BAFF Regulates T Follicular Helper Cells in Autoimmunity

Christine Morel Coquery
Los Altos, CA

M.S., Baylor University, 2011
B.S., University of California San Diego, 2005

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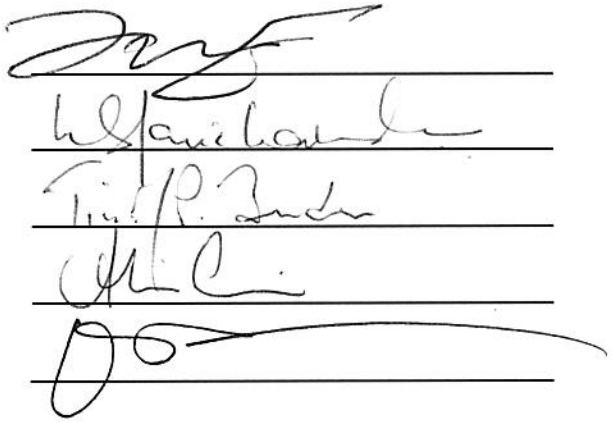

ABSTRACT

T follicular helper (T_{FH}) cells are critical for the development of protective antibodies (Ab) via germinal center (GC) B-cell responses; however, uncontrolled T_{FH} cell expansion activates autoreactive B-cells to produce antibodies that cause autoimmunity. The mechanisms that control T_{FH} cell homeostasis remain largely unknown. The work described here shows that the receptor B Cell Maturation Antigen (BCMA) has a critical role in $CD4^+$ T cells for regulating T_{FH} cell responses in autoimmune lupus. Under lupus-prone or inflammatory conditions, T_{FH} cells express BCMA and the BAFF receptor BR3. In BCMA-deficient animals, T_{FH} cells accumulate in the spleen. Mixed bone marrow chimeras and adoptive transfer studies demonstrated that BCMA deficiency in T cells was sufficient to promote the abnormal expansion of T_{FH} cells, leading to germinal center formation and autoantibody production. The accumulation of BCMA deficient T_{FH} cells was mediated by BAFF signals transduced through BR3. We identified increased frequencies of both splenic-dendritic cells (DCs) and neutrophils, both potent BAFF producers, which co-localized in the T cell zone. DCs and neutrophils induced IFNγ production by T_{FH} cells that in turn increased BAFF expression in DCs. Blocking BAFF or IFNγ *in vivo,* as well as neutrophil depletion, reduced T_{FH} cell accumulation and improved autoimmunity in BCMA deficient animals. Moreover, we observed elevated circulating $CD4^+$ T cells and T_{FH}-like cells that express BR3 (but not BCMA) in SLE patients, which correlated with serum BAFF and IFNγ titers. These findings identify a new BCMA-BAFF axis that controls T_{FH} cell homeostasis and suggests the balance between BCMA and BR3 signaling in T_{FH} cells serves as a checkpoint of immune tolerance.

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LIST OF ABBREVIATIONS

Activation Induced Cytidine Deaminase (AID)

Antigen (Ag)

Analysis of Variance (ANOVA)

Antigen Presenting Cell (APC)

A Proliferation Inducing Ligand (APRIL)

Arbitrary Units (A.U.)

Achaete Scute Homologue 2 (Ascl-2)

B Cell Activator of the TNF Family (BAFF)

B Cell Activator of the TNF Family Receptor (BAFF-R or BR3)

Bcl-2 Associated Agonist of Cell Death (Bad)

Bcl-2 Like Protein 11 (Bim)

Basic Leucine Zipper Transcription Factor (Batf)

B Cell Lymphoma 6 (Bcl-6)

B Cell Maturation Antigen (BCMA, *Tnfrsf17*)

PR Domain Zinc Finger Protein 1 (Blimp-1)

Complement 3 (C3)

Class II, Major Histocompatibility Complex Transactivator (CIITA)

Complementary DNA (cDNA)

