultured with BAFF or anti-IgM alone or in combination. The data show that BAFF coupled with BCR signaling results in a significant increase in survival when compared to B cell signaling alone. Another interpretation of the data could be that BCR is increasing the B cells mainly by proliferation and the addition of BAFF could be resulting in an increase in survival of these cells resulting in a synergistic increase in the total number of cells. By examining proliferative effect of BAFF on B cells, we were able to show that BAFF by itself does not cause proliferation of B cells (Fig 3.5). However, in combination with anti-IgM signaling, proliferation could be detected both by BrdU and CFSE assays (Fig 3.5). Thus, BAFF shows coupling to BCR cross linking in both proliferation and prevention of apoptosis in B cells. These data indicate that BAFF signaling is in many ways coupled to BCR signaling. We further tried to decipher which B cell populations are targeted by BAFF for survival and proliferation. BAFF seems to indiscriminately protect all B cell populations from spontaneous apoptosis with and without BCR stimulation (Fig 3.3). DNA replication by BAFF coupled to BCR stimulation is also

seen in all B cell populations (Fig 3.11). BAFF was also found to stimulate B cell activation as judged by increased CD25 and CD69 cell surface expression and this was further enhanced in combination with BCR signaling (Fig 3.12). We further showed that BAFF signaling is not linked to BTK signaling since the survival effects of BAFF were not affected by the absence of BTK (Fig 3.13). These *in vitro* studies gave us a better understanding of the effects of BAFF on B cells in culture which could help in clarifying the process by which BAFF results in autoimmune disease generation.

Chapter 4

BAFF enhances frequency of An1 cells *in vitro*

Introduction

Many of the newly generated B cells express a clonal immunoglobulin on their surface that bind to an antigenic determinant that is normally expressed by the cells. This could potentially lead to autoreactivity and thus autoimmune disease development. However due to as yet ill-defined tolerance mechanisms, B cells that express auto-reactive immunoglobulin receptors are tolerized through three different mechanisms. The autoreactive cells either undergo receptor editing so that they no longer bind to self-antigens, (Nemazee and Burki 1989; Tiegs, Russell et al. 1993; Chen, Prak et al. 1997), are eliminated through the induction of apoptosis (Hartley, Crosbie et al. 1991; Hartley, Cooke et al. 1993; Chen, Nagy et al. 1995), or are rendered unresponsive to potentially activating signals through their BCR (Goodnow, Crosbie et al. 1988). These unresponsive cells have been termed anergic B cells. Anergic cells persist in the periphery and have lowered IgM expression and a reduced life span of only 2 to 3 days. These anergic B cells have the potential of becoming activated by external stimuli or by T cell help (Sekiguchi, Jainandunsing et al. 2002; Seo, Fields et al. 2002). An abundance of autoreactive cells leads to autoimmune disease such as Systemic Lupus Erythematosus (SLE) or Rheumatoid Arthritis (RA). Recently, a population of naturally occurring anergic B cells has been defined in wild type mice (Merrell, Benschop et al. 2006). These naturally occurring anergic B cells that were previously thought to be immature B cells known as transitional type 3 (T3 cells) have now been renamed as An1 cells. Since it is known that excess BAFF results in autoimmune disease, we hypothesized that BAFF may be causing an increase in this An1 population. BAFF may be doing this by increasing survival or differentiation of the An1

cells, leaving the organism susceptible to autoimmune disease. In order to test the hypothesis, we had to evaluate whether an excess of BAFF could lead to an increase in the An1 cells. The first step was to identify the An1 population in C57BL/6 wild type mice. This was done using anti-IgM, anti-CD23 and anti-AA4.1 antibodies that have been shown to be adequate for identifying An1 cells (Merrell, Benschop et al. 2006). Once that was accomplished, the next step was to test and see whether BAFF had any role in increasing the development of this population. Our results indicate that BAFF does cause an increase in the An1 population. That this population has anergic properties was established by measuring calcium flux, which showed a lowered ability to mobilize calcium upon BCR stimulation. This finding presents an exciting possibility of another link between BAFF and autoimmune disease. Since anergic B cells have the ability to revert to the autoreactive phenotype by , an abundance of anergic cells present the risk of eliciting autoreactive responses and secreting autoantibodies resulting in disease.

Results

Identification of An1 cells

Our first goal was to identify the An1 cells according to the original study that described them (Merrell, Benschop et al. 2006). One of the antibodies used in that study targeted CD93 which is a marker that is expressed on transitional type 1 (T1) and transitional type 2 (T2) B cells. Recently it was suggested that transitional type 3 (T3) also express this marker on their surface (Thomas, Srivastava et al. 2006) and these cells were later identified as the An1 cells. In our studies we used a commercially available antibody AA4.1, which recognizes a different epitope of the same protein CD93, also know as ClqRP (C-type lectin-like transmembrane protein). The An1 cells have been described as AA4.1hi, CD23+ and IgM lo cells (Fig 4.1). Thus the purified B cells were stained with antibodies to AA4.1, CD23 and IgM and were analyzed by flow cytometer. The cells were first analyzed by the forward/side scatter plot, which can be used to separate out the lymphocytes using the lymphocyte gate (forward-scatter high and side-scatter low). The cells within the lymphocyte gate were then plotted on CD19 and AA4.1. The CD19hi and the AA4.1hi cells were gated and then analysed on CD23 and IgM (Fig 4.1 B).). The AA4.1hi, CD23 positive and IgM low cells were described as the An1 cells (Fig 4.1C).

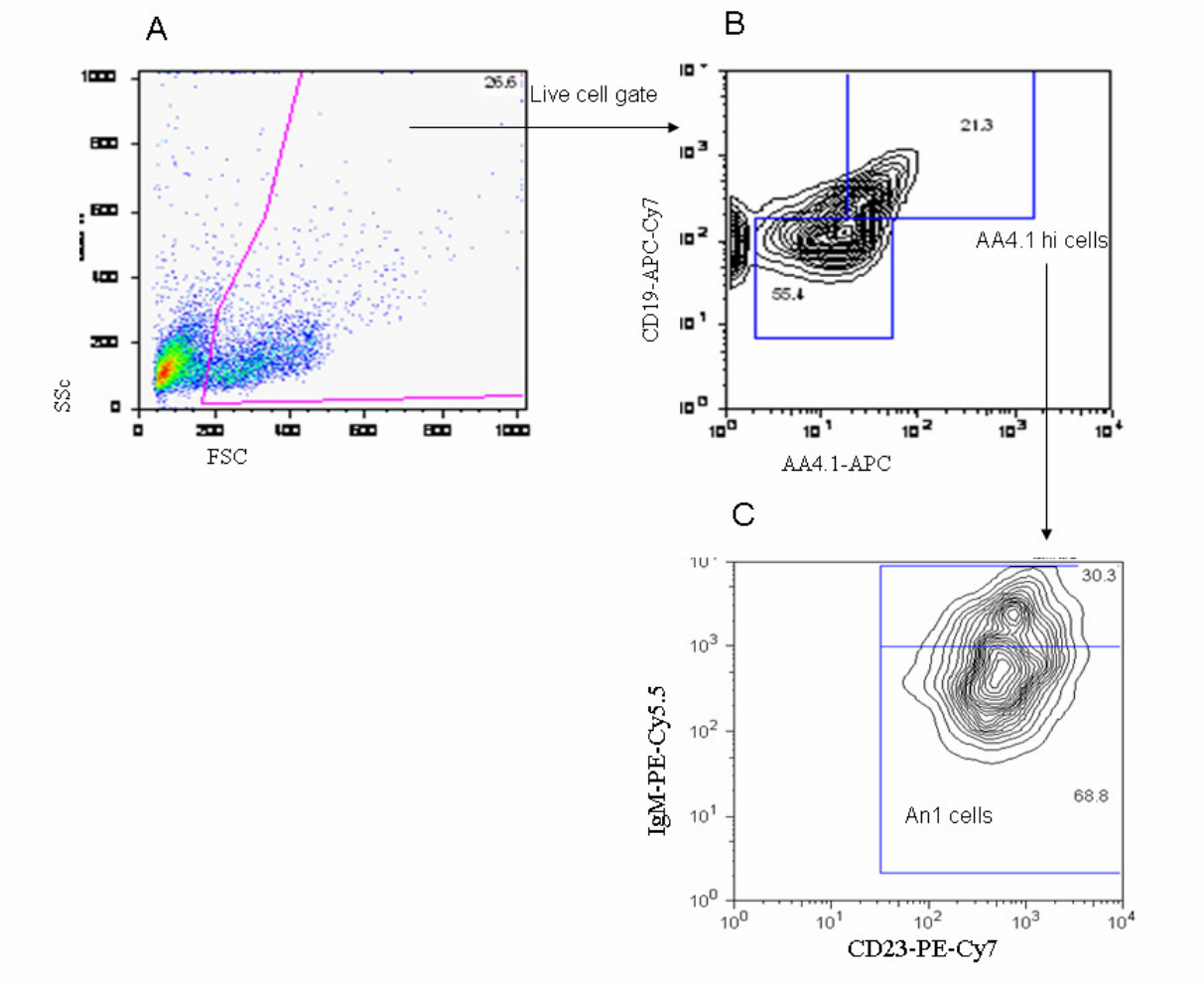


Figure 4.1 Identification of An1 cells

Splenic B cells were purified from C57Bl/6 mice and analyzed for the expression of AA4.1, CD23 and IgM to identify An1 cells. The cells were first gated on lymphocyte gate (A), then on AA4.1 hi cells (B) and then finally analyzed for the expression of IgM and CD23 (C).

BAFF enhances frequency of T3 or An1 cells

Various studies have tried to decipher how BAFF may be involved in autoantibody production and autoimmune disease development. Recently, it was shown that excess BAFF allow autoreactive cells to compete with normal cells (Lesley, Xu et al. 2004). Since anergic B cells are basically autoreactive cells that have become silent, we wanted to test the hypothesis that BAFF may be involved in increasing these cells. In order to test this, splenic purified B cells were cultured with or without BAFF for 48hrs and then stained with anti-CD19, anti-AA4.1 and anti-CD23 antibodies for identifying the An1 cells.

The results showed that in the control splenic B cell population, around 31.4% of the cells are AA4.1hi and of these, about 50% are IgM low, CD23+, which are the An1 cells (Fig 4.3). Thus around 15% of the total control cell population developed into An1 cells. The BAFF treated cells showed that around 50% of the cells were AA4.1hi and 70% of those were An1 cells (Fig 4.3). Thus around 35% of the total BAFF treated B cell population could be identified as An1 cells. This indicates that about twice as many BAFF treated cells are An1 cells compared to the control cultures.

BAFF increases frequency of T3 or An1 cells from T2 and Mature B cells

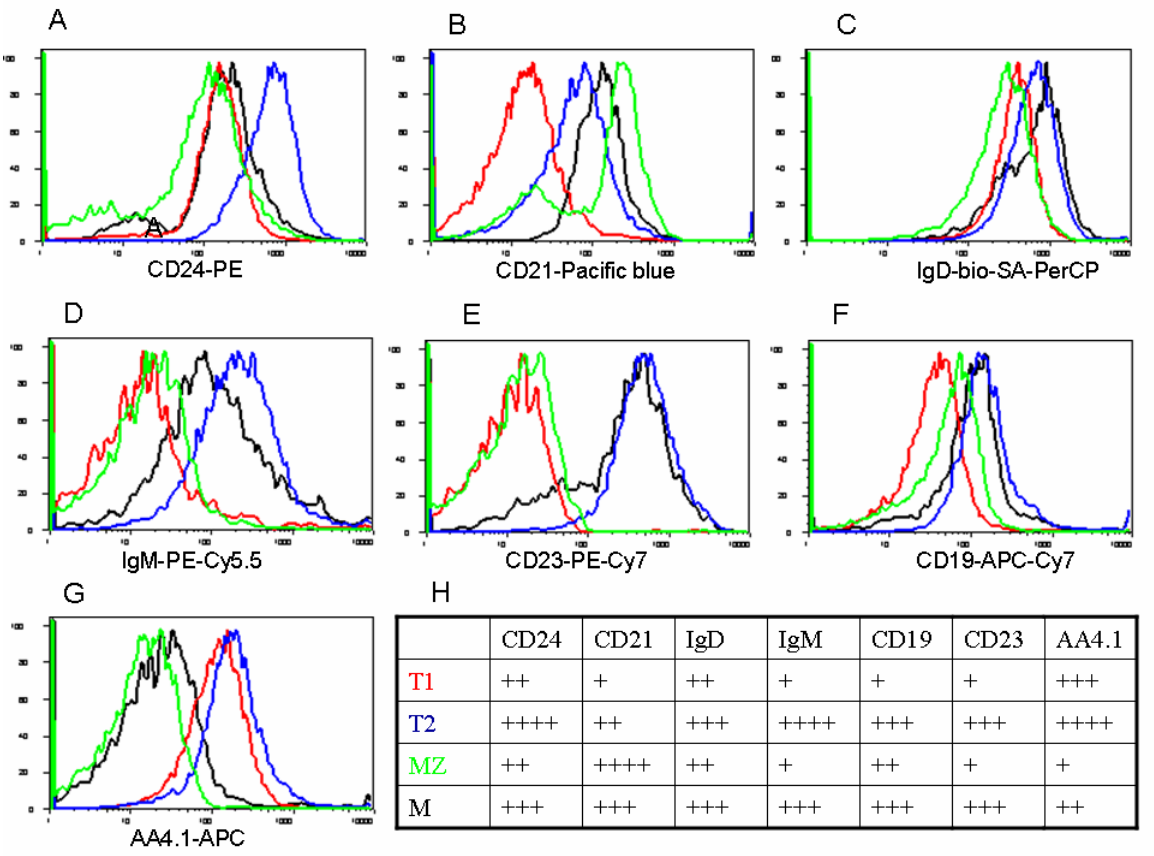
We next wanted to find exactly which cell population gives rise to the An1 cells under the influence of BAFF. In order to address this, splenic B cells were sorted to give the four major B cell populations: Mature, T1 (transitional 1), T2 (transitional 2) and MZ (Marginal Zone) B cells. The cells were sorted based on AA4.1 and CD23 expression. T1 and T2 cells are AA4.1hi while the mature and MZ cells are AA4.1 low. T1 and MZ are

CD23 low and can therefore be separated from the T2 and mature cells. After sorting the four different populations, the identity was confirmed using antibodies to several different markers expressed on the surface of these populations, such as CD24, CD21, IgD, IgM, CD23, CD19, and AA4.1 (Fig 4.2). The summary of the expression profile of each of the sorted populations is shown in Fig 4.2 and Table H. The results conform to what is already known about the marker expression profiles of these different B cell subsets. The immature transitional type I (TI) population express CD24 and IgD at moderate levels, very low of the other markers and high levels of AA4.1 as can be seen in the table. Mature (M) B cells express most of the markers at high levels except AA4.1. Transitional type 2 or T2 express CD24, IgM and AA4.1 at very high levels, IgD, CD19 and CD23 at high levels and CD21 at moderate levels. Marginal zone or MZ cells express CD21 at very high levels, CD24, IgD and CD19 at moderate levels and all the other markers at low levels.

Once it was confirmed that the cells have been properly sorted into the four different populations, they were cultured in the presence or absence of BAFF for 48hrs. The cells were then stained with antibodies against CD23, AA4.1 and IgM and analyzed using flow cytometry to determine whether any differentiation had taken place. The results indicate that 15.1% of T2 cells from control cultures were AA4.1 high; of these 52.8% were IgM low, consistent with the An1 marker profile (Fig. 4.4A). In contrast, 29.3 % of the T2 cells from BAFF treated cultures were AA4.1 high with similar percentages of IgM lows, suggesting that BAFF had increased the production of An1 cells from T2 progenitors by 2 fold. In mature B cell cultures, BAFF also led to an increase of AA4.1 high cells from 19.5% in the control cultures to 33.4% in the BAFF treated cultures. About 68.4% of the AA4.1hi cells in

the control cultures and about 74.5% in the BAFF treated cultures consisted of An1 cells (Fig 4.4 B). This data again indicates that about twice as many An1 cells are present in the mature B cell culture in the presence of BAFF compared to control. A summary of the percentage of An1 cells from each of these cells types in the presence or absence of BAFF is shown in Table 4.4 C. Since the An1 cells are also present in the untreated samples, this indicates that the presence of these cells is not due to effects on differentiation by BAFF. Therefore, since BAFF has such strong survival effects on all other B cell populations, the increase in the An1 cells can be attributed to promotion in survival of these cells by BAFF.

One caveat in this experiment was that sorted T2 and mature B cells had similar distribution of AA4.1 expression after 48hrs of culture although they were initially started off as either AA4.1 hi T2 or AA4.1 lo mature. One explanation for this could be that due to cell culture effects (48hrs), the expression of AA4.1 was downregulated so that the purified T2 began to resemble the mature that have low AA4.1 expression. Added to this, was the spontaneous differentiation of the An1/T3 cells so that the T2 and M ended up with somewhat similar distributions of AA4.1 lo and AA4.1 hi cells.



H

	CD24	CD21	IgD	IgM	CD19	CD23	AA4.1
T1	++	+	++	+	+	+	+++
T2	++++	++	+++	++++	+++	+++	++++
MZ	++	++++	++	+	++	+	+
M	+++	+++	+++	+++	+++	+++	++

Figure 4.2 Sorted populations of B cells express markers at levels indicative of their differentiation stage.

Purified splenic B cells were sorted into mature (M, black), transitional type II (T2, blue), transitional type I (T1, red) and marginal zone (MZ,green), based on expression of CD23 and AA4.1 expression. After the sort, the cells were stained with antibodies to (A) CD24, (B) CD21, (C) IgD, (D) IgM, (E) CD23, (F) CD19, (G) AA4.1 and CD21 to ensure that the sort has been successful. Table (H) shows a summary of the expression profile of each of the sorted T1, T2, MZ and M B cell populations. (++++ : very high expression; +++ : high expression; ++ : moderate expression, +: low expression).

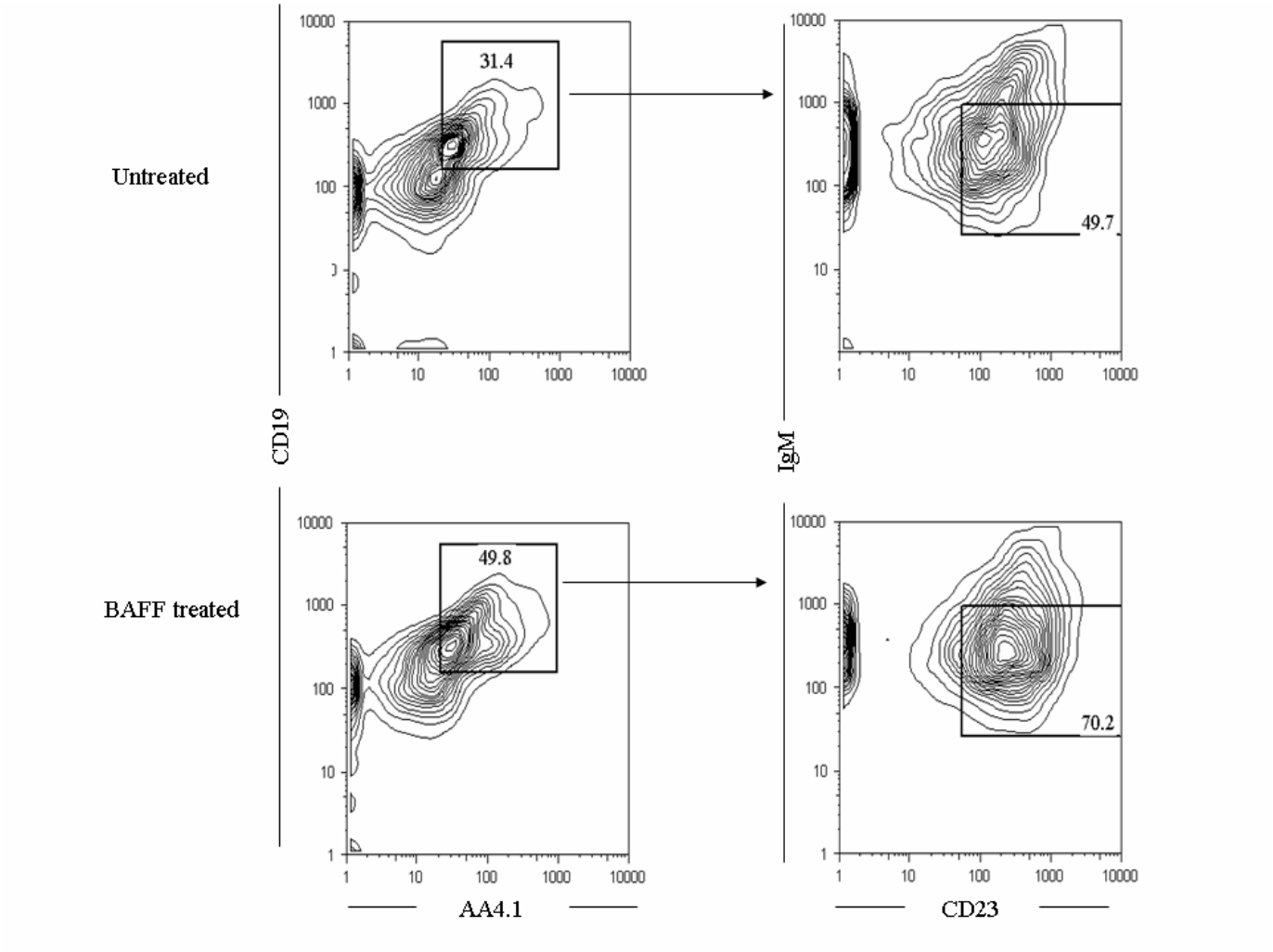
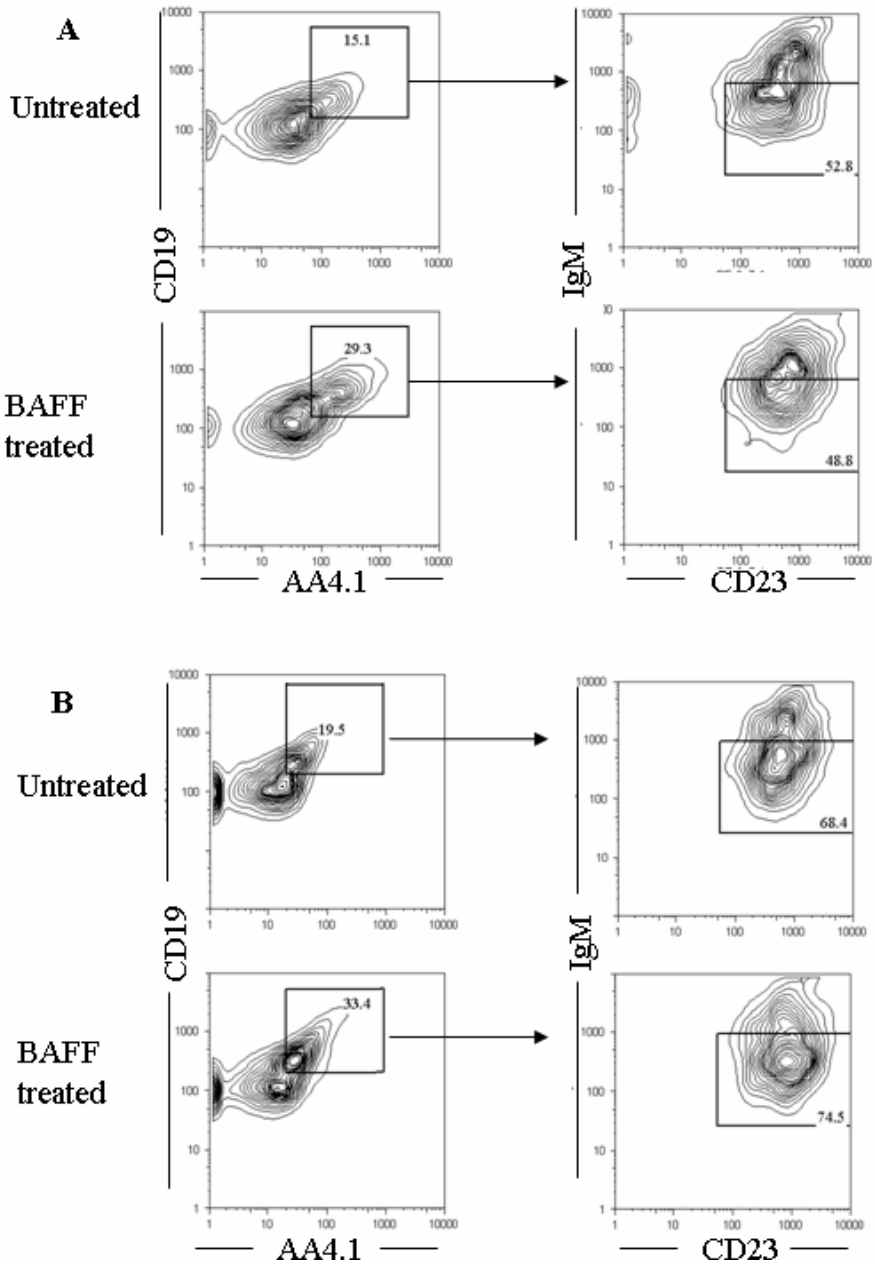


Figure 4.3 BAFF increases frequency of An1 cells

Flow cytometric analysis of purified primary splenic B cells containing mixed population of cells cultured in the absence or presence of BAFF for 48hrs. Data shown as contour plots with the squares in the left panels showing AA4.1 hi cells and the squares in the right panels showing CD23 + and IgM low cells. N = 3 to 4


A
Untreated
BAFF treated
15.1
52.8
29.3
48.8
CD19
IgM
AA4.1
CD23
B
Untreated
BAFF treated
19.5
68.4
33.4
74.5
CD19
IgM
AA4.1
CD23

C

An1 cells as % of total lymphcytes:

	Untreated	BAFF treated
Splenic B cells	15.6	34.9
Sorted T2 cells	8	14.3
Sorted Mature cells	13.4	24.8

Figure 4.4 BAFF increases frequency of An1 cells from both T2 and Mature B cells
Flow cytometric analysis of purified primary splenic B cells containing sorted T2 cells (A) and sorted mature cells (B) cultured in the absence or presence of BAFF for 48hrs. Data shown as contour plots with the squares in the left panels showing AA4.1 hi cells and the squares in the right panels showing CD23 + and IgM low cells. Table (C) shows a summary of the An1 cells as a percentage of total lymphocytes. N = 3 to 4

An1 cells emerging from BAFF treated B cells are truly anergic

In order to test whether the An1 cells truly exhibit anergic properties, we measured the ability of these cells to mobilize intracellular calcium, which is an effective measurement for anergy since calcium mobilization is an early aspect of antigen receptor signaling in B cells. It has been shown that anergic B cells have no further increase in intracellular calcium upon stimulation (Benschop, Aviszus et al. 2001). The Indo-1 fluorescent dye can be used to measure the levels of intraceullar free Ca in live cells. As the cells are activated and the calcium concentration in the cytoplasm increases, the dye binds more calcium. Indo-1 functions by changing spectral properties such that it emits at 390 nm (violet) when bound to Ca++ and at 500 nm (green) when unbound. The color change from green to violet that Indo-1 undergoes can be plotted dynamically by programming the flow cytometer to display the two signals as a ratio of bound and free indo-1 against time. This allows measurement of changes in intracellular free calcium concentration [Ca (2+)]. In addition, the cells were also stained with antibodies to separate the An1 cells from the rest of the population. After establishing basal calcium levels, the cells were stimulated with anti-IgM Fab'2 to initiate BCR signaling. Calcium flux was measured on the gated live cell population.

The calcium began to flux at around 70s after the addition of stimulating antibody (Fig 4.5). The untreated mature B cells showed an increase in [Ca (2+)] i value of about 0.14 at the highest level compared to the baseline. The untreated T2 and An1 reached about 0.10 and the An1 showed a change of only 0.07 in the [Ca (2+)] i value when compared to baseline (Fig 4.5 A). This indicates that the An1 cells have a blunted calcium compared to

the other cell types in the control culture. This reinforces what has already been shown, namely that the An1 cells do have an anergic nature and do not respond well to activation.

Furthermore, BAFF treated An1 have an increase in [Ca (2+)]I of about 0.075 when compared to baseline while the untreated An1 cells reach around 0.065. This indicates that the An1 cells in the BAFF-treated cultures have blunted Calcium response in comparison with An1 cells from control cultures (Fig 4.5 C). These results indicate that BAFF is promoting an increase in An1 cells that are truly anergic as seen by their reduced capacity to mobilize intracellular calcium.

In addition, the mature BAFF-treated cells showed a blunted response compared to the mature untreated cells. This was seen by the low increase of [Ca (2+)] i of about 0.14 compared to baseline in the untreated mature B cells while the BAFF treated mature B cells showed an increase of 0.10 compared to baseline (Fig 4.5 B). This indicates that BAFF could be promoting some level of anergy in the mature B cells as well.

These results implicate that BAFF may be employing a global method for autoreactive B cells to undergo an anergic state and escape clonal deletion.

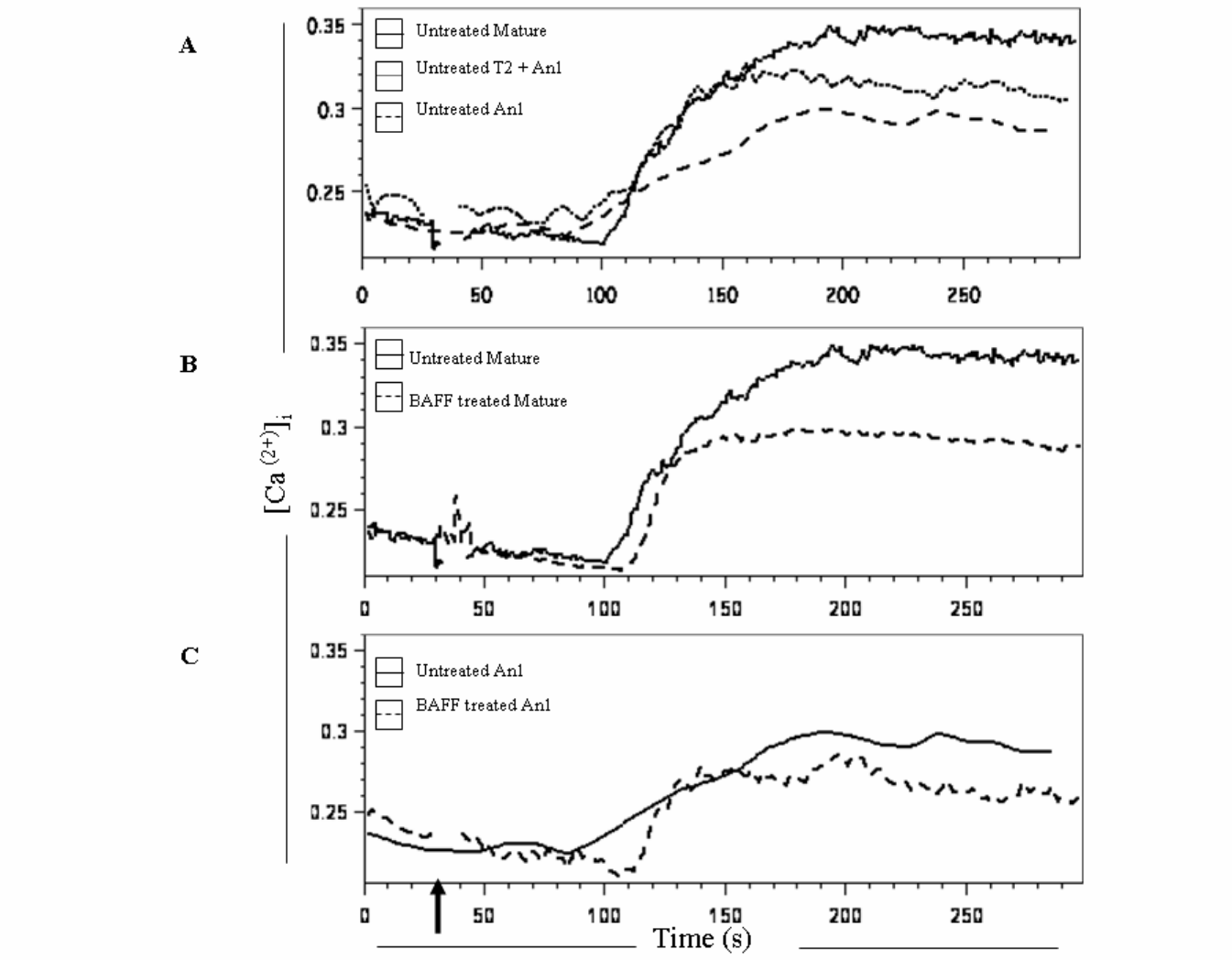


Figure 4.5 BAFF treated cells show lower calcium flux

Flow cytometric analysis of calcium flux assay on purified splenic B cells cultured in the presence or absence of BAFF for 72hrs. Baseline calcium flux was established before stimulation with anti-IgM as shown with the arrow. Change in ratio of intracellular free calcium [Ca (2+)]i measured against time is shown. Cell populations were separated based on differentiation markers.

(A) Calcium flux in untreated Mature, T2 + An1, and An1 cells.

(B) Calcium flux in untreated and BAFF treated mature cells.

(C) Calcium flux in untreated and BAFF treated An1 cells.

Summary

Our data present a unique approach to understanding the link between BAFF and autoimmune disease. The recent finding of the presence of anergic B cells in wild type mice led us to hypothesize that BAFF may be involved in the production and maintenance of this population. An1 cells are anergic B cells that have autoreactive properties. Thus any connection between BAFF and An1 cells could lead to a new and powerful connection between BAFF and autoimmunity. Our studies show that BAFF does cause an increase in the An1 population from normal wild type B cells *in vitro* (Fig 4.3A). One of our key findings was that BAFF is capable of increasing the proportion of An1 cells *in vitro*, primarily derived from the T2 (Fig 4.3B) and the mature B (Fig 4.3C) cell populations. This increase in An1 cells can be attributed to a promotion in survival by BAFF. Since the An1 cells are also present in the untreated samples, the possibility that BAFF is causing differentiation of the An1 cells from the mature and T2 cells, can be ruled out. Finally, these An1 cells were shown to be truly anergic by their reduced ability to flux calcium. Interestingly, BAFF treatment was found to induce a state of unresponsiveness even in the mature B cells, driving them towards an anergic phenotype (Fig 4.4). Since it is possible that anergic B cells could undergo reversal and become activated by external stimuli, high numbers of these cells pose a valid threat of autoimmune disease development for the organism.

Chapter 5

Effects of BAFF on B cell development *in vivo*

Introduction

Lupus prone mice such as NZBxNZW F_1 and MRL/lpr develop lupus erythematosus spontaneously and have elevated levels of BAFF in blood (Gross, Johnston et al. 2000). In addition, BAFF transgenic mice have expanded secondary lymphoid organs and higher immature T2 and mature cell numbers. Eventually, the BAFF transgenics develop systemic lupus erythematosus, and symptoms related to increased levels of DNA specific autoantibodies (Mackay, Woodcock et al. 1999). Studies of BAFF injected mice have surprisingly shown an increase in IgM titres in otherwise unstimulated mice. Since B cells do not normally produce antibodies in the absence of antigen stimulation this provides additional evidence to show that BAFF injection may favor the development of autoimmune B cells. BAFF injections also enhance responses to Pneumovacs Z3 vaccine, a T cell independent antigen, as well as DNP-BSA, a T cell dependent antigen (Do, Hatada et al. 2000). From our *in vitro* studies we have shown that BAFF leads to increased production of An1 cells that have anergic properties. The next important step was to carry these experiments over to an *in vivo* system and see whether the physiological milieu within the mice would still allow the same effect of BAFF. We hypothesized that an excess of BAFF could lead to an increase in anergic B cells, perhaps by supporting their survival.

In order to test whether the presence of excess BAFF results in an increase in An1 cells *in vivo*, it was necessary to produce large quantities of biologically-functional, soluble, recombinant human BAFF. We had previously used commercially-available BAFF for some of our *in vitro* studies, but this source was cost-prohibitive for the planned *in vivo* studies. In addition, most of these commercial BAFF preparations have been expressed in bacterial

cells. We wanted to use eukaryotic SF9 cells to express BAFF in order to ensure proper glycosylation of the protein and to avoid the effects of any bacterial products that might contaminate the commercial bacteria-derived preparations.

In this chapter, we describe the preparation of recombinant BAFF and the effects of BAFF injected into C57Bl/6 mice on B cell development and responsiveness *in vivo*.

Production of recombinant BAFF

The first step in generating purified recombinant human (rh) BAFF was to construct a his-tagged BAFF vector containing the extracellular domain of BAFF. Once the vector plasmid was constructed it was transfected into cells to produce recombinant baculovirus, which was then used to infect SF9 cells for recombinant BAFF protein production (see Methods chapter for the experiment details). The SF9 cells were then lysed to release the recombinant BAFF protein from within the cytoplasm of the cells. The lysate was analyzed with SDS-PAGE and Western blot to ensure that the correct protein band is obtained. The molecular weight of the control BAFF protein is approximately 20kDa. The silver stain and the Western blot showed that the protein was recognized specifically by mouse anti-hBAFF antibody confirming that the expressed heterogeneous protein was BAFF. The histidine-tagged BAFF (Lane 2-4 in Fig 5.2) runs slightly higher compared to the control BAFF (Lanes 8,9 in Fig 5.2) as indicated by the red arrows.

Purification of the BAFF from the lysate was done using a nickel affinity column. The nickel resin is capable of binding with high affinity and selectivity to 6xHis-tagged recombinant fusion proteins. The lysate was allowed to bind to the nickel resin overnight. After the overnight binding, any unbound protein was washed from the column, using a wash buffer containing 20mM imidazole. Proteins bound to the column are eluted by competition with a high concentration of imidazole (200 mM) in the elution buffer.

SDS PAGE and western blot analysis was performed in addition to silver staining in order to ensure correct size and purity of the eluted and dialysed BAFF (Fig 5.1 and 5.2). The silver staining showed all the non-specific proteins that were bound to the column and that were eluted along with the BAFF protein. Thus the lysate (Lane 1 in Fig 5.1 A) showed the highest amount of protein evident in the dark bands that could be seen in all size ranges. Following overnight incubation, much of the his-tagged BAFF protein bound to the nickel column so that the flow-through (Lane 2, Fig 5.1A) was left with less protein than the lysate. The early wash (Lane 3, Fig 5.1A) looked lighter than the previous two lanes indicating that it had less protein than the lysate or the flow through. The bands in the lane reflect the non-specific proteins that were loosely held in the resin and came off with the washes. The later wash (Lane 4, Fig 5.1A) showed hardly any bands and indicated that the column was much cleaner and devoid of the non-specific proteins that were weakly associated with the nickel resin. The optical density (OD) was measured and it also indicated the absence of any protein in these later wash fractions.

Once the elutions were started, the imidazole began to displace the bound protein from the column and the OD started increasing. A single band could be seen in the early elution lane, elution #35 (Lane 5, Fig 5.1A) which as appeared to be the recombinant BAFF protein since it ran a little higher than the control BAFF with a molecular weight of around 25 kDa. The next few elution fractions were represented by elution fraction # 43 (lane 6, Fig 5.1A). This lane showed that beside the BAFF band, there were many more protein bands, which indicated that there were still some non-specific proteins present in the column that also

eluted out. The imidazole concentration in the elution buffer is high (200mM) which is why many more of the non-specific proteins that were bound to the column started coming off. The wash buffer contained only 20mM imidazole which is why a lot of the non-specific proteins still remained bound to the column. Elution fraction #45 (lane 7, Fig 5.1A) showed much less non-specific protein impurities than the previous elutions. This is because most of the proteins had already come off. Thus at the beginning of the elutions, BAFF could be found in its most pure form. As the elutions proceeded, more and more of the non-specific proteins that were still bound to the column started coming off together with the BAFF protein.

In order to measure the amount of BAFF protein in each of the elution fractions, a Western blot was performed (Fig 5.1B). The lysate shows higher BAFF protein content than the flow through or the wash. The earlier elution fraction (Lane 6) shows very faint bands of BAFF indicating that the elution of the BAFF protein had just started. The next few elution fractions (lane 7 and 8, Fig. 5.1B) show a clear BAFF band. As more elution fractions are collected the amount of BAFF protein increases as seen by the progressively thicker band (Lane 8-14, Fig 5.1B). Lane 14 has the thickest band indicating that the elution of the protein is at its peak. The band intensity starts diminishing as the elution fractions progress indicating that whatever was bound to the column had been eluted. The last few elutions therefore show much less of the BAFF protein (Lane 16-19). Thus from the silver stain and the Western blot, it can be seen that the later fractions that contain the higher amount of

BAFF protein also have more impurities than the earlier fractions, which have less BAFF protein but are more pure.

Once the BAFF protein was eluted, it had to be dialyzed in order to remove the high concentration of imidazole. The elution buffer was removed by using a sodium phosphate/sodium chloride buffer by dialysis. By running a SDS PAGE analysis and Western blot of the pre- and post-dialysis samples (Lanes 8 and 9, Fig 5.2) it can be seen the intensity of the band remains about the same, indicating very little loss of the protein during the dialysis procedure. By comparing the intensity of the bands with the control BAFF, the amount of protein can also be estimated. Thus the generated rhBAFF (lane 9, Fig. 5.2) is between the 20ng and the 10ng band (lane 3 and 4, Fig 5.2) and is estimated to be 15ng. Since 1µl was loaded, the concentration of the purified rhBAFF protein is estimated to be 15 ng/µl or, 15 µg/ml.

One caveat for the production of recombinant protein is the possibility of contamination with endotoxins. In the event that there is an endotoxin contamination, several steps can be taken to remove or neutralize the toxin. The agent used mostly for this purpose is PMB, a polycationic peptide antibiotic that binds to the anionic lipid A portion of LPS, the most prevalent and potent endotoxin (Wakelin, Sabroe et al. 2006). One method of detecting such contaminations would be by producing a protein using the same procedures as the recombinant protein of interest that would serve as the 'negative control'. Our BAFF recombinant protein was expressed in SF9 insect cell line, which allows less chance for such contamination. Since bacterially derived recombinant proteins are notorious for endotoxin contamination, this provides an additional advantage of using a eukaryotic cell line for the

expression of recombinant proteins. However, there is nevertheless a possibility of contamination from the nickel column or from any other experimental apparatus that is used for the purification of BAFF. One way that such a contamination could be evaluated is by looking at the total cell numbers or proliferation of cells stimulated with the recombinant BAFF and comparing with results from LPS stimulated cells. LPS can cause very strong activation of the cells, and therefore the fwd/ssc of the cells are much higher for LPS stimulated cells. One evidence to show that the recombinant BAFF is not contaminated with endotoxin is that the fwd/ssc of the BAFF treated cells vary very little from the untreated cells, showing most of the cells to be either resting or slightly activated, which is typical of BAFF. No large blasting cells can be observed, which indicates that the preparation is endotoxin free.

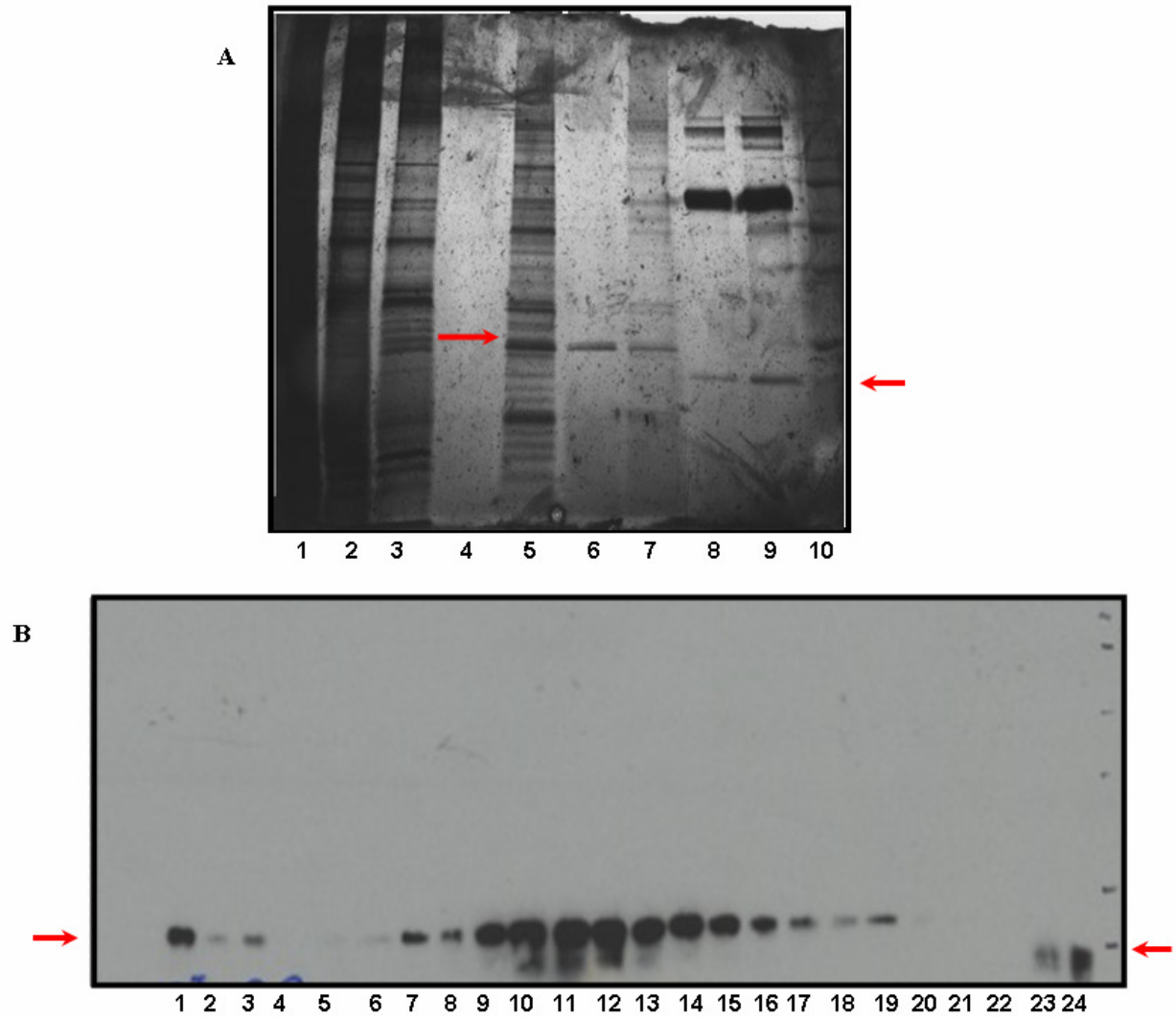


Figure 5.1 Purification of rhBAFF

The protein from the different purification steps were run on 10% SDS gel and were then analysed by silver staining and by Western blot. A) Silver staining of the purification steps of rhBAFF by elution from Nickel column. Lane 1, Lysate; Lane 2, Flow through; Lane 3, Early wash; Lane 4, Late wash; Lane 5, Eluent fraction #35; Lane 6, Eluent fraction #43; Lane 7, Eluent fraction #45; Lane 8, BAFF control 20ng; Lane 9, BAFF control 40ng; Lane 10, Protein ladder. B) Western blotting analysis of purification steps of BAFF by elution from Nickel column. Lane 1, Lysate; Lane 2, Flow through; Lanes 3 and 4, Wash; Lanes 5-22, Eluent # 25-59 (all odd numbers of eluents), Lane 23, BAFF control 20ng; Lane 24, BAFF control 40ng.

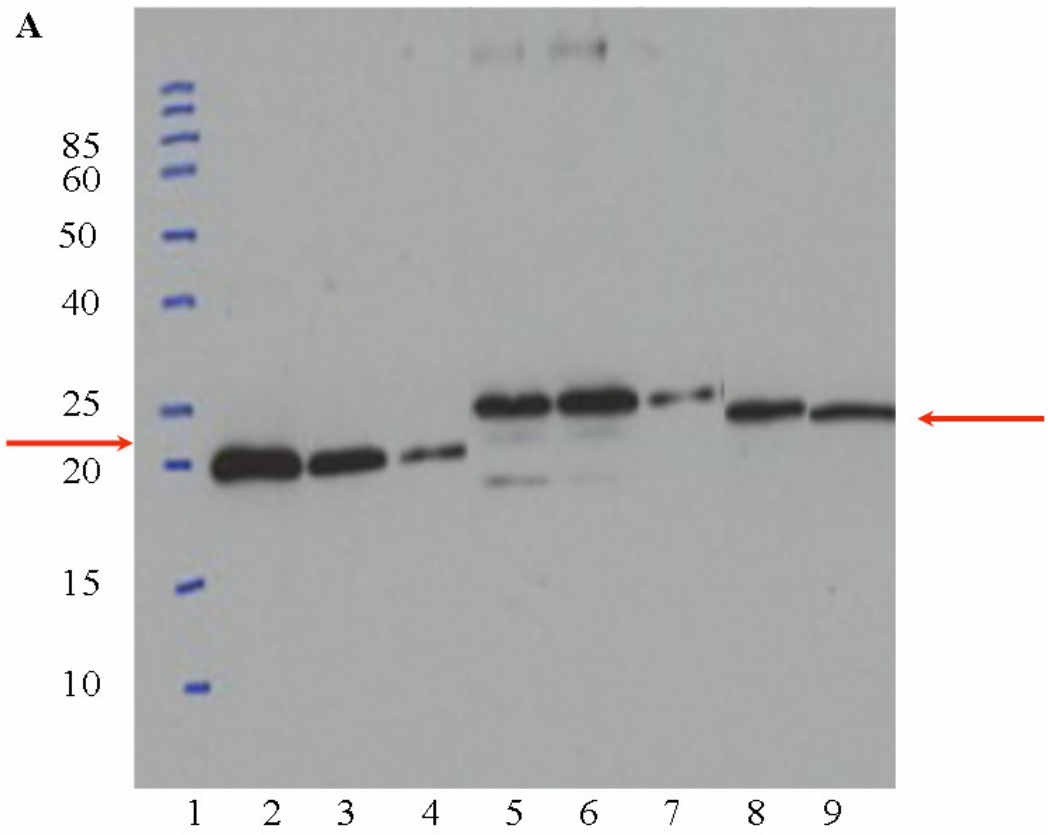


Figure 5.2 Western blot analysis shows correct BAFF band

Western blot analysis to show specific binding of hsBAFF to anti-BAFF antibody. Lane 1, Protein molecular weight markers; Lane 2, BAFF control 40ng; Lane 3, BAFF control 20ng; Lane 4, BAFF control 10ng; Lane 5, Lysate; Lane 6, Flow through; Lane 7, Wash; Lane 8, Recombinant hsBAFF pre-dialysis; Lane 9, Recombinant hsBAFF post dialysis.
BAFF control is commercial BAFF

Purified rhBAFF is biologically functional: In order to ensure that the purified rh BAFF is functional, purified B cells were cultured in the presence or absence of BAFF for 48hrs and then assayed for survival. The survival assay included staining the cells with Annexin V, which marks the apoptotic cells. The percentage of Annexin negative cells, which are the live cells, was measured by flow cytometry (Fig 5.3). A significant increase in Annexin negative cells was observed under BAFF treatment conditions as compared to untreated conditions. This indicates that the purified rhBAFF protein is biologically functional since increase in survival is a well-documented trait in the effects of BAFF on B cells (Batten, Groom et al. 2000; Rolink, Tschopp et al. 2002; Rauch, Tussiwand et al. 2009).

Three different doses, 80ng, 120ng and 240ng, of the recombinant protein were used. Results showed that the percentage of the surviving cells varied from 71 % and 74% between the different doses of the purified rh BAFF. Thus, compared to only 40% surviving cells in the untreated population, the purified rh BAFF showed effective protection against spontaneous apoptosis. The increasing doses of the purified rh BAFF show a consistent slight trend in increase in the average percentage of surviving cells. However, since this increase is not significant, the smallest dose of rhBAFF (80ng) show near maximum efficacy for *in vitro* studies. The activity of the purified rh BAFF was compared with the commercial BAFF that was previously used in the lab. There were around 10 to 15% more survival of the B cells in culture under the treatment of the commercial rh BAFF compared to the purified rh BAFF. However, given the effectiveness of the purified rh BAFF which increased survival ability of the B cells to about 2 fold higher than the untreated shows that it has sufficient bioactivity for our *in vivo* studies.

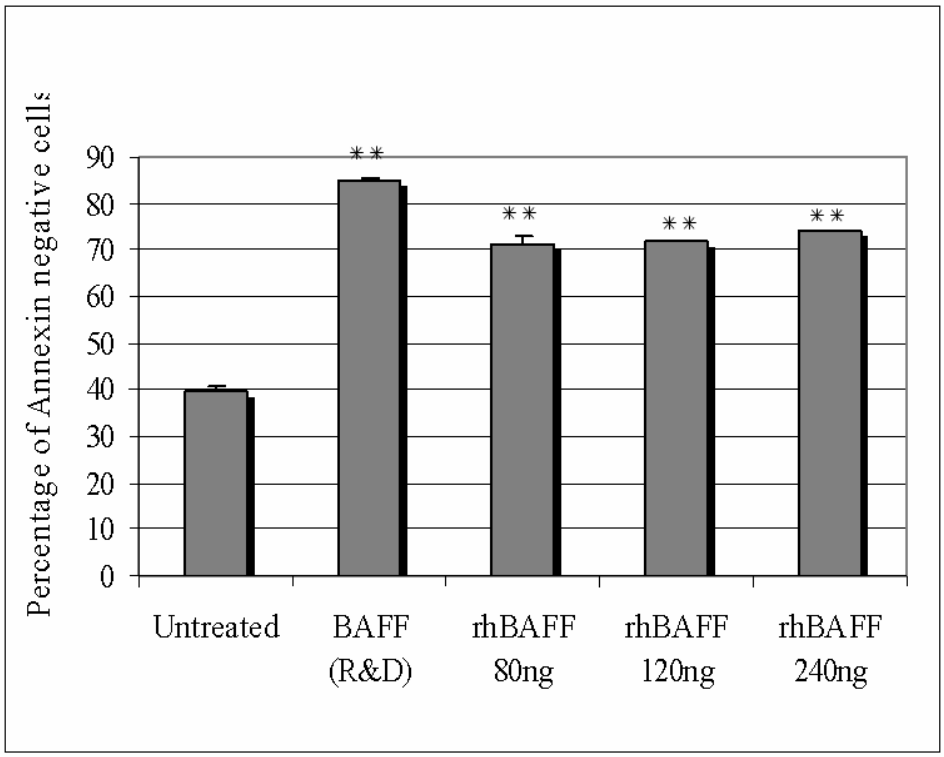


Fig.5.3 RhBAFF is bioactive

In order to test whether the purified recombinant human BAFF is biologically active, a survival assay was done. Purified splenic B cells were cultured with or without different doses of rhBAFF for 48hrs. 60ng of commercial BAFF from R & D was used as a control. Survival was measured with Annexin V which detects apoptotic cells. Percentage of Annexin V negative cells are shown.

Statistical test was performed by comparing average of triplicates of commercial or purified rh BAFF treated samples with average of untreated controls using the Student's *t* test.

** Represents p value of less than 0.01

Determining correct dosage of BAFF to use in the in vivo studies: Before we started the *in vivo* studies, it was necessary to evaluate the optimal dosage of treatment needed to see an effect of the purified rh BAFF on B cells. This was done using three different dosages of the BAFF: 10µg, 15µg and 20µg injected peritoneally into C57Bl/6 mice. The control mice of the same age were injected with equivalent amounts of phosphate buffer. The mice were injected for three consecutive days and were sacrificed on the fourth day for analysis. The splenic B cells from these mice were purified and analyzed for total B cell numbers. The results showed that the total number of B cells ranged from 20 million to 33 million in the control mice, and 46 million to 56 million in the purified rh BAFF injected mice. This indicates that the BAFF injected mice have on average a greater total number of B cells than the PBS injected mice. Although all three BAFF injected mice had higher levels of B cells than the PBS injected controls, there was no correlation between the dosage and B cell levels. This indicates that amongst the 3 doses tested, the lowest dose of BAFF, 10 µg, is sufficient to show a maximal response on B cells *in vivo*.

After determining the dosage of the purified rh BAFF to be injected in the mice, several different assays were carried out.

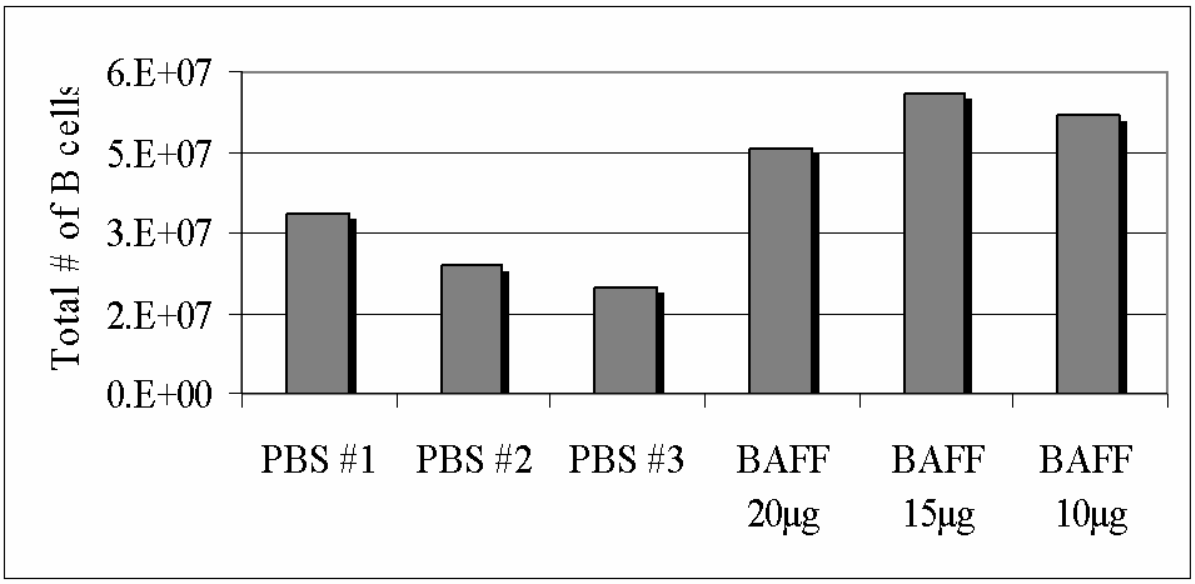


Fig 5.4 Dose response shows that 10µg of BAFF is enough for *in vivo* studies:
In order to determine the optimal dose of BAFF injection for the *in vivo* injections, mice were injected with 10ug, 15ug or 20ug BAFF or equivalent amount of PBS for 3 days and then sacrificed the 4th day. Purified splenic B cells were then analyzed for total B cell numbers by Trypan blue exclusion.

Results

BAFF increases total B cell numbers

The first step in the analysis of the effects of the purified rh BAFF *in vivo* was to ensure that the survival activity of the protein had remained intact. In order to test this, three mice were injected with 10 μg of the purified rh BAFF for three consecutive days along with three control mice which were injected with the phosphate buffer saline (PBS) alone. The mice were sacrificed on the fourth day. Splenic B cells were then purified and analyzed for total B cell numbers by trypan blue exclusion. The results show that the BAFF injected mice had greater total number of B cells, about 51 million on average, compared to the control PBS injected mice which had about 25 million on average (Fig 5.5 A). This indicates that the generated BAFF had the same protective effect on B cells against spontaneous apoptosis *in vivo* as has been shown *in vitro* (Fig. 5.3). The greater total number of B cells in the presence of BAFF also demonstrates how BAFF changes the homeostasis of B cell populations even in the short time span of 3 days. The standard error bars indicate that there was variability between the mice in each group. Although age matched mice of the same background were used, there could still be some physiological differences between them (variations such as low grade infections, stress level, activity, etc) leading to the variability in the total number of the splenic B cells.

Although the splenic cells were also stained with CD4 and CD8, there was no difference seen between the BAFF treated and the control mice. Since the T cells are the only other cells that express the BAFF-R, only the T cells were examines besides the B cells.

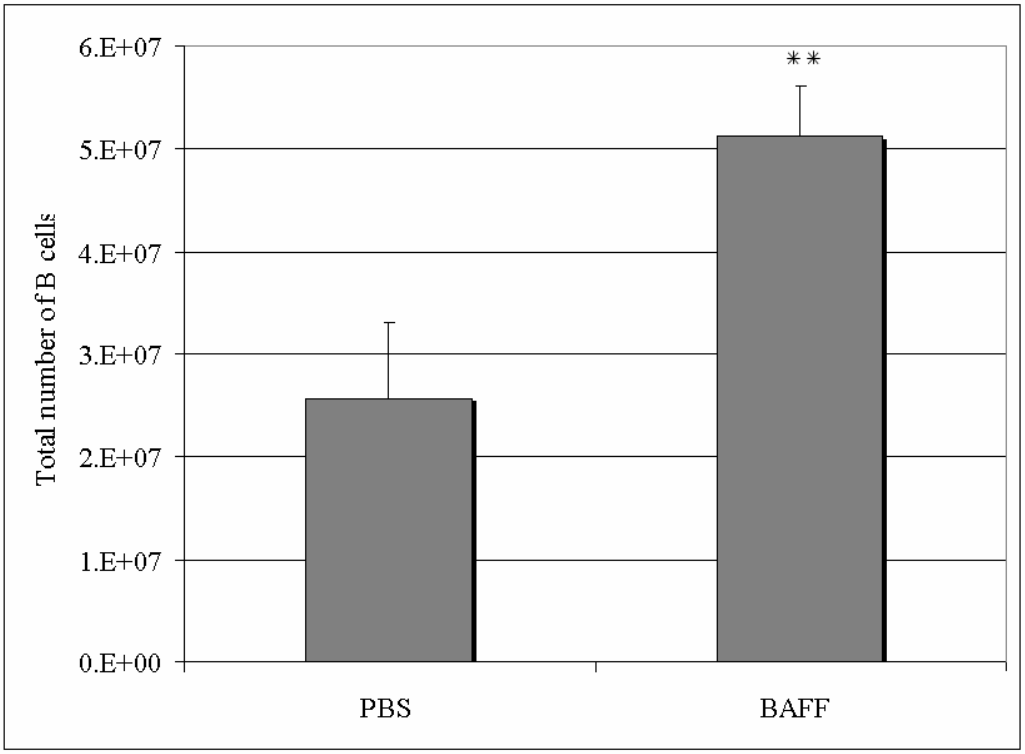


Figure 5.5 BAFF increases total B cell numbers: Mice were injected with 10ug BAFF or equivalent amount of PBS for 3 days and then sacrificed the 4th day. Purified splenic B cells were analyzed for total B cell numbers by Trypan blue exclusion

Statistical test was performed by using the Student's *t* test.

** Represents p value of less than 0.01

BAFF enhances frequency of An1 cells in vivo

The most interesting finding of our *in vitro* studies was the increase in frequency of the An1 cells from mature and T2 cells by BAFF. We wanted to evaluate whether this will also hold true *in vivo*. Unlike *in vitro* stimulations involving single ligands, the cells *in vivo* are constantly being bombarded by numerous other stimuli from chemokines and cytokines present within the organism. Therefore, it was essential to examine whether BAFF could still induce the increase in the An1 population in the presence of the other external stimuli that may be going on *in vivo.* The differentiation markers for identifying An1 cells include AA4.1, IgM and CD23. An1 cells can therefore be differentiated by first gating on the AA4.1 hi cells and then looking at the CD23hi and IgM low population (Merrell, Benschop et al. 2006). Using this strategy on the BAFF and PBS injected mice it was seen first of all that there is a general increase in the percentage and total number of AA4.1 hi cells (Fig 5.6 A and B). Since AA4.1 is an identifying marker for An1 cells, this suggests that BAFF may be causing an increase in An1 cells also *in vivo.* However, since AA4.1 hi cells also include the T1 and T2 cells, it was necessary to separate out the An1 cells using additional markers. Once the An1 cells were teased out by the gating strategy described above using anti-CD23 and anti-IgM antibodies, it was seen that there is a general trend in increase in these cells. Although nearly all the mice show an increase, a significant increase was seen in the total number of the An1 cells in one set of mice amongst the three different sets of mice examined in the three separate experiments (Fig 5.6 C and D). This could be due to variability between the mice due to underlying physiological conditions in the individual mice. In addition, this could also be due to the fact that the development of An1 cells is a longer

process and therefore may be detected in larger quantities after a few more days of BAFF injection. Thus, although the data looks promising in its implication of the effect of BAFF on the increase in frequency of An1 cells, it needs to be repeated with longer incubation periods and with a greater number of replicates to determine if the differences observed are significant.

We also examined all other B cell subsets in the untreated and BAFF injected mice. The only consistent difference was seen in the mature B cells which showed a trend in a higher percentage of cells in the BAFF injected mice compared to the control, although this increase was not seen to be statistically significant. Because of an overall expansion in the total number of B cells in the BAFF injected mice, all of the subsets showed a higher total number of cells. These results indicate that *in vivo* treatment with BAFF causes enhanced survival of all the B cell subsets.

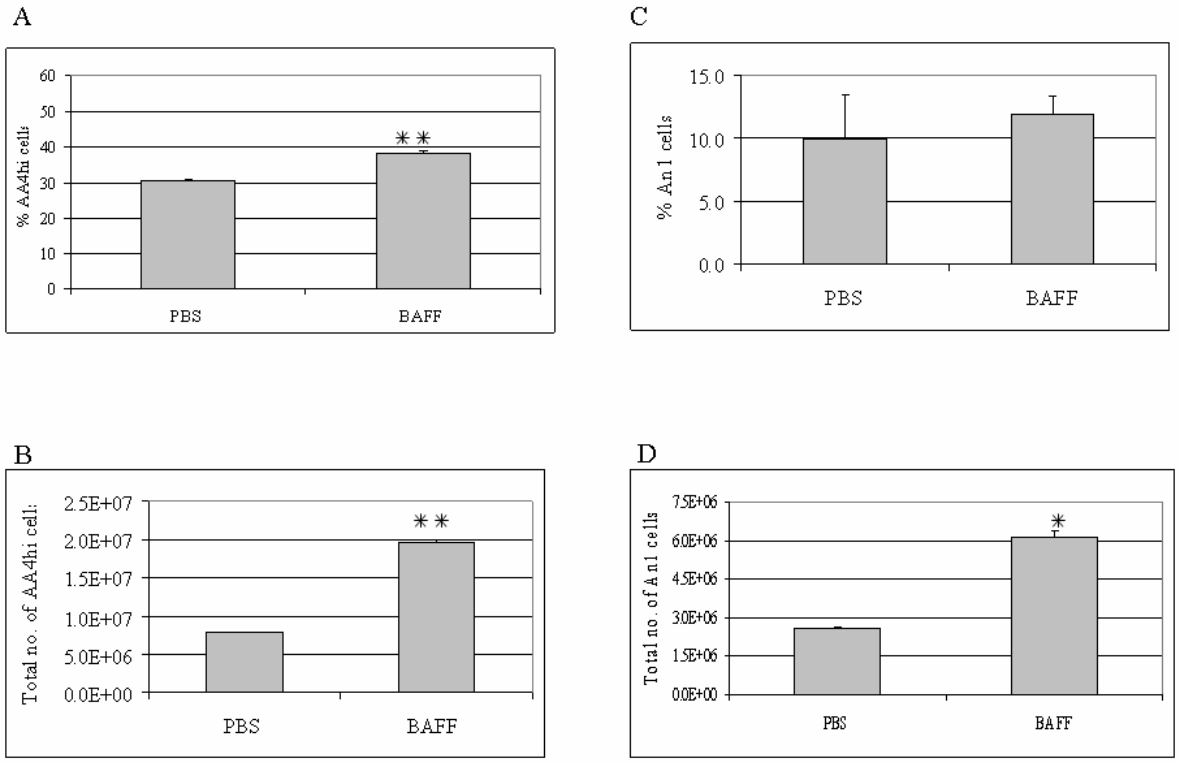


Figure 5.6 BAFF increases the frequency of AA4.1hi cells and An1 cells

Mice were injected with 10ug BAFF or equivalent amount of PBS for 3 days and then sacrificed the 4th day. Purified splenic B cells were stained with antibodies against AA4.1, CD23 and IgM and analyzed by flow cytometry for (A) Percentage of AA4.1hi cells (B) Total number of AA4.1hi cells (C) Percentage of An1 cells (D) Total number of An1 cells

BAFF lowers calcium flux in B cells.

Anergic B cells remain silent and are unresponsive to external stimuli. In order to examine whether BAFF is promoting the development of these An1 cells, we wanted to test whether BAFF has any influence in increasing B cell unresponsiveness *in vivo*. Calcium flux is an effective method for measuring anergy since it is an early aspect of B cell signaling. This study directly addresses the question of whether BAFF can cause anergy and therefore increase the An1 population. The cells were extracted from the BAFF injected mice and were cultured in media alone overnight. This was done to allow the cells to synchronize to the same cycle so that any new signaling can occur in all the cells at the same time. After overnight culture, the cells from the BAFF injected and the PBS injected mice were harvested from the plates and were loaded with Indo-1-AM, a fluorescent calcium indicator. The calcium flux was measured by the change in the ratio of the fluorescence emission of the indo-1 dye over time. The measurements were taken by first establishing basal calcium levels and then stimulating the cells with anti-IgM Fab'2 to initiate BCR signaling. The data shows that cells from the BAFF treated mice have lowered calcium flux compared to the PBS treated mice. At 170 seconds, the cells of the PBS injected mice showed an increase in the [Ca (2+)]i value of about 0.03 from baseline while the cells from the BAFF injected mice showed an increase of 0.02 compared to baseline (Fig 5.7).

This indicates that the BAFF treated mice have reduced ability to mobilize calcium and are anergic in nature.

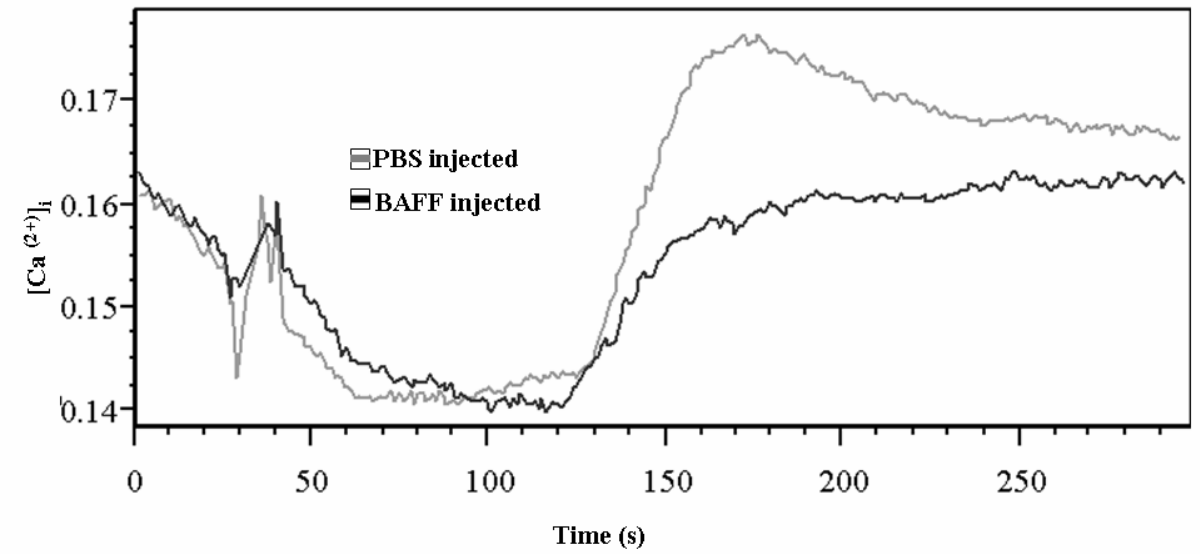


Figure 5.7 BAFF lowers calcium flux in purified B cells *in vivo*:
In order to measure effect of BAFF on B cell responsiveness, mice were injected with 10ug BAFF or equivalent amount of PBS for 3 days and then sacrificed the 4th day. Purified splenic B cells were analyzed for calcium flux by Indo-1-am loading. After basal calcium level was recorded for 30 seconds, the cells were stimulated with anti-IgM Fab'2 and calcium level was recorded for another 250 seconds.

Summary

Our *in vitro* studies have shown that BAFF can stimulate the development of An1 cells from mature and T2 cells. In order to test whether this could also be happening *in vivo* we needed to inject BAFF into mice and compare with PBS injected mice. To serve this purpose we generated purified recombinant human BAFF in the lab. The purity of the purified rhBAFF was determined by silver stain and Western blotting. The results show that the purified rhBAFF is of the right size although it runs higher than control BAFF due to being his-tagged. The purified rhBAFF was tested for bioactivity and a good response was obtained in which about twice as many cells were protected from spontaneous apoptosis *in vitro* (Fig 5.3). Once this BAFF was injected into mice, we saw that the survival activity of the rhBAFF remained intact (Fig. 5.5). Interestingly we were able to measure an increase in AA4.1hi cells in general and An1 cells in particular (Fig 5.6). In addition we were also able to capture the reduced ability of the BAFF stimulated cells to flux calcium resulting in hyporesponsiveness of the B cells to BCR stimulation (Fig 5.7). This indicates that the An1 cells generated by the BAFF injected mice are truly anergic. Although high basal intracellular calcium levels are reported to be present in anergic cells, our data did not show any such difference between the basal calcium levels of BAFF injected and control mice.

Since the An1 cells are essentially silent autoreactive cells, an increase in this population poses a potential risk for autoimmune disease upon sudden activation of the cells by external stimuli. Thus, this provides a mechanism for the established link between BAFF and autoimmunity.

Chapter 6

Microarray to study global gene expression of BAFF treated cells

Introduction

DNA microarrays provide a powerful tool for obtaining global gene expression pattern of cells under different treatments. Several microarray studies have been carried out to examine cell signaling within B cells. The B cell single ligand screen project was an effort to do a comprehensive study of the responses of primary B lymphocytes to a panel of 32 different input single ligands by microarray expression studies (Lee, Sinkovits et al. 2006). However, the single ligand screen project was done on very early timepoints in culture, which is not sufficient for measuring the expression pattern of late expressing genes. Other studies have focused on B cell lymphomas and other disease associated B cells or cell-lines to analyse the skewing of the signaling in the diseased versus healthy cells.

The main purpose for our microarray studies was to get an overall understanding of the effect of BAFF on primary B cells. By looking at the broad array of gene expression profiles we hoped to gain insight into the mechanism of BAFF induced autoimmune disease.

Microarray design and analysis

Experimental approach: The microarray study was designed to understand B cell signaling by the stimulation of several important ligands, with BAFF being the main focus. The experimental set-up in these gene expression studies included the ligands BAFF, anti-CD40 and anti-IgM alone, as well as in combination, in order to mimic *in vivo* physiological conditions. Anti-IgM is effective in cross-linking the B cell receptor (BCR) and therefore mimics antigen binding to B cells. CD40L is a member of the TNF family and is another important ligand for B cells. CD40L on T cells binds to CD40 on B cells and provides the costimulatory signal for the activation of B cells, which results in B cell proliferation and differentiation. LPS signals through toll-like receptor 4 (TLR4) on B cells and bypasses the BCR in the activation of B cells. Looking at the gene expression profile of these ligands by themselves and in combination with BAFF may help to elucidate whether BAFF interacts with any of these signaling pathways.

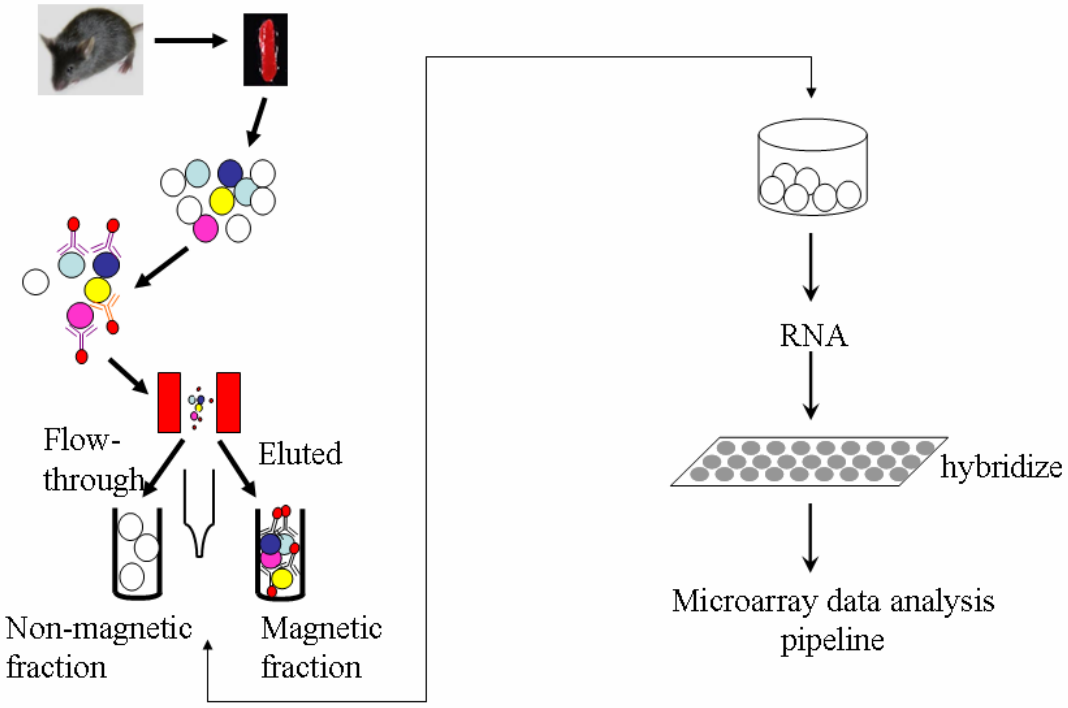
In order to ensure proper controls, it was important to include unstimulated samples at all the timepoints so that ligand specific effects could be differentiated from cell culture effects. In addition, there were quadruplicates of each sample so that error introduced by experimental bias could be reduced. Purified B cells were cultured in media with the appropriate ligand stimulation and were harvested at three different timepoints. These included an early timepoint of 6 hrs and later timepoints of 24hr and 48hr. The earlier timepoint is useful in capturing the expression pattern of genes that are regulated during the earlier phases of signal tranduction and the 24hr and 48hr timepoints are useful for the later

expressing genes that are involved in events such as cell division and apoptosis. Also, the earlier timepoint is useful in eliminating any undesired cell culture effects, such as cell death, that may confound the patterns seen in later timepoints.

Preparation of samples: Primary B cells were isolated from the spleen of 6 to 10 week old C57BL/6 male mice and purified using the MACs sorter (Fig. 6.1). The microarray experiment consisted of a total of 28 samples which consisted of the purified B cells cultured with: 1) media alone; 2) BAFF; 3) Anti-CD40; 4) Anti-IgM; 5)LPS; 6)BAFF + anti-IgM; 7) anti-CD40 + anti-IgM; 8) BAFF + anti-CD40 + anti-IgM; and 9) BAFF + anti-CD40. Each of these stimulations were carried out for three timepoints, 6hr, 24hr and 48hr, and a 0hr media alone treatment was also included. Four separate replicates were prepared independently to lower bias introduced by experimental error. The RNA was then amplified, labeled with fluorescent dyes, hybridized to microarray chips and scanned using an Axon scanner (see Methods chapter for experiment details). The data from the scanner were stored as jpeg files or tiff images.

The microarray data were collected and analyzed in several steps (Fig 6.2). The data were first filtered to remove unreliable values and then inter-array and intra-array normalized to enable comparison between the scans. The data were then statistically filtered to find significant genes. After clustering the data to find patterns of gene expressions, the data were finally processed through CLASSIFI, a bioinformatics tool. Each of these steps is outlined in more detail below.

Data acquisition: The total spleen RNA (TSR) and the sample RNA were labeled with the fluorescent dyes Cyanine 3 (Cy3) and Cyanine 5 (Cy 5), respectively, and mixed for microarray hybridization. Raw microarray data scans consist of the monochrome images of the green Cy3 channel and the red Cy5 channel, which can be combined to give a composite false-color image of the array (Fig.6.3). Green spots correspond to features that were expressed more in the total spleen sample labeled with Cy3, while the red features correspond to those that are expressed more in the experimental sample labeled with Cy5. Features that are yellow represent a similar level of expression in both channels. By comparing the scans, it can be seen that most of them have the same overall pattern indicating that the sample preparation is reproducible. Although a lot of the genes are unchanged as seen by yellow spots, there are some spots that show marked changes in expression in just a few of the scans, thus displaying ligand specificity. Such examples of specific effects by ligands are shown by the two red spots in the BAFF stimulated and the AIG stimulated scans that is either not seen or is very dim in the other samples (arrows in Fig 6.3). Once these raw data were obtained, they were processed through a rigorous data analysis pipeline (Fig 6.2).



http://www.afcs.org/reports/ContentBcell/ContentFrameBCell.htm

Figure 6.1 B cell enrichment and microarray analysis

Purification of splenic B cells is shown. The splenic cells were first treated with red blood cell (RBC) lysis buffer and then mixed with anti-CD43 and MAC-1 antibodies conjugated to magnetic beads. These antibodies bind to non B cells (colored cells), so that when the cells are passed through the magnetic column, the non-B cells remain in the column while the B cells (white cells) pass through and are collected. These purified B cells remain "untouched" and therefore inactive by this procedure and can be used for the Microarray experiment. The purified B cells were then cultured in the appropriate media with different ligand treatments for alloted timepoints. Next, the cells were harvested before extracting the RNA for the microarray experiment. The RNA is then converted to cDNA, labeled with fluorescent dye and hybridized to 17K oligo spotted array chips.

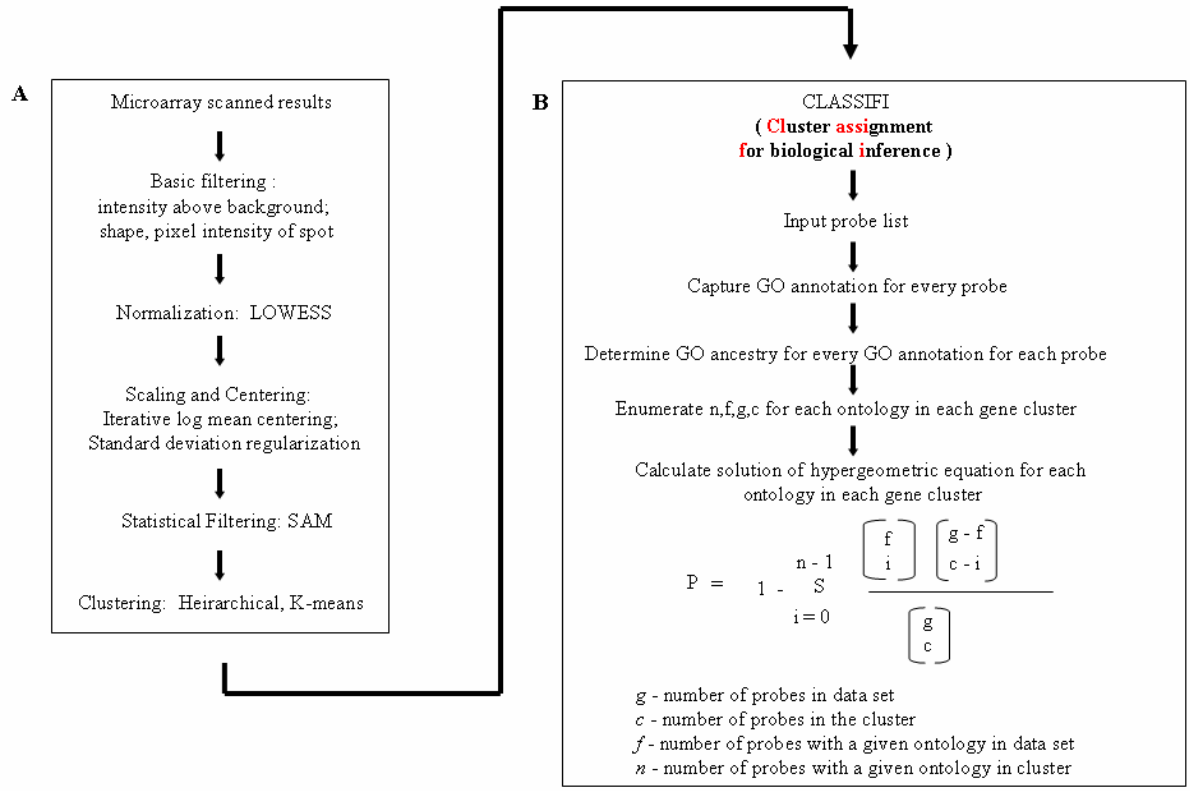


Fig. 6.2 Microarray data analysis pipeline:

The Microarray data is processed through several data analysis steps.

The data is finally processed using CLASSIFI. This bioinformatics tool involves the hypergeometric equation shown in the figure.

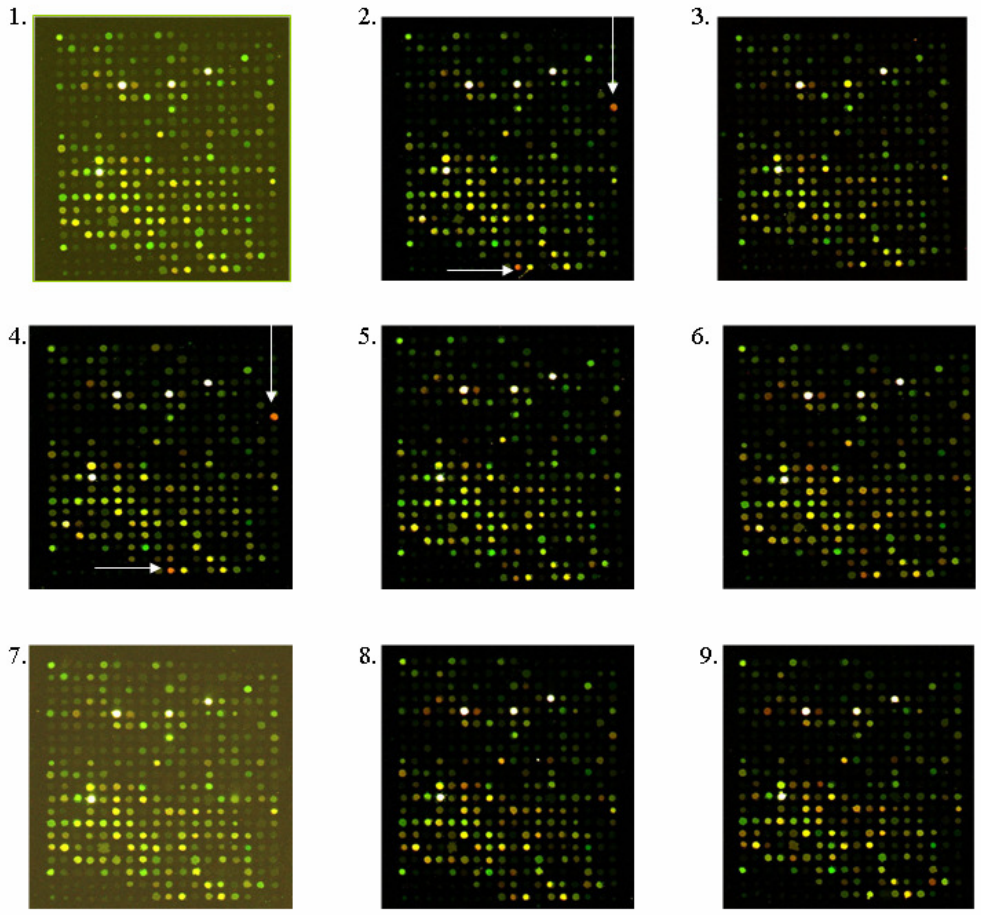


Fig. 6.3 Raw Microarray scans of purified B cells. Microarray was performed on purified B cells cultured with single ligands or in combination to understand the effect of these ligands on the function and homeostasis of B cells. The following treatments were applied to the cells and representative microarray scans of each of them are shown:
1) Untreated, 2) BAFF, 3) anti-CD40, 4) Anti-IgM, 5) BAFF + anti-IgM, 6) anti-CD40 + anti-IgM, 7) BAFF + anti-CD40, 8) BAFF + anti-CD40 + anti-IgM, 9) LPS. Higher expression in the B cell sample in comparison to total spleen is shown by red, a lower expression is shown by green, and equivalent expression by yellow. White is due to overexposure and is filtered out.

Most of the spots are yellow (equal expression) and the overall pattern is the same in all the scans showing reproducibility of the data. Arrows pointing to red spots (higher expression) show specificity of ligand treatment.

Filtering: The images obtained from the microarray scanner was analyzed using the Axon Genepix Pro 3.0 software which recognizes spots on the microarray as being valid or invalid based on a number of criteria including the spot diameter, contour, and the number of pixels in the spot that are saturated. The software then calculates the absolute intensity values for the two channels, green and red. The filtering step also includes getting rid of those values that are not above background levels of fluorescence.

Intra-array normalization: The filtered data is then taken through a normalization step known as Lowess. The normalization step is necessary in order to make it possible to conclude from the data that the differences in the spot intensities are due to true differential gene expression and not due to bias and error introduced by the experimental method. So normalization simply eliminates experimental bias. There are several ways that experimental bias can be introduced. These include systemic experimental error from labeling and hybridizing, differential incorporation of the Cy3 and Cy5 labels, uneven hybridization, or uneven printing of spots on the array. Emission of the Cy3 and Cy5 dye could also be differentially measured and there could also be differential image processing from the two dyes. In order to take out all these experimental variations, we carried out Lowess normalization, which does a correction for this bias. Thus, the pre-normalization plot, obtained before the Lowess normalization was carried out, shows that most of the spots are biased towards Cy3 (Fig. 6.4A). Since the y axis represents the log ratio of Cy5/Cy3, any

spot above 0 represent greater Cy 5 intensities compared to Cy3 and any spot below 0 represent greater Cy3 intensities compared to Cy 5. We want the spots to be equally distributed about the 0 axis since if the Cy5 and Cy3 channels are behaving similarly, then the data should appear symmetrically about the horizontal line. Any deviation from this horizontal line represents differential response of one of the channels, in this case Cy3. Lowess is an algorithm that computes the local regression of each of the features or spots across the data set and calculates the normalized log ratio by subtracting the fitted value on the local regression from the raw log ratio. So after the Lowess algorithm is applied, the post normalization plot shows equal distribution about the x-axis indicating that the differential response of the two channels has been corrected (Fig 6.4B).

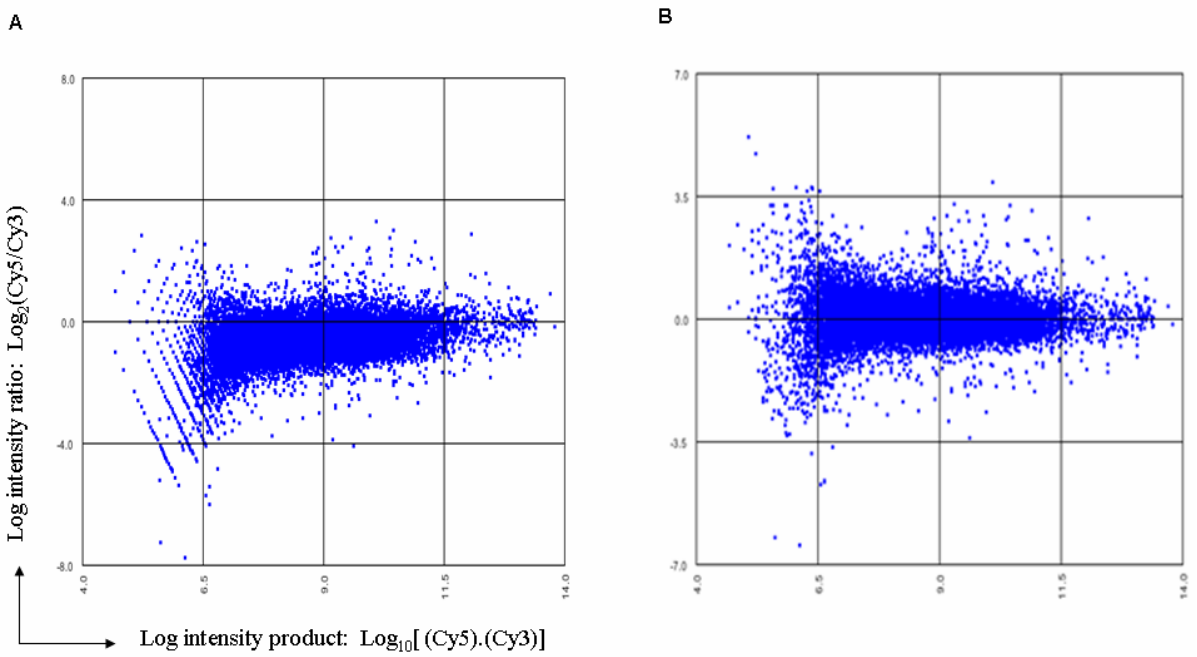
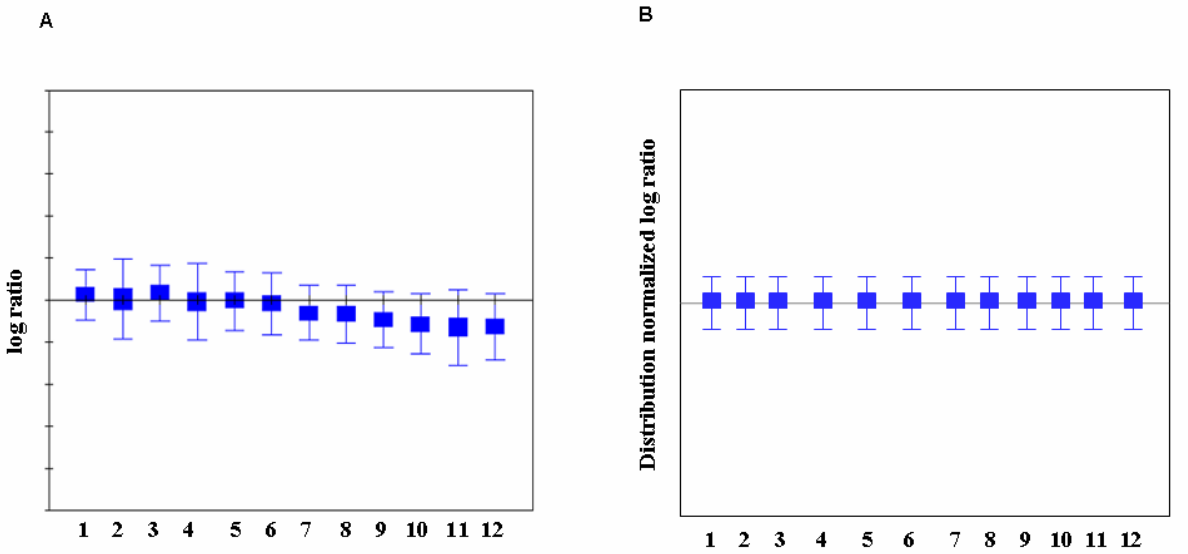


Fig. 6.4 Lowess (Locally weighted polynomial regression) normalization

The microarray data were adjusted using the Lowess normalization tool present in the TIGR Midas suite. Unequal distribution of the data around the x-axis is seen in the pre-normalization plot (A). After applying Lowess, the data becomes equally distributed about the x-axis as shown in post-normalization plot (B).

Inter-array normalization: After the intra-array normalization step by Lowess, the data was further processed through an inter-array normalization. This includes scaling and centering that allows arrays to be compared between each other. The assumption is that major variations in the distributions between arrays are due to experimental conditions and not due to the treatment differences. Scaling is done to ensure that the means of all the distributions of the data are equal. This is done by subtracting the mean log ratio of all the data on the array from each of the log ratio measurements on the array. After scaling is completed, the mean of the measurements on each array will be zero. Centering is done to ensure that the means and the standard deviations of all of the distributions of the data are equal. This is achieved by subtracting the mean measurement of the array from each of the measurements on the array and then dividing by the standard deviation. After centering, the mean of the measurements on each array will be zero and the standard deviation will be 1 (Fig 6.5 B). In the MIDAS suite, the tools that were used for scaling and centering are known as iterative log mean centering normalization and standard deviation regularization.



http://www.umanitoba.ca/afs/plant_science/psgendb/doc/MIDAS/MIDAS.pdf

Fig. 6.5 Box graph to depict Iterative Log Mean Centering and Standard Deviation regularization

The microarray data is further normalized using Iterative Log Mean Centering and Standard deviation regularization. The unequal distribution of the raw log ratios of the different arrays (1-12 on the x-axis) is shown in the box plot (A). The center of each box represents the mean value of the distribution, the size of the box represents the standard deviations of the distribution and the two horizontal lines bracketing the box represent the extreme values of the distribution. The data has been distribution normalized in (B) by iterative log mean centering and standard deviation regularization allowing the normalized arrays to be compared with one another.

Statistical Filtering: After normalizing, scaling and centering, the data was further processed using SAM (Statistical Analysis of Microarrays). The importance of SAM is that it identifies the genes that show statistically significant difference in expression between sample groups. When a large data set is being analysed, there is a possibility of genes showing a low p-value when in fact this is just an artifact. SAM performs bootstrap analyses on microarray data and is available in some microarray data analysis software programs packages as an easy to use excel plug-in. A valuable feature of SAM is that it has a good false discovery rate (FDR) algorithm. FDR is the proportion of genes that have been identified by chance as being significant. The way that SAM works is by doing an assimilation of gene specific t tests. Each gene is assigned a score on the basis of its difference in gene expression relative to the standard deviation of repeated measurements for that gene (Tusher, Tibshirani et al. 2001). Genes that have a score greater than an adjustable threshold are deemed potentially significant. SAM is an interactive algorithm since it allows users to eyeball the distribution of the test statistic and then set thresholds for significance after looking at the distribution (Fig 6.6).

The results of SAM are shown in Table 6.1. Each row represents the number of genes that are significantly changed compared to the untreated sample. So for example the first row consists of 24hr and 48hr BAFF treated cells which were compared to untreated samples of the same timepoint. The results show that at 24hr, BAFF treatment results in 104 differentially-expressed genes and at 48hr only 2 differentially-expressed genes. In contrast, AIG shows 2411, CD40L resulted in 4031 and LPS shows 4235 differentially-expressed genes. This indicates that BAFF is the weakest ligand amongst the treatments tested in the

microarray experiment. The results of BAFF treated cells also indicate that expression of genes is much higher at the 24hr timepoint than the 48hr timepoint at the culture conditions that were used for the experiment. Since gene expression is highest at 24hrs, this appears to be the most important timepoint during which BAFF effects B cells *in vitro*.

The table further shows the number of differentially-expressed genes in the treatments by the combination of ligands. Thus BAFF + CD40L gives 1980, BAFF + Anti-IgM 2822, CD40L + Anti-IgM 2647 and BAFF + CD40L + Anti-IgM 2419 differentially-expressed genes at 24 hrs. Each of the ligand treatments (except Anti-IgM) show lower number of significant genes at 48hrs, indicating that under the *in vitro* cell culture conditions, the activity of the ligands on gene expression appear to be highest at 24 hrs. In addition to calculating the number of significant genes by comparing treated versus untreated it is also possible to find the number of significant genes of one treatment versus another treatment. Thus for example, Anti-IgM, and Anti-IgM + BAFF resulted in 4921 differentially-expressed genes.

In order to compare one treatment against another, the following method is used: First, the list of genes that are significantly differentially expressed compared to control are compiled together. Next, those genes that appear in duplicate are discarded. Last but not least, the gene list is used to pull the expression data from all the different treatment conditions. So, for example, in order to compare the anti-IgM treatment with the BAFF + anti-IgM treatment, the 2411 significant genes under Anti-IgM treatment are compiled with the 2822 genes under the BAFF + Anti-IgM treatment. After deleting the duplicates which appear in both Anti-IgM and BAFF + Anti-IgM treatment, we are left with 4921 genes. The

expression data of these 4921 genes can now be pulled in order to evaluate how these genes are expressed differentially under the two different treatments. (Table 6.1)

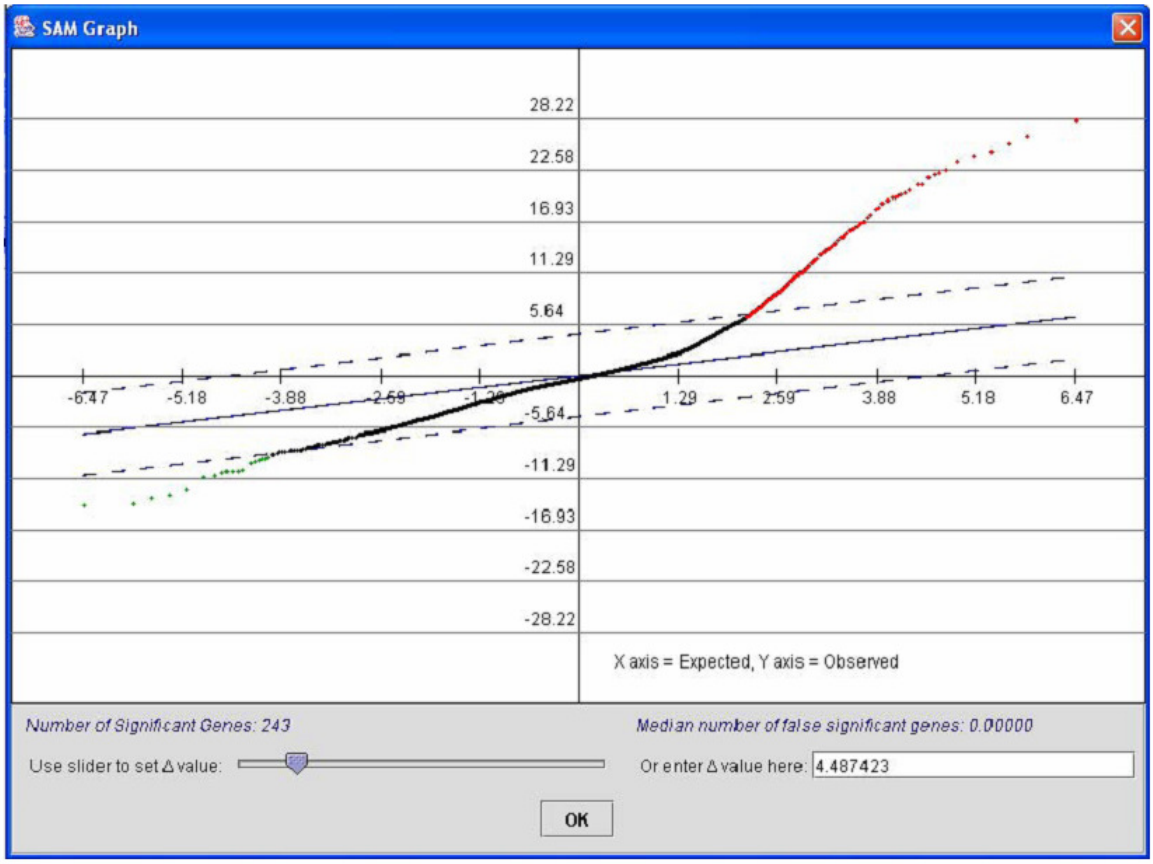


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Fig. 6.6 SAM or Significance Analysis of Microarrays

SAM is an algorithm that identifies significant genes within Microarray data and is useful because it also provides the False Discovery Rate (FDR). Since SAM is an interactive algorithm, users are allowed to set thresholds for significance through the tuning of the slider or directly entering the Δ (delta) value after looking at the distribution. As the Δ changes, the number of significant genes and the median number of false significant genes are calculated and shown. Genes beyond the blue dashed line are the significant genes, genes above the threshold are positive significant genes and those that are below are negative significant genes.

	24hr	48hr
BAFF	104	2
CD40L	4031	212
BAFF + CD40L	1980	1204
Anti-IgM	2411	2660
BAFF + Anti-IgM	2822	1855
CD40L + Anti-IgM	2647	2637
BAFF + CD40L + Anti-IgM	2419	1263
LPS	4235	1163
Anti-IgM, Anti-IgM + BAFF	4921	

Table 6.1 Results of SAM (signifance analysis of Microarray)

Table showing summary of SAM analysis with the number of differentially-expressed genes resulting from each of the ligand treatments when compared against the untreated control cultured for the same time from the microarray data analysis of purified B cells.

Clustering: After the filtering step the data were clustered based on expression pattern similarities using two different clustering methods. The central notion of cluster analysis is the degree of similarity / difference between individual objects being clustered. Clustering allows the genes to be arranged into a tree (or dendogram) so that similar profiles appear close together in the tree and dissimilar profiles appear further apart. This allows simplification of the data. In addition, groups of similar genes are revealed which can then be further studied. The two main methods of clustering that were used in our studies were hierarchical and k-means clustering.

Hierarchical clustering uses hierarchical methods to cluster the data depending on distance matrix of the spot patterns. There are four main steps in the algorithm for hierarchical clustering. The first step is to calculate the distance matrix and find the nearest entries. In the second step, the entries or genes are joined together in the tree to form a new cluster. The distance between the newly formed cluster and the other genes and clusters are then calculated. These steps are repeated until all the genes and clusters are linked. The similarity of genes' expression patterns are assessed by the Pearson correlation coefficient. A Pearson correlation coefficient indicates the relationship between two sets of numbers and indicates both how the two sets are related and the strength of that relationship. Therefore if gene A increases over time and gene B is proportional to it but shows a decrease in expression, the correlation value to show divergence is -1. In contrast, if both A and B increase proportionally over time, then their correlation will be 1. If gene A and B have absolutely no relation to each other, then their correlation is 0. The Pearson correlation coefficient is sensitive to not only direction of change, but also magnitude of change.

In order to determine the distance between the clusters, average linkage clustering is used. In average linkage clustering, the distance between two clusters is defined as the average of distances between all pairs of objects from each group. Hierarchical clustering using Pearson correlation and average linkage is the most commonly used method for microarray data analysis. Using hierarchical clustering on Anti-IgM, Anti-IgM + BAFF, the dendogram obtained is shown in Fig 6.8.

K-means clustering is used for identifying groups of genes that have similar expression profiles. The similarity between two expression profiles is defined based on the Euclidean distance between the two profile vectors. The smaller the Euclidean distance between the two expression profiles, the greater the similarity between the profiles. The clustering procedure consists of four steps. The first step involves the algorithm randomly selecting *K* profile vectors as the initial seeds. *K* is the number of clusters (i.e., gene groups) that is determined by the user. Step 2 of the algorithm assigns each gene expression profile to the closest seed based on Euclidean distance. So genes that are assigned to the same seed will belong to the same cluster. Step 3 calculates the centroid of each cluster with the expression profiles of the genes in the cluster. The final step includes the algorithm updating the positions of the seeds with the cluster centroids and repeating Steps 2 and 3 until the algorithm converges (i.e., the seeds no longer move and the gene assignments no longer change) (MacQueen, J.B., et al. 1967).

The results of both hierarchical and k-means clustering have to be validated. There are different ways to validate the results from the clustering. These include visually determining whether the genes in the same cluster have similar expression profiles, or by

determining whether the genes in the same cluster have the same or complementary biological functions. Both hierarchical clustering and k-means clustering was performed on 'Untreated versus BAFF' and 'Anti-IgM versus BAFF + Anti-IgM' treatments. By visual examination of the dendrograms and by looking at their expression levels, several clusters were suggested. The hierarchical clustering of 'Untreated versus BAFF' yielded 21 clusters and that of 'Anti-IgM versus BAFF + Anti-IgM' yielded 71 clusters. The K-means clustering of Untreated versus BAFF was set to give 20 clusters

CLASSIFI: After clustering the data, it was run through a bioinformatics tool known as CLASSIFI (Cluster Assignment for Biological Inference) (Lee, Sinkovits et al. 2006) (Fig. 6.2). CLASSIFI calculates the biological significance of the co-clustering of genes that have similar biological properties using Gene Ontology (GO) annotation. This is based on the idea that genes involved in the same biological process are often expressed coordinately. The Gene Ontology Consortium is an effort to unify the description or annotation of gene products based on three basic categories (ontologies) - cellular component, molecular function or biological process. Cellular component describes the anatomical structure such as rough endoplasmic reticulum or nucleus. Biological process terms in the broader sense include cellular physiological process or signal transduction while the more specific terms include terms like pyrimidine metabolic process. Molecular functions at the broader level describe activities such as binding activity or catalytic activity that occur at the molecular level while more narrow terms include terms like adenylate cyclase activity or Toll receptor binding. The ontologies have been assembled in the form of directed acyclic graphs of parent-child relationships where one GO term may have multiple parents. For example, the cellular component of a gene may be described as nucleolus, which in turn is a component of the nucleus. Each GO term has a unique numerical identifier and a term name. Thus, each gene that is well characterized will have multiple ontologies associated with it that describe its location, biological role and biochemical function within the cells. CLASSIFI uses the Gene Ontology annotation to define the functional properties of all the genes/probes in the microarray data set. A cumulative hypergeometric distribution analysis is then applied to

determine whether the probability of the co-clustering of a particular ontology has occurred by chance.

Calculating this hypergeometric distribution p-value involves several steps (Fig 6.2). The first step is to upload the input file (Table 6.2), which consists of the probe list with gene cluster assignments. CLASSIFI then queries the database and captures the primary GO annotations for every probe in the dataset. CLASSIFI then further queries the database to capture complete GO ancestry for every primary GO annotation for each probe. Next CLASSIFI enumerates n, f, g, and c for each gene ontology in each gene cluster - n = number of probes with associated GO ID in the gene cluster, f = number of probes with the associated GO ID in the data set, g = number of probes in the data set, and c = number of probes in the cluster. Using these values, the solution of the hypergeometric equation is calculated. Finally, CLASSIFI orders ontologies based on p values.

The function of CLASSIFI is to allow specific biological functions to be assigned to the different clusters depending on whether the probability of the co-clustering of genes with that specific biological function is below a threshold value. The threshold value was determined using a Bonnferoni correction which gives a reasonable estimate for most datasets. The significance cutoff is determined by dividing 0.05 with the number of datapoints used in the calculations (the number of hypotheses tested), which is equivalent to the number of rows in the data set.

The CLASSIFI interface is shown in figure 6.7. The input file for CLASSIFI is generated following microarray data clustering and consists of 3 columns, one with the probe IDs, one with the gene names and the third one with cluster IDs (Table 6.2). Once the input file is uploaded into CLASSIFI, and the query is submitted, the results appear shortly depending on how big the file is. There are three output files generated by CLASSIFI which are the go file, output file and top file. The go file consists of all the primary GO annotations of each of the genes in the clusters as well as the GO parentage associated with these primary annotations. This file is useful in understanding which probes/genes are responsible for the gene cluster classification. The output file consists of all the enumerated variables that are used for the hypergeometric equation including the total number of probes, the total number of probes with the associated GO ID, the number of probes in the cluster, and the number of probes with the associated GO ID in the cluster (Table 6.4). The GO IDs for each gene cluster are ranked from lowest to highest. The GO type representing the Gene Ontology type, which could be either Molecular Function, Biological Process or Cellular Component (location), are shown. The third file which is known as the top file is a list of the GO IDs that give the lowest p value in each of the gene clusters (Table. 6.3).

There are two aspects to the importance and usefulness of using CLASSIFI. Microarray data consists of both experimental and biological noise, which could result in obscuring cluster classifications. These limitations are dealt with by CLASSIFI since it uses statistical methods to identify the significant clustering of the genes. In addition, CLASSIFI functions as a hypothesis generating tool. Using the gene annotations for biological function or

location, hypotheses about the physiology of the cluster can be deduced. These hypotheses must then be validated using further studies.

The CLASSIFI method has been explained in my thesis in a user friendly manner so that anyone wanting to use this tool can get a quick basic overview. CLASSIFI determines statistical significance of the co-clustering of genes, thereby giving biological meaning to the data.

Other web based tools for analyzing microarray data using the GO ontology consortium include:

- EASE: This tool rapidly calculates over-representation statistics for every possible Gene Ontology term with respect to all genes represented in the data set. Although each term used for the calculation is defined, the process of the calculation itself has not been defined leaving a 'black box' for the user.
- FunCluster: Functional analysis of the gene expression data by detecting co-regulated biological processes through a co-clustering procedure involving biological annotations and gene expression data. It is a standalone R package. This analysis tool does not involve statistical analysis.
- GENECODIS: web based tool which co-clusters genes according to their co-occurrence in different sources of information such as GO, KEGG, SwissProt. This tool is used to determine annotations that are significantly associated to a list of genes under study with respect to a reference list. In order for this tool to be used, a reference list is needed which means that some pre-conceived ideas about the genes is required.

- GoHyperGAll: This uses the R/BioC function to computes the p values using a hypergeometric distribution test for all GO nodes for a sample of genes with overrepresentation of certain GO terms. Basic knowledge about R and BioConductor is necessary to use this tool.
- GOrilla: Web based tool that identifies enriched GO terms in ranked list of genes or search for enriched GO terms in a target list of genes compared to a background list of genes. This tool requires having some preconceived ideas about the genes.
- GOTM: web based tool for the analysis and visualization of sets of interesting genes based on Gene Ontology hierarchies. Inputs and outputs are not very user friendly. The calculations used for statistical analysis have not been shown.

  These tools are similar to CLASSIFI but either require some basic knowledge of the programming, some preconceived idea about the results of the data or do not explain the process of calculation for the statistics.

Fig. 6.7 CLASSIFI The CLASSIFI interface is shown. Data files can be uploaded as input files containing probe ids', gene names and cluster IDs.
http://pathcuric1.swmed.edu/pathdb/classifi.html

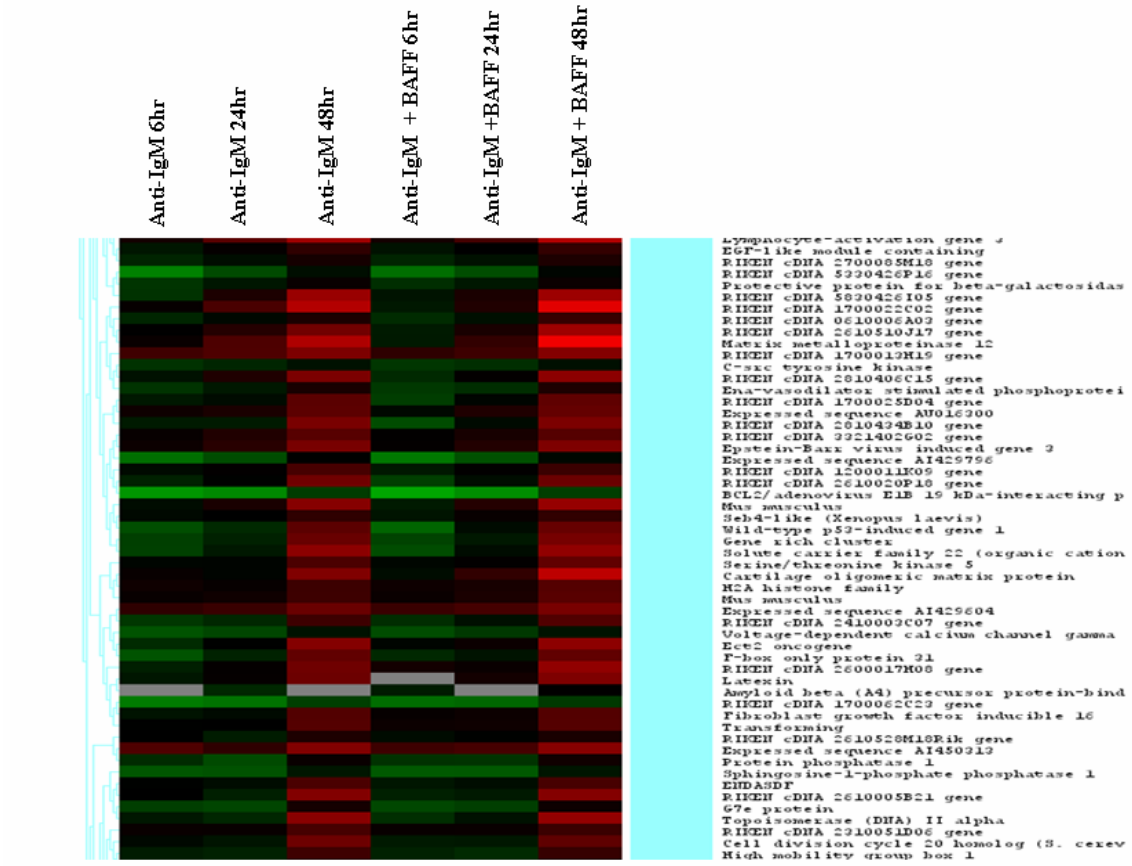

Fig.6.8 Excerpt from expression results from genes in the "Cell division" cluster in the Anti-IgM versus Anti-IgM + BAFF microarray results

Results from the Microarray data analysis pipeline have yielded a cluster, which has been characterized with the term "Cell division" by the bioinformatics tool, CLASSIFI. In this cluster, both Anti-IgM alone and Anti-IgM + BAFF show upregulation of the genes at the 48hr timepoint with some of the genes showing slightly higher levels of expression in the Anti-IgM + BAFF cluster. An higher expression in comparison with untreated controls is shown by red, a lower expression is shown by green, and no difference in expression by

black. Part of the graphical result of the dendogram (blue) is shown.

AK010453	RIKEN cDNA 2410008J01 gene	1
AK012634	GrpE-like 2	1
NM_019408	Nuclear factor of kappa light polypeptid	1
NM_010296	GLI-Kruppel family member GLI	1
AK005084	NADH dehydrogenase (ubiquinone) 1 alpha	1
M94350	Immunoglobulin lambda chain	1
AK010426	RIKEN cDNA 2410006H10 gene	1
NM_019976	Differential display and activated by p5	1
NM_026428	RIKEN cDNA 1810027P18 gene	1
AK004661	RIKEN cDNA 1200009B18 gene	10
AK020046	RIKEN cDNA 6030404E16 gene	10
NM_030675	Cerebral cavernous malformations 1	10
NM_008091	GATA binding protein 3	10
BC004678	Expressed sequence AW123240	10
AK002411	RIKEN cDNA 0610009J05 gene	10
BC007177	Cyclin L	10

Table 6.2 CLASSIFI input file

CLASSIFI input file is composed of the probe ID, the gene name and the cluster ID (left to right). This file can be directly uploaded into the CLASSIFI web interface and is processed within minutes. A subset of rows for two of the clusters in the input files is shown as an example.

GO id	g	f	c	n	Expt	Prob	GO type	GO name	Cluster ID
GO:0051301	4921	58	306	24	3.607	8.66E-15	biological_process	cell division	37
GO:0006260	4921	43	125	13	1.092	1.88E-11	biological_process	DNA replication	60
GO:0005840	4921	53	24	5	0.258	4.35E-06	cellular_component	Ribosome	5
GO:0005830	4921	17	35	4	0.121	4.78E-06	cellular_component	cytosolic ribosome (sensu Eukaryota)	6
GO:0006091	4921	91	30	6	0.555	1.41E-05	biological_process	generation of precursor metabolites	3
GO:0016229	4921	2	41	2	0.017	6.77E-05	molecular_function	steroid dehydrogenase activity	13
GO:0009200	4921	2	45	2	0.018	8.18E-05	biological_process	deoxyribonucleoside triphosphate	56

Table 6.3 Excerpt of the top file from CLASSIFI analysis of Anti-IgM vs. BAFF + Anti-IgM microarray results

Manual analysis of the hierarchical clustering of BAFF versus BAFF + Anti-IgM microarray data resulted in 71 clusters. CLASSIFI Topfile lists the GO ids giving the lowest p value in each gene cluster. Out of a total of 71 clusters, 7 clusters with the lowest p value are shown. The four topmost clusters have the lowest p value and are below the bonnferonni threshold. GO ID = a unique Gene Ontology identifier that corresponds to a defined molecular function, biological process, or cellular component, g = number of probes in the data set, f = number of probes with the associated GO ID in the data set, c = number of probes in the cluster, n = number of probes with associated GO ID in the gene cluster. Expt = Expected number of probes of a given GO ID in a given cluster of size n based on random distribution. Prob = the probability that the GO ID co-clustering pattern has occurred by chance. Only those clusters with a Bonferroni-corrected significant or marginal p-value for a specific GO term are listed.

GO id	g	f	c	n	expt	prob	GO type	GO name
GO:0051301	4921	58	306	24	3.607	8.66E-15	biological_process	cell division
GO:0000087	4921	35	306	17	2.176	3.29E-12	biological_process	M phase of mitotic cell cycle
GO:0007067	4921	35	306	17	2.176	3.29E-12	biological_process	mitosis
GO:0000279	4921	44	306	18	2.736	2.78E-11	biological_process	M phase
GO:0000278	4921	53	306	18	3.296	1.04E-09	biological_process	mitotic cell cycle
GO:0007049	4921	157	306	32	9.763	1.06E-09	biological_process	cell cycle
GO:0005694	4921	63	306	18	3.917	2.35E-08	cellular_component	chromosome
GO:0000775	4921	12	306	8	0.746	8.13E-08	cellular_component	chromosome, pericentric region
GO:0043228	4921	235	306	33	14.613	6.10E-06	cellular_component	non-membrane-bound organelle
GO:0043232	4921	235	306	33	14.613	6.10E-06	cellular_component	intracellular non-membrane-bound
GO:0005515	4921	665	306	67	41.351	2.22E-05	molecular_function	protein binding
GO:0000793	4921	8	306	5	0.497	4.32E-05	cellular_component	condensed chromosome

Table 6.4 Excerpt of CLASSIFI output file for cluster 37 of Anti-IgM versus BAFF + Anti-IgM microarray results

CLASSIFI output file lists the resulting p values for every GO term in each gene cluster. Cluster 37 from Anti-IgM versus BAFF + anti-IgM microarray data analysis is shown here. It has the GO annotation of “Cell division”. GO ID = a unique Gene Ontology identifier that corresponds to a defined molecular function, biological process, or cellular component, g = number of probes in the data set, f = number of probes with the associated GO ID in the data set, c = number of probes in the cluster, n = number of probes with associated GO ID in the gene cluster. Expt = Expected number of probes of a given GO ID in a given cluster of size n. Prob = the probability that the GO ID co-clustering pattern has occurred by chance.

Results:

BAFF in the presence of anti-IgM causes greater expression of genes involved in cell division and DNA replication compared to anti-IgM alone.

Once the clustered data was processed through CLASSIFI, several interesting cluster classifications came up. In the Anti-IgM versus BAFF + Anti-IgM dendogram, there were 71 clusters designated by visual examination. The threshold p-value cutoff by the Bonnferonni correction for the 4921 genes would be 0.05/4921, which equals 1.016×10^{-5}. Only 4 out of the 71 clusters had a p value less than this cutoff. Two of the clusters have GO annotations of 'Ribosome' and 'Cytosolic ribosome' (Table 6.3). These are organelles that are involved in protein translation, which are well-characterized physiological processes within the cells, and may not present anything novel. The other two clusters have the CLASSIFI designations of 'DNA replication' and 'cell division' (Table 6.3). These may be interesting since our *in vitro* studies have shown BAFF to be involved in proliferation. Cluster 37 which was annotated as the Cell division cluster showed a very low p value of 8.66 E -15 (Table 6.3). The next lowest p value of 1.88E-11 was shown by cluster 60 which has the GO name of DNA replication. By carefully looking at the dendogram of cluster 37 and 60, it can be seen there is higher expression of the genes under the treatment of both anti-IgM and BAFF + anti-IgM (Fig 6.8).

CLASSIFI is an important hypothesis-generating tool since it links cell physiology, including function and location, with the genes in the cluster. Thus the hypothesis that can

be generated by the low p-values of these two clusters is that anti-IgM induces B cell proliferation by both cell division and DNA replication. Some genes that are present in the clusters include Cyclin D3, Cyclin A2, Cyclin G2, Cyclin B2, Cell division cycle 25 homolog (Table 6.5). Cyclins control the progression of cells through the cell cycle by regulation of cyclin dependent kinases. Our *in vitro* studies have shown BAFF to enhance anti-IgM induced proliferation. Since the upregulated genes are also present in the BAFF + anti-IgM treatment, it may be interesting to study the function of these proteins in addition to others present in the clusters in order to understand how BAFF may be involved in anti-IgM induced cell division and DNA replication in B cells.

	GO ID	Probe ID	Gene Name	Cluster ID	P Value
A	GO:0051301	NM_009773_NCBI_refseq	Budding uninhibited by	37	8.66E-15
	GO:0051301	NM_028232_NCBI_refseq	RIKEN cDNA 3300001M08 gene	37	8.66E-15
	GO:0051301	NM_007632_NCBI_refseq	Cyclin D3	37	8.66E-15
	GO:0051301	NM_026410_NCBI_refseq	RIKEN cDNA 2610036L13 gene	37	8.66E-15
	GO:0051301	NM_023117_NCBI_refseq	Cell division cycle 25 homolog B (S. cer	37	8.66E-15
	GO:0051301	NM_019771_NCBI_refseq	Destrin	37	8.66E-15
	GO:0051301	NM_009828_NCBI_refseq	Cyclin A2	37	8.66E-15
	GO:0051301	NM_012025_NCBI_refseq	Rac GTPase-activating protein 1	37	8.66E-15
	GO:0051301	NM_017461_NCBI_refseq	Septin 1	37	8.66E-15
	GO:0051301	NM_017464_NCBI_refseq	Neural precursor cell expressed	37	8.66E-15
	GO:0051301	NM_007635_NCBI_refseq	Cyclin G2	37	8.66E-15
	GO:0051301	NM_007630_NCBI_refseq	Cyclin B2	37	8.66E-15
	GO:0000087	NM_009773_NCBI_refseq	Budding uninhibited by	37	3.29E-12
B	GO:0000087	NM_028232_NCBI_refseq	RIKEN cDNA 3300001M08 gene	37	3.29E-12
	GO:0000087	NM_026410_NCBI_refseq	RIKEN cDNA 2610036L13 gene	37	3.29E-12
	GO:0000087	NM_023117_NCBI_refseq	Cell division cycle 25 homolog B (S. cer	37	3.29E-12
	GO:0000087	NM_009828_NCBI_refseq	Cyclin A2	37	3.29E-12
	GO:0000087	NM_017464_NCBI_refseq	Neural precursor cell expressed	37	3.29E-12
	GO:0000087	NM_007635_NCBI_refseq	Cyclin G2	37	3.29E-12
	GO:0000087	NM_009791_NCBI_refseq	Calmodulin binding protein 1	37	3.29E-12
	GO:0000087	NM_007630_NCBI_refseq	Cyclin B2	37	3.29E-12
	GO:0000087	NM_010892_NCBI_refseq	NIMA (never in mitosis gene a)-related	37	3.29E-12

Table 6.5 Genes that are associated with "cell division" and "Mitotic cell cycle" related GO terms

List of genes detected using specific cDNA probes found in Gene Cluster # 37 that are associated with the term "cell division" (GO:0061801) and "M phase of Mitotic Cell Cycle" (GO 0000087).

BAFF has very subtle effects on B cells:

CLASSIFI analysis of BAFF versus Untreated shows very few clusters with significant p values (Table 6.6). Hierarchical clustering revealed 21 clusters by visual examination. The Bonnferonni correction for this data set was (0.05/1025) or 0.48 e-5 and only 3 clusters show p values below this Bonnferoni correction. These clusters have the classification of "cytosolic ribosome"; "membrane" and "response to stimulus." These classifications are very broad terms in general and are therefore difficult to study. Although Cluster #3 has a p-value slightly above the Bonnferonni Correction, it is an interesting cluster to examine because of the CLASSIFI designation of "receptor activity" (Table 6.6). The expression of the genes in the Receptor activity cluster show lower expression of the genes in relation to untreated controls (Fig 6.9). There are several interesting genes that are detected using specific cDNA probes associated with GO terms that are related to signaling (Table 6.7). These include interleukin 10 receptor, nuclear receptor subfamily 3, G protein coupled receptor 35 or GPR 35, Thioredoxin interacting protein and others. IL-10 is important in the regulation of the expression of BAFF. Thioredoxin interacting protein (TXNIP) is a novel proapoptotic beta-cell gene that is also found to be expressed in T cells. This is known to be pro-apoptotic although its function in B cells is not known. Nuclear receptors are a family of receptors that can directly bind to DNA and direct the expression of adjacent genes. Prostaglandin E receptor 4 (subtype EP4) acts in Langerhans cells by increasing their costimulatory molecules and enhancing their ability to stimulate T cells in vitro. G protein coupled receptor 35 or GPR 35 is important in kynurenine pathway which elicits calcium

mobilization. All these proteins could be interesting to study in order to understand more about signaling by BAFF in B cells.

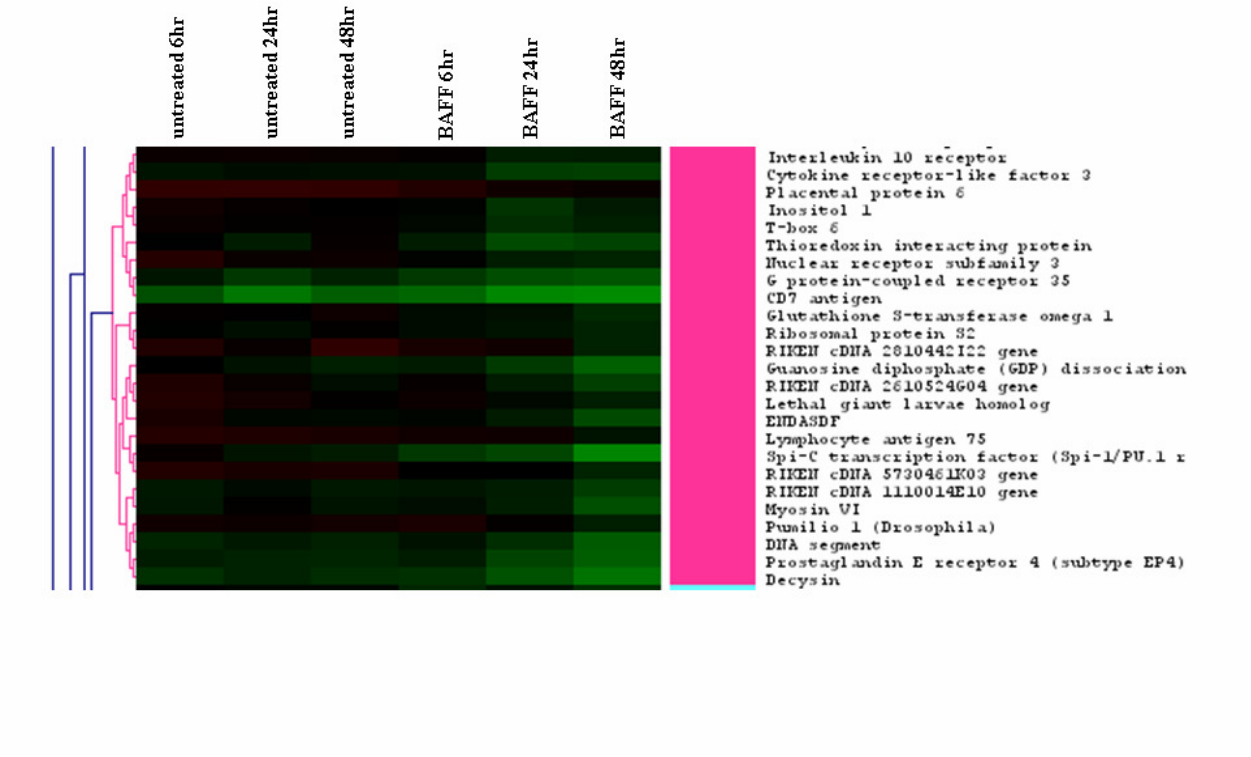

Figure 6.9 Expression of genes in the "Receptor activity" cluster in the Untreated versus BAFF dendogram

Results from the Microarray data analysis pipeline has yielded a cluster which has been termed "Receptor activity" by the bioinformatics tool, CLASSIFI. Treatment with BAFF results in downregulation of the genes at 24hr and 48hr compared to untreated. An increase in expression is shown by red, a decrease is shown by green, and no change in expression by black. The dendogram (pink) is the graphical result of the hierarchical clustering.

GO id	g	f	c	n	Expt	Prob	GO type	GO name	Cluster ID
GO:0005830	1025	10	97	7	0.946	5.24E-06	cellular_component	cytosolic ribosome (sensu	2
GO:0016020	1025	178	19	12	3.3	8.79E-06	cellular_component	membrane	16
GO:0050896	1025	65	42	11	2.663	2.57E-05	biological_process	response to stimulus	15
GO:0004872	1025	54	25	7	1.317	1.75E-04	molecular_function	receptor activity	3
GO:0007186	1025	18	11	3	0.193	6.89E-04	biological_process	G-protein coupled receptor protein	17
GO:0043234	1025	74	89	15	6.425	9.72E-04	cellular_component	protein complex	14
GO:0042423	1025	2	42	2	0.082	1.64E-03	biological_process	catecholamine biosynthesis	10
GO:0005740	1025	11	97	5	1.041	2.00E-03	cellular_component	mitochondrial envelope	19
GO:0003677	1025	65	57	10	3.615	2.08E-03	molecular_function	DNA binding	7
GO:0030135	1025	5	66	3	0.322	2.33E-03	cellular_component	coated vesicle	9
GO:0006281	1025	2	58	2	0.113	3.15E-03	biological_process	DNA repair	21

Table 6.6 Excerpt of Topfile from CLASSIFI analysis of BAFF versus Untreated microarray results

Topfile lists the GO ids with the lowest probability for all the gene clusters in the BAFF versus Untreated microarray data. The top 3 represent the clusters with p values below the Bonnferonni correction. GO ID = a unique Gene Ontology identifier that corresponds to a defined molecular function, biological process, or cellular component, g = number of probes in the data set, f = number of probes with the associated GO ID in the data set, c = number of probes in the cluster, n = number of probes with associated GO ID in the gene cluster. Expt = Expected number of probes of a given GO ID in a given cluster of size n based on random distribution. Prob = the probability that the GO ID co-clustering pattern has occurred by chance.

GO:0005515	NM_008348_NCBI_refseq	Interleukin 10 receptor	3	1.75E-04
GO:0005515	NM_023719_NCBI_refseq	Thioredoxin interacting protein	3	1.75E-04
GO:0005515	NM_008173_NCBI_refseq	Nuclear receptor subfamily 3	3	1.75E-04
GO:0007165	NM_008348_NCBI_refseq	Interleukin 10 receptor	3	1.75E-04
GO:0007165	NM_008173_NCBI_refseq	Nuclear receptor subfamily 3	3	1.75E-04
GO:0007165	NM_022320_NCBI_refseq	G protein-coupled receptor 35	3	1.75E-04
GO:0007165	NM_008965_NCBI_refseq	Prostaglandin E receptor 4 (subtype EP4)	3	1.75E-04
GO:0007154	NM_008348_NCBI_refseq	Interleukin 10 receptor	3	1.75E-04
GO:0007154	NM_008173_NCBI_refseq	Nuclear receptor subfamily 3	3	1.75E-04
GO:0007154	NM_022320_NCBI_refseq	G protein-coupled receptor 35	3	1.75E-04
GO:0007154	NM_008965_NCBI_refseq	Prostaglandin E receptor 4 (subtype EP4)	3	1.75E-04

Table 6.7 Genes that are associated with GO terms related to receptor activity

List of genes detected using specific cDNA probes found in Gene Cluster # 3 in BAFF versus Untreated microarray analysis. The GO terms are associated with the term “protein binding” (GO:0005515), “signal transduction” (GO 0007165), “cell communication”(GO 0007154) and “cellular process”(0009987).

Genes that are involved in anergy are upregulated by BAFF treatment

Our *in vitro* studies have shown that BAFF enhances the development of anergic B cells. In order to evaluate whether stimulation by BAFF has any effect on the upregulation of genes that have been found to be involved in anergy we looked at the expression profile of these previously characterized genes.

There are 7 genes that were reported to be upregulated in anergic B cells: Egr1 (early growth response 1), Egr2 (early growth response 2), Nab2 (Ngfi-A binding protein 2), Pcp4 (Purkinje cell protein 4), Cd72, Crisp3 (cysteine-rich secretory protein 3 and Nrgn (neurogranin) (Glynne, Ghandour et al. 2000). Nrg, Pcp4 and Cd72 are inhibitors of B cell signaling, Nab2 acts as a negative regulator by repressing many genes and Egr 1 and Egr2 are pro-mitotic genes. Out of these 7 genes, only 4 were present in the spotted microarray that were used in our studies. These genes did not appear in the list of differentially expressed genes resulting from SAM analysis indicating that the effect of BAFF on these genes was either very subtle or was obscured by sample variability. Examination of these genes before SAM-based filtering showed higher expression of Egr1, Nab2 and CD72 under treatment with BAFF for 48hrs when compared to untreated controls (Fig. 6.10). Unfortunately, these differences did not reach statistical significance due to the high variability observed in these samples.

Therefore, in order to confirm the trends in expression of these genes observed in the microarray data, real time PCR was performed on RNA extracted from purified B cells that were cultured with or without BAFF for 16hrs and 24hrs. All three genes showed

upregulation with BAFF treatment at 24 hrs (Fig. 6.11). Because of the detrimental effects of cell culture on the cells, these expression studies were not done beyond 24hrs of culture. Upregulation of these genes in the presence of BAFF suggests that BAFF may be enhancing anergy in B cells through effects on the expression of these anergy-associated genes.

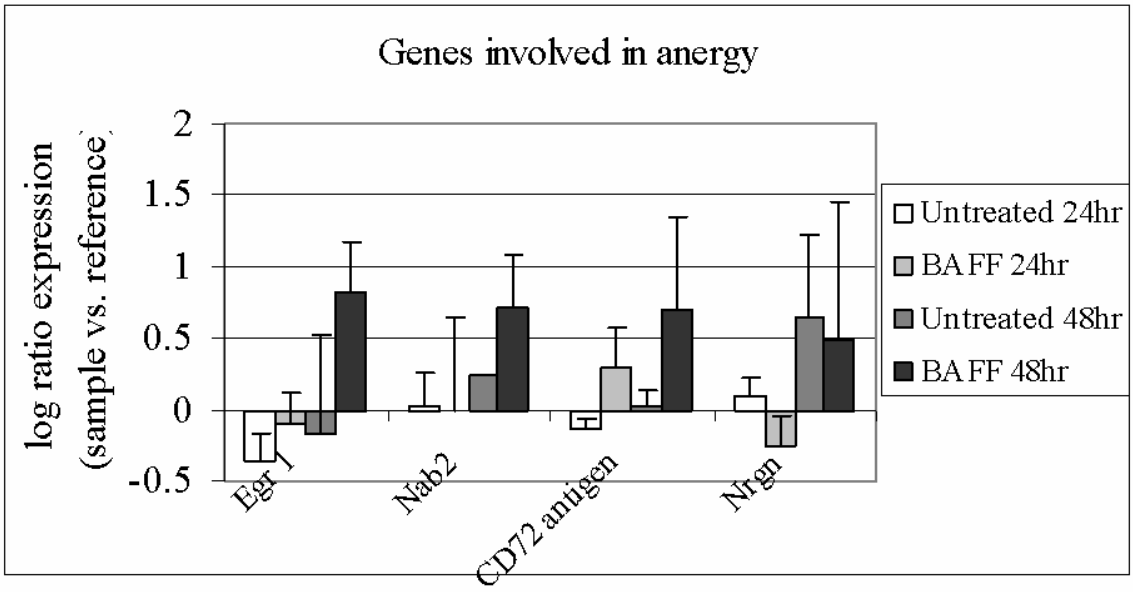

Figure 6.10 Microarray data show increased expression of genes involved in anergy by BAFF treatment

Expression of anergic genes in microarray data of purified B cells cultured in media alone or in media + BAFF for 24hr and 48hr is shown. Egr1, Nab2 and CD72 are upregulated in response to BAFF treatment by 48hrs. Log ratio expression of sample versus reference is shown. Error bars indicate SD, n=3

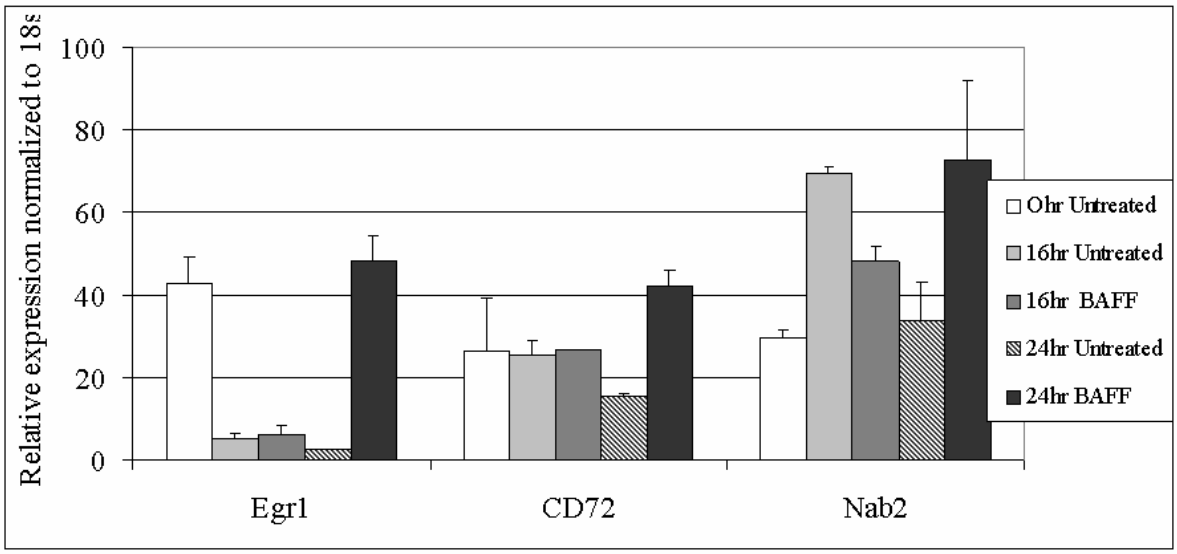

Figure 6.11. Real time PCR confirms Microarray data

Real time RT PCR was performed to evaluate the expression of 3 anergy related genes that were seen to be upregulated by BAFF in the Microarray data. The genes showed upregulation by 24hrs with BAFF treatment. The data was normalized to 18s RNA as a reference. Error bars indicate SD, n=3

Summary

Our data showed that BAFF has very subtle effects on gene expression. A similar finding was reported in the single-ligand screen performed by the AFCS (Alliance for Cellular Signaling), which showed that amongst 32 ligands, BAFF-stimulated samples had one of the lowest number of genes affected. In contrast, CD40L and anti-IgM had the highest number of genes affected. That this is repeated in our studies confirms the validity of our data. One way of increasing the statistical power and thereby the number of differentially expressed genes would be by increasing the number of replicates so that even genes that have very subtle changes can be picked up. The BAFF + anti-IgM versus anti-IgM clustering shows interesting CLASSIFI annotations such as “cell division” and “DNA replication” that could yield the important role of BAFF regulated genes involved in the enhancement of anti-IgM induced proliferation. Microarray and QPCR analysis of untreated versus BAFF-treated B cells have also revealed that anergic genes are upregulated in response to BAFF, which provide a potential mechanism in support of our *in vitro* data.

Chapter 7

Discussion

Biological functions of BAFF

One of the most studied functional properties of BAFF is its effect on the survival of B cells. This is a very important aspect of the effect of BAFF on B cell biology, since an increase in survival has a direct effect on B cell homeostasis. Our studies show that both the commercial and the lab generated BAFF have a direct survival effect on B cells (Fig. 2.2, Fig. 2.3, Fig 3.4). Work by various groups have also shown an increase in survival by B cells (Batten, Groom et al. 2000; Do, Hatada et al. 2000; Rolink, Tschopp et al. 2002). Although most studies report the survival effect of BAFF mainly on mature and T2 cells, both in vitro and in vivo (Batten, Groom et al. 2000) our studies show that BAFF increases survival of each of the differentiation stages of B cells (Fig 3.6). This finding has also been reported by another group which showed that each of the five consecutive stages of B-cell development, including immature B cells in the bone marrow, as well as the T1 and T2 cells and mature B cells in the spleen, and the activated cells derived from the mature B cells upon stimulation, are all directly targeted and acted upon by BAFF {unpublished data discussed in (Rolink and Melchers 2002).

Intraperitoneal injections of 10μg of BAFF per mouse, also showed an increase in total B cell numbers (Fig. 5.5), similar to what has been reported by others (Moore, Belvedere et al. 1999). Other studies that indicated the crucial role of BAFF for B cell survival include studies on BAFF-deficient mice. These mice exhibited a reduced number of B cells (Gross, Dillon et al. 2001; Schiemann, Gommerman et al. 2001; Thompson, Bixler et al. 2001). Similar results were obtained with injection of soluble decoy receptors of BAFF,

such as BCMA-Ig and TACI-Ig. The results from these mice also showed reduced number of peripheral B cells (Gross, Dillon et al. 2001; Pelletier, Thompson et al. 2003). In addition, BAFF transgenic mice, which overexpress BAFF, have a dramatically increased number of B cells (Mackay, Woodcock et al. 1999; Khare, Sarosi et al. 2000). Thus, our studies, together with various others, demonstrate the crucial role of BAFF in B cell survival.

We also investigated the effect of BAFF on B cell proliferation. Although BAFF by itself did not induce any proliferation, there was a significant increase in anti-IgM mediated proliferation upon BAFF stimulation (Fig. 2.5). These results indicated that BAFF costimulated BCR induced B cell proliferation. Work by others has also shown BAFF to be incapable of stimulating B cell growth by itself, but costimulating anti-IgM induced growth (Schneider, MacKay et al. 1999). Furthermore, our BrdU incorporation studies have shown that BAFF in the presence of BCR cross-linking promotes DNA replication in each of the different populations of B cells. BAFF by itself also showed a small but significant increase in the total number of cells in each of the different populations, but this appears to be primarily due to increased survival of the cells (Fig. 2.11).

The first clue on the effect of BAFF on B cell differentiation came from BAFF-deficient mice which showed a severe block in B cell development from the splenic T1 stage onwards. The immature B cells in the bone marrow as well as the development of B1 cells appeared to have remained unaffected (Gross, Dillon et al. 2001; Schiemann, Gommerman et al. 2001). However, re-evaluation of these mice suggested that there is no real blockade in B cell development since B cells with high IgD, which is indicative of mature and T2 cells, was found. These cells have reduced CD21/35 and CD23 differentiation markers which is why

they were harder to detect. In addition, these cells were still capable of mounting an antigen specific antibody response and could form germinal centers (Gorelik, Cutler et al. 2004). One study has also reported the importance of BAFF in the differentiation of MZ B cells (Schneider, Takatsuka et al. 2001). It has been reported that BAFF up-regulates Pax5 and its downstream target CD19 which is a major component of the B cell co-receptor complex and is therefore important for B cell differentiation (Hase, Kanno et al. 2004). Our survival studies have also shown a decrease in the percentage of MZ cells in the anti-IgM and the BAFF + anti-IgM treated cultures indicating that there may be some level of differentiation (Fig 3.8 iii and Fig 3.9 iii). This needs to be resolved in the future by sorting the MZ population and culturing with anti-IgM in order to compare with anti-IgM + BAFF treatment. Any change in the expression markers showing a more mature phenotype would indicate differentiation.

Our *in vitro* studies have shown that BAFF promotes an increase in T3/An1 cells which may be linked to differentiation (Fig. 4.3 and Fig. 4.4). T3 cells have previously been described as a developmental intermediate between T2 and mature B cells (Allman, Lindsley et al. 2001). However, these cells have very recently been shown to be anergic B cells present within the normal wild-type B cell pool (Merrell, Benschop et al. 2006). The An1 cells generated by BAFF treatment of splenic B cells *in vitro* show reduced calcium flux, indicating their true anergic nature (Fig. 4.5). These cells were seen to arise primarily from T2 and mature B cells. In addition, mice injected peritoneally with BAFF, had a significantly higher total number of AA4.1 hi cells, which include the An1 cells (Fig. 5.6 A and B). The total number of An1 cells also showed a general increase in the BAFF

treated mice (Fig. 5.6 C and D). Once the calcium flux of the B cells from the BAFF injected mice were measured, it was seen that these cells also have reduced ability to flux calcium (Fig. 5.7). This indicated that B cells from the BAFF injected mice have become anergic in nature. Thus the results from both our *in vitro* and *in vivo* data support the proposal that BAFF enhances the An1 population. At this point we are unable to decipher whether this increase in An1 cells is due to differentiation of the T2 and mature B cells or whether it is simply an increase in survival by BAFF. However, regardless of the mechanism of this increase in An1 cells, this novel finding has important implications in finding a missing link between BAFF and autoimmunity.

The fluorochromes that were used with the antibodies to identify the An1 cells included APC-Cy7 conjugated to CD19 and APC conjugated to AA4.1. APC-Cy7 is a tandem dye that functions by the transfer of energy from APC to Cy7 by FRET (Fluorescence Resonance Energy Transfer) resulting in a longer wavelength emission. However, APC-Cy7 can degrade in the presence of light, fixation and elevated temperatures into the parent components, APC and Cy7 and emit at the wavelength specific for APC resulting in false positives. Thus, the data that was generated using CD19-APC-Cy7 and AA4.1-APC could result in the samples showing higher percentage of AA4.1 + cells due to leak of fluorescence from the CD19-APC-Cy7 degraded products.

We have additional data to support that there is higher proportion of AA4.1 + cells in the presence of BAFF compared to untreated cultures using different staining reagents. One set of experiments were done by staining with anti-CD24-PE, anti-IgD-PerCP, and anti-AA4.1-APC and the results showed about a 2 fold higher proportion of the AA4.1 hi

cells in the BAFF treatment compared to the untreated samples. This was seen in 2 separate experiments. Another staining group which included the above reagents in addition to CFSE also showed similar results. Other staining reagents used have all shown an increase in the proportion of AA4.1hi cells by BAFF treatment, although the increase seen was more subtle. These results indicate that BAFF does have a true effect on AA4.1 hi cells which include the An1 cells. Although, the AA4.1hi cells also include the T1 and the T2, the fact that BAFF does enhance the An1 population can be further determined by the calcium flux experiments (Fig 4.5, 5.7) which showed an induction in unresponsiveness even in the mature B cells. This indicates that BAFF truly initiates anergy in B cells.

The observation that BAFF promotes survival and increases the proliferating pool of cells stimulated with anti-IgM and yet also impairs BCR responses, may seem contradictory. However, BAFF may be promoting the increase in the An1 cells and impairing BCR responses only when present in pathologically increased amounts as a feedback mechanism to dampen BCR signaling. In physiologically appropriate levels, BAFF may be functioning as the positive regulator of B cells by promoting survival and modest effects of activation and proliferation in the presence of BCR signaling. We could also speculate that BAFF may have stronger dampening or anergic responses at later timepoints, after 72hrs incubation, indicating that a longer presence of BAFF may be more favorable for anergy.

BAFF and BCR

Most peripheral B cells die after BCR is knocked out regardless of BAFF signaling. This indicates the importance of the BCR in the survival of resting B cells even in the absence of antigen as well as the need for tonic BCR signaling (Lam, Kuhn et al. 1997). On the other hand, if there is not enough BAFF, such as in the BAFF knockouts, there is impaired B cell maturation and reduced total number of cells (Schiemann, Gommerman et al. 2001). Our studies have shown that BAFF increases survival of B cells both in the absence and presence of BCR signaling (Fig. 2.2, 2.3 and 2.4). This indicates that there is cross talk between the two signaling pathways. Work by others has shown that BCR ligation up-regulates the expression of BAFF-R on B cells which is evidence towards the interplay between BAFF and BCR (Smith and Cancro 2003). The increase in expression of the receptor results in B cells becoming increasingly more sensitive to BAFF signals as they mature. A recent study has also shown that tonic B cell signaling supplies the NFκB substrate, p100, which is necessary for the BAFF signaling pathway. The study showed increase in p100 expression in B cell extracts after crosslinking of the BCR using anti-IgM and this increase was blocked by an inhibitor of the classical NF-κB pathway (Stadanlick, Kaileh et al. 2008). Thus, our studies and work by several groups show an intricate relationship between BAFF and the BCR signaling pathway. Fine tuning of the BCR signaling pathway by BAFF could result in tipping the scales of selection leading to autoimmunity.

Thus the two pathways interact by feeding into each other. BCR signaling results in providing the p100 substrate necessary for signaling by BAFF via the non-canonical NFκB pathway (Stadanlick, Kaileh et al. 2008). On the other hand, BAFF signaling through TACI initiates the classical NFκB pathway (Mackay and Schneider 2008) which could reinforce stimulation of this pathway by BCR.

Both our *in vitro* and our *in vivo* data have shown BAFF to downmodulate BCR induced calcium flux. BCR signaling that leads upto calcium flux include the following steps: The first step includes phosphorylation of the ITAMs by the receptor-associated Src-family tyrosine kinases, such as Blk, Fyn, or Lyn. This is followed by high affinity binding between the phosphorylated ITAM and the SH2 domain of Syk tyrosine kinase. Syk than phosphorylates and interacts with the adaptor molecule BLNK, also known as BASH or SLP-65. BLNK then helps recruit Tec kinases such as BTK (Bruton's tyrosine kinase), which in turn phosphorylates and activates the enzyme phospholipase C- γ2 (PLC- γ2). PLC- γ2 than cleaves the membrane phosphotidyl inositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can then bind to calcium channels in the endoplasmic reticulum (ER) membrane, opening the channels and allowing Ca^{2+} to enter the cytosol from stores in the ER. This causes increase in intracellular Ca^{2+} concentration. In order for BAFF to have an adverse effect on calcium flux mediated by BCR, it can be modulating any of the steps that lead upto the calcium flux. One example could be a negative regulatory role on Lyn or Syk, which could lead to an inhibition of calcium flux.

One evidence showing involvement of BAFF in regulating calcium flux was presented by Hase et. al. This group have shown that BAFF is capable of upregulating a B

cell specific transcription factor Pax5/BSAP (Pax5/B cell-specific activator protein) which directly targets CD19. Since CD19 is a major component of the B-cell coreceptor complex, this could provide a link between BAFF and BCR signaling, particularly in the upstream events leading to calcium flux. CD19 has a positive effect on BCR signaling by recruiting PI3K which in turn enhances calcium flux. BAFF was shown to synergistically enhance phosphorylation of CD19 following BCR ligation (Hase, Kanno et al. 2004). CD19 phosphorylates and recruits Vav and phosphatidylinositol-3-Kinase (PI3K) which in turn enhances calcium flux. This study was done by incubating human or murine B cell lines with BAFF transfected murine pre-B-cell line while our studies were performed either by *in vitro* stimulation of primary murine cells or *in vivo* injections of wild type mice with BAFF. Although the results shown in the study are in contrast to our studies which have shown BAFF to cause hyporesponsiveness in the B cells upon BCR ligation, the fact that BAFF is capable of regulating CD19 provides an interesting perspective on how BAFF may be modulating calcium flux and therefore BCR responsiveness.

Thus pretreatment of the cells with BAFF before stimulation with BCR can result in a very different response compared to simultaneous treatment with BAFF and BCR. Pre-treatment can result in accumulation of components of one pathway which can enhance or inhibit signaling from the subsequent treatment. Once example of a condition where cells could see BAFF prior to antigen would be the T1 cells in the bone marrow which have not yet experienced peripheral autoantigens. This would allow BAFF to initiate unresponsiveness or anergy in the cells, allowing them to avoid clonal deletion and persist in the periphery.

Microarray studies on B cells

Our microarray experiment was designed to investigate the effects of BAFF on the basic physiology and functional properties of B cells at a systems level. The main interpretation of the experimental results from the microarray data analysis is that BAFF is a very subtle inducer of gene expression at the timepoints that have been tested. Using the significance threshold to reduce the false discovery rate in SAM (Significance Analysis of Microarray), lead to only 104 genes at 24 hrs and only 2 genes at 48hrs of BAFF treatment that had significant p values (Table 6.1). This was 10 to 40 fold lower than the other ligand treatments.

The fact that BAFF stimulation resulted in such subtle effects on gene expression could have been due to a technical issue. However, given that the data was processed through a rigorous data analysis pipeline which normalized the data and should have filtered out most of the experimental and biological noise, allows us to rule out technical problems. The type of analysis should also not have been the cause for the limited effects of BAFF on gene expression, since the same analysis was used for other ligands which showed a much broader and stronger effect on gene expression. It is important to take into account that the effects of BAFF begin even before isolation of the cells and this background stimulation could mask any effects of BAFF during culture. Patke et. al, have addressed this issue by culturing the cells for 12 – 16hrs to reduce the background BAFF signaling from endogenous BAFF before signaling with BAFF (Patke, Mecklenbrauker et al. 2006). One interpretation that can be drawn from the subtle effects of BAFF on gene expression seen in the microarray data is

that BAFF may be primarily affecting genes that are not directly involved in transcription or mRNA stability. Having direct effects on proteins such as modifications, translocations, etc. would result in no changes in gene expression levels which would be difficult to pick up in a microarray experiment.

One way of increasing the number of genes that show significant p values would be by decreasing the threshold of significance, however that would result in an increase in the false discovery rate. That BAFF stimulation results in such limited effects on gene expression is also seen by the microarray data generated by the AFCS (Alliance for Cellular Signaling), involving stimulation of primary B cells with BAFF (Lee, Sinkovits et al. 2006). Thus the main inference from the BAFF results in the microarray data is that it functions by causing very subtle changes in gene expression.

The *in vitro* and *in vivo* data revealed that BAFF is causing an increase in the An1 cells which led us to look at the effect of BAFF on specific genes involved in anergy, before the stringent significance cut-off by SAM. The expression data showed that BAFF is causing an increase in the expression of the anergic genes, CD72, Egr2 and Nab2 (Fig. 6.10). In order to confirm the trends in expression of these genes observed in the microarray data, real time PCR was performed on RNA extracted from purified B cells. All three genes showed upregulation with BAFF treatment (Fig. 6.11) thus confirming the microarray expression data. Upregulation of these anergy associated genes in the presence of BAFF suggests that BAFF may be enhancing anergy in B. These results could imply that BAFF may be stimulating these anergic genes which in turn could have a dampening effect on other genes involved in B cell signaling.

In addition to the genes involved in anergy, many targets of BAFF, including the anti-apoptotic genes, were evaluated before the SAM stringency cutoff, but they did not show any significant changes on the microarray. NFkB2 and Rel A showed higher gene expression in the BAFF treated cells compared to untreated, but again this increase in gene expression did not pass the significance cutoff.

The limited effects on gene expression by BAFF is also seen by the microarray data generated by the AFCS (Alliance for Cellular Signaling), involving stimulation of primary B cells with BAFF (Lee, Sinkovits et al. 2006). In the data set analyzed by Lee et. al, the effect of 32 different ligands on purified B cells were measured over a time course upto 4 hrs. The data was processed using an analysis pipeline consisting of filtering, normalization and SAM analysis, which was very similar to our analysis pipeline. The results showed that the number of genes that were differentially expressed compared to the untreated control were about 200 fold less in the BAFF treated cells (about 10) compared to the anti-IgM treated cells (about 2000). A stringent pipeline with a high threshold for significance could result in more false negatives, a failure to detect true differences in gene expression._Thus, the threshold may need to be lowered in order to be able to pick up more genes that are differentially expressed by these weaker ligands. This should be done with the caveat in mind that the false discovery rate will be high resulting in false positives so that the gene expression results will have to be verified using other methods such as real time PCR.

Similar to our microarray data, anti-IgM stimulation in the Lee et. al. dataset showed a very strong effect on the differential expression of genes. After the application of SAM analysis, the microarray data from Jamie Lee et. al., was subjected to categorical clustering

based on expression of the genes in response to three different ligands, CD40L, anti-IgM and LPS which gave 19 different clusters. This method of clustering is different from the hierarchical and k-means clustering that were used in our microarray data analysis, which is why the clusters that emerged from this data set under anti-IgM treatment is very different from the clusters from our dataset. After the categorical clustering, CLASSIFI was applied which allowed the annotation of the 19 different clusters. One of the clusters which showed a significant p value had genes involved in molecular functions such as actin binding, cytoskeletal protein binding, actin monomer binding and regulation of transcription. This cluster is similar to the 'cell division' and 'DNA replication' clusters that were obtained by applying CLASSIFI to our microarray data. In addition our microarray data also yielded a cluster called 'cytoskeletal organization', although this did not have a significant p value.

Microarray data was also generated by Patke et. al., from BAFF treated cells. Cells were cultured for 12-16hrs in FBS supplemented media to reduce background BAFF signaling caused by endogenous BAFF. Live cells were than separated over a Ficoll gradient (Cedar Lanes) and purity assessed by trypan blue exclusion. After this, cells were stimulated in culture medium using BAFF. RNA was isolated from unstimulated B cells or after 36hrs of treatment with 25ng/ml of BAFF. This indicates that this data has no time-matched untreated control i.e., 36 hr untreated sample. This could be problematic because the readings of the differential expression of genes in the 36hr BAFF treatment will include genes that are differentially expressed by culture conditions alone. Raw expression data were analyzed using Microarray Analysis software (version 5.1 Affymetrix). Data were normalized to a target intensity of 500. There was no statistical filtering applied, compared to our data which

was processed through a statistical filter known as SAM (Significance analysis of Microarray). Differentially expressed genes were identified using GCOS software. GCOS (GeneChip Operating Software) is main analysis software for Affymetrix data analysis. GCOS provides a scatter plot with fold-difference lines on it. Any two chips form the same experiment can be plotted against each other to give the fold change. If no replicates have been used, than there may be too much random noise that can not be ruled out. However if there is 10x or 30x fold change, where one of the signals is reasonably high, than the list of genes can be used to analyze further. 377 transcripts showed a change of twofold or more. Approximately three quarters of these genes were up-regulated upon BAFF stimulation, and the rest were downregulated. It is important to note that there were no replicates used for generating the data, which is in contrast to data generated by our group which was generated from 4 replicates for each treatment. Although there was only a 2 fold change seen by the BAFF treatment, this is at least 5 fold lower than what is recommended by the GCOS software to weed out random noise. The GoMiner gene ontology (GO) tool was used on the 377 differentially expressed genes. GoMiner takes as input two lists of genes: the total set of genes that are present on the array, and the set of genes that show differential expression which is flagged by the user. The two-sided Fisher's exact test is then applied which is a test of the null hypothesis that the category is neither enriched in, nor depleted of, flagged genes with respect to what would have been expected by chance alone. The data is then displayed within the GO ontology hierarchy, both as a directed acyclic graph (DAG) or as the tree structure. The most important parameter is the enrichment or depletion of a category with respect to flagged genes. The relative enrichment is indicated by blue numbers for total

flagged genes and by red and green for over and underexpressed genes, respectively. Using these methods, two clusters were identified, the first cluster containing mRNA related to glycolysis and the second cluster contained proteins controlling cell cycle, chromosome condensation and mitosis.

The cell cycle progression proteins include cyclin D and cyclin E, Cdk4, Mcm2 and 3, and the proliferation marker Ki67 and Survivin. The upregulation of these proteins by BAFF were independently confirmed by protein expression analysis. Our data brought up only one gene involved in cell cycle control, cdc 25 homolog B, which was downregulated by BAFF. This could be due to differences in culture conditions between the microarray data generated in our lab compared to the Patke et.al., data. Since our data included endogenous BAFF effects on the B cells this may have masked subsequent treatment with BAFF. It is also very difficult to reproduce microarray data given the complexity of the studies and the many steps involved.

We have used the bioinformatics tool, CLASSIFI, which was helpful in identifying a cluster of genes with a p value slightly above the cutoff for the calculated Bonnferonni correction. This cluster, which was classified as the “receptor activity” cluster, consists of an interesting set of genes which includes IL-10 receptor, G protein coupled receptor 35 (GPR 35) and nuclear receptor subfamily 3. Since the microarray data showed lower expression of these genes in relation to untreated, this indicated that the treatment of BAFF may be causing a down modulation of these genes. IL-10 causes an increase in the production of BAFF. A decrease in the IL-10 receptor could be a method by which B cell homeostasis is maintained by providing a negative feed back loop. Thus, increase in IL-10 results in an increase in

BAFF which in turn down regulates expression of the IL-10 receptor. Another gene that showed lower expression by BAFF compared to untreated was Thioredoxin interacting protein (TXNIP) which is a pro-apoptotic gene also found to be expressed in T cells. Downregulation of this pro-apoptotic gene is consistent with the anti-apoptotic effects of BAFF on B cells. Previous work has shown that BAFF prevents apoptosis by the downregulation of the pro-apoptotic Bcl-2 family member Bim (Craxton, Draves et al. 2005). Other genes that were present in this cluster included nuclear receptor subfamily 3 and G protein coupled receptor 35 (GPR 35). Nuclear receptors can directly bind to DNA and regulate the expression of adjacent genes. GPR35 is important in the kynurenine pathway which elicits calcium mobilization and is therefore also important in signaling (Guo, Williams et al. 2008) . Thus, regulation of these genes by BAFF may reveal new ways by which BAFF communicates with the BCR signaling pathway.

Since clustering of the microarray data and subsequent application of CLASSIFI revealed very few clusters with significant p values, we decided to look at the list of genes before clustering. In order to obtain the list of significant genes by 24hr BAFF treatment versus untreated, reran SAM. For this SAM run, there were 104 genes with the FDR of 21.6%. Some of the genes present on the list included:

G1RP (G1 related zinc finger protein): The mRNA for this gene is upregulated in myeloid precursor cells following the removal of IL-3 from the culture. This suggested that the gene may regulate growth factor withdrawal-induced apoptosis of myeloid precursor cells (Baker and Reddy 2000). This could be an interesting gene to look at since it may also play a pro-

apoptotic role in B cells and was seen to lower expression in the presence of BAFF compared to untreated cells.

Mus musculus golli-interacting protein: Characterization of the overexpressed and purified protein revealed that it acts as a Mg^{2+} dependent phosphatase showing its importance in signaling (Bamm and Harauz 2008). This could be playing a negative regulatory role in BAFF signaling since stimulation by BAFF led to its downregulation.

Cell division cycle 25 homolog B: This protein plays an important role in centriole duplication and an absence of it results in cells accumulating in the G2 phase (Boutros and Ducommun 2008). This gene is expressed at lower levels in the presence of BAFF indicating that BAFF is not involved in cell division. This is similar to what we have shown in our *in vitro* data, namely that BAFF by itself does not show any effect on proliferation.

TGF-beta1-induced anti-apoptotic factor: The murine TGF-beta1-induced anti-apoptotic factor 2 or TIF2 resists death caused by TNF (Tumor necrosis factor) (Carey and Chang 1998). TIF2 gene expression is increased in human breast cancer and lymphoid leukemia cells. This would be an interesting protein to look at since BAFF has been known to be associated with several different forms of cancer. This gene is downregulated by BAFF at 24hrs which is opposite to what would be expected if BAFF does play a role in preventing apoptosis in cancer cells. It would be interesting to see how BAFF regulates this gene at later time points.

Annexin A3: Annexins are a class of proteins that are regulated by Ca^{2+} and are involved in membrane-related events such as the regulated organization of membrane domains and/or membrane-cytoskeleton linkages, certain exocytic and endocytic transport steps and the regulation of ion fluxes across membranes (Gerke, Creutz et al. 2005). Annexin A3 is thought be involved in the modulation of membrane binding and permeabilizaion. Other Annexin members which possess membrane permeabilizing activity include Annexin A7 which was reported to be a calcium channel, Annexin A5 which allows divalent cations to cross membrane and Annexin A5 which also allows monovalent cations to cross the membrane (Hofmann, Raguenes-Nicol et al. 2000). Since our studies have shown a lowering of calcium flux by BAFF, this could be an interesting protein to study. It could provide the link by which BAFF modulates BCR mediated signaling.

By comparing the microarray expression data from BAFF treated against BAFF + anti-IgM treated cells, several interesting observations could be made. CLASSIFI analysis revealed two clusters with the GO annotations of 'DNA replication' and 'Cell division' that have significant p values. Both of these clusters showed higher expression of some genes by anti-IgM in the presence or absence of BAFF treatment at the 48hr timepoint. Our in vitro data showed increase in the pool of proliferating cells by BAFF and anti-IgM. These clusters contain various genes involved in proliferation such as Cyclin D3, Cyclin A2, Cyclin G2, Cyclin B2 and Cell division cycle 25 homolog (Cdc 25 homolog). Thus, studying these

genes would be important in finding the relevant players in the anti-IgM induced proliferation of B cells.

Model of the action of BAFF in autoimmune disease

Our studies have revealed a unique connection between BAFF and autoimmune disease. We have shown that BAFF promotes an increase in the An1 cell population. We therefore proposed that by expanding this population, BAFF causes an accumulation of these self-reactive cells. Anergic B cells are neither edited nor deleted and persist in the periphery. Since the majority of self-reactive B cells become anergic, it is essential to maintain anergy in order to prevent autoimmunity. The development of autoimmune disease in BAFF transgenic mice could therefore be explained by an increase in the An1 autoreactive B cell population upon BAFF stimulation. The presence of these An1 cells in the periphery can prove to be dangerous for the organism since anergy may be reversed by autoantigen removal (Gauld, Benschop et al. 2005). It has been shown that anergy can also be reversed by ligand stimulation such as by CD40 stimulation. When anergic B cells from tolerant HEL/anti-HEL double-transgenic mice were incubated with a membrane preparation from activated T cell clones expressing CD40, the anergic B cells were activated and started proliferating and secreting antibodies (Eris, Basten et al. 1994)). We can speculate that this could also be true in T cell independent stimulation such as by TLR stimulation of the anergic B cells. Thus, the presence of greater numbers of anergic B cells could pose the threat of autoimmune disease from sudden triggers that cause reversal of anergy.

Previous studies have shown that mice transgenic for a DNA reactive BCR exhibit a dramatic increase in mature autoreactive B cells when they are given exogenous BAFF (Hondowicz, Alexander et al. 2007). Immature T1 cells that are strongly self-reactive

undergo negative selection before they can express sufficient BAFF-R which would enable them to survive. However, self-reactive B cells with low affinity for self-antigen are positively selected, acquire expression of BAFF-R on their cell surface and are able to survive in response to increased levels of BAFF. Studies have shown a correlation between increased levels of BAFF and disease progression. Mice that are transgenic for BAFF develop autoimmune disease-like symptoms. Elevated anti-nuclear antibodies in the serum, a hallmark of Lupus, are present at high levels in BAFF transgenic mice (Mackay, Woodcock et al. 1999; Khare, Sarosi et al. 2000). Elevated levels of BAFF have also been found in the serum of human patients with RA, SLE and SS (Mariette, Roux et al. 2003; Stohl, Metyas et al. 2003).

Due to negative selection, which is the attempt to remove cells that are reactive to self antigen, only about 10% of the newly generated bone marrow cells are able to exit as transitional cells. Of these bone marrow émigrés, only about half are able to survive and join the follicular or marginal zone pools (Allman, Ferguson et al. 1993; Rolink, Andersson et al. 1998). Selection of B cells is divided into two phases. The first phase of positive selection occurs in the bone marrow and relies on BCR strength alone to determine whether a cell faces elimination versus further differentiation and exit from the bone marrow. One model suggests that the second phase of negative selection, which occurs in the peripheral transitional B cell pools, not only relies on the BCR signal strength but also to the available amounts of BAFF for the cells to survive and mature and home to the follicular or marginal zonal pools ready for activation (Cancro 2006). Evidence from the BAFF knockouts have indicated that newly formed immature B cells emerging from the bone marrow are very

susceptible to elimination in the absence of BAFF (Schiemann, Gommerman et al. 2001). Kinetic studies in mixed bone marrow chimeras have also demonstrated that mature primary B cells in the follicular and marginal zones also display a dependence on BAFF for their survival and compete for available BAFF in order to survive (Harless, Lentz et al. 2001; Hsu, Harless et al. 2002). Lesley et al. have shown that autoreactive cells that normally have a very short half life in the presence of limiting amounts of BAFF are rescued by the presence of excess BAFF (Lesley, Xu et al. 2004). Cyster et al. has also shown that B cells with an autoreactive BCR transgene were able to persist in the absence of competition, but were excluded from splenic follicles and died when other B cells of normal receptor diversity were present (Cyster, Hartley et al. 1994). The study also showed that overexpression of bcl-2 (b cell lymphoma proto oncogene), a downstream effector of BAFF, led to increased survival of these cells although they were still excluded from the follicular niches. The work that followed this has shown that this reduced competitiveness of autoreactive B cells is due to an increased dependence on BAFF (Lesley, Xu et al. 2004). Thus, excess BAFF allows those autoreactive B cell to survive that normally would have undergone elimination (Mackay, Woodcock et al. 1999; Khare, Sarosi et al. 2000). In the absence of excess BAFF, self reactive B cells are unable to compete with wild type cells and are eliminated. Interestingly, it has been shown that cells with low or intermediate affinity to self-antigens are more responsive to BAFF and are preferentially recruited to the MZ compartment (Thien, Phan et al. 2004). This model suggests that the transitional stage is an elastic checkpoint and the threshold for negative selection is homeostatically adjusted by the amount of BAFF present. Thus excess amounts of free BAFF in the presence of lymphopenia or reduced bone marrow

output, leads to less competition and more survival, perhaps setting the stage for autoimmunity.

One method of disrupting negative selection would be by increasing survival of self-reactive B cells and allowing them to mature and persist in the periphery. Niiro and Clark et al. have speculated that BAFF may be overriding death signals and tipping the balance for survival of B cells (Niiro and Clark 2002). BAFF transgenic mice have an overabundance of mature, transitional type 2 (T2) and marginal zone B cells (Batten, Groom et al. 2000; Rolink, Tschopp et al. 2002). Work by others report that recombinant soluble BAFF has a direct survival effect on transitional and mature B cell populations *in vitro* (Do, Hatada et al. 2000; Thompson, Schneider et al. 2000; Hsu, Harless et al. 2002). The fact that BAFF is important in B cell survival can also be seen in BAFF deficient mice, which show reduced number of B cells from the immature transitional type 1 (T1) stage onwards (Schiemann, Gommerman et al. 2001). Also, in TACI-Ig mice (mice transgenic for antibodies against TACI) or in mice that have been treated with TACI-Ig, there is a reduction of immature transitional type 2 (T2) and mature B cells (Gross, Johnston et al. 2000). BAFF also stimulates the pro-survival oncoproteins Bcl-2 and Bcl-x_L and decreases the expression of genes that are pro-apoptotic such as Bak (Batten, Groom et al. 2000; Do, Hatada et al. 2000; Amanna, Clise-Dwyer et al. 2001). Our data also revealed an increase in survival of all the transitional, marginal zonal, and mature B cell populations by BAFF. These observations suggest that negative selection takes place in the presence of limiting amounts of BAFF but is disrupted in the presence of excess BAFF by an increase in survival of the autoreactive cells.

Thus, the model suggests that BAFF allows the survival of cells that are normally targeted for or are susceptible to apoptosis and have shorter half-lives so that a higher number of self-antigen specific B cells are able to hang around longer. This increases the reservoir of autoreactive B cells, which may then lead to autoimmunity. Our novel addition of the enhancement of the An1 population by the presence of BAFF adds to the existing model and provides us with a more in depth perception of how BAFF could be involved in autoimmune disease.

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