Carboxyfluorescein Succinimidyl Ester (CFSE)

Proto-Oncogene MAF (C-Maf)

Cercopithecus Aethiops Kidney 7 (COS7)

c-AMP Response Element Binding Protein (CREB)

Class Switch Recombination (CSR)

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Combined Variable Immunodeficiency (CVID)

Dendritic Cell (DC)

Deoxyribonucleic Acid (DNA)

Diptheria Toxin Receptor (DTR)

Double Stranded DNA (dsDNA)

Enzyme Linked Immunosorbent Assay (ELISA)

ETS Domain Containing Protein 1 (Elk1)

Extracellular Signal Regulated Kinase (ERK)

Fetal Bovine Serum (FBS)

Follicular Dendritic Cell (fDC)

Follicle (FO)

Field of View (FOV)

Fluorescence Minus One (FMO)

Forkhead Box Protein 1 (Foxp1)

Forkhead Box Protein 3 (Foxp3)

Forward (Fwd)

Germinal Center (GC)

Green Fluorescent Protein (GFP)

Glucocorticoid-Induced TNFR Family Related Gene (GITR)

Healthy Donor (HD)

Inducible T Cell Co-Stimulator (ICOS)

I Kappa Beta Kinase (Iκκ)

Interleukin (IL)

Immunofluorescence (IF)

Interferon (IFN)

Immunoglobulin (Ig)

Intraperitoneal (i.p.)

Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome (IPEX)

Interferon Regulatory Factor (IRF)

Intravenous (i.v.)

C-Jun N-Terminal Kinase (JNK)

Lymphocyte Activation Gene 3 (LAG-3)

Lymphocytic Choriomeningitis Virus (LCMV)

Lymphocyte Function Associated Antigen-1 (LFA-1)

Lymph Node (LN)

Lipopolysaccharide (LPS)

Molar (M)

Monoclonal Antibody (mAb)

Induced Myeloid Leukemia Cell Differentiation Protein 1 (Mcl-1)

Mean Fluorescence Intensity (MFI)

Major Histocompatibility Complex (MHC)

Messenger RNA (mRNA)

Mantle Zone (MZ)

Neutrophil Extracellular Trap (NET)

Neurolipin 1 (Nrp-1)

Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κβ)

Optimized Antibody Cocktail (OAC)

Ovalbumin T Cell Receptor Class II (OT-II)

Paired Box Protein 5 (PAX-5)

Peripheral Blood Mononuclear Cell (PBMC)

Phosphate Buffered Saline (PBS)

Plasma Cell (PC)

Pigeon Cytochrome C (PCC)

Programmed Cell Death 1 (PD-1)

Polymerase Chain Reaction (PCR)

Proviral Integrations of Moloney Virus 2 (Pim2)

Polymorphonuclear (PMN)

Red Pulp (RP)

Roswell Park Memorial Institute Medium (RPMI)

Real Time PCR (RT-PCR)

Reverse (Rev)

SLAM Associated Protein (SAP)

Standard Error of the Mean (SEM)

Signaling Lymphocyte Activation Molecule (SLAM)

Somatic Hypermutation (SHM)

Small Interfering RNA (siRNA)

Signal Transducer and Activator of Transcription (STAT)

Systemic Lupus Erythematosus (SLE)

SLE Disease Activity Index (SLEDAI)

Single Stranded DNA (ssDNA)

Transmembrane Activator and CAML Interactor (TACI)

T Box Transcription Factor (T-bet)

T Cell Receptor (TCR)

T Follicular Helper (T_{FH})

T Follicular Regulatory (T_{FR})

T Helper Cell (Th)

Transforming Growth Factor Beta (TGFβ)

Tumor Necrosis Factor (TNF)

Tumor Necrosis Factor Receptor Superfamily (TNFRSF)

TNF Receptor Associated Factor (TRAF)

Wild-type (WT)

X-Box Binding Protein 1 (xbp-1)

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CHAPTER 1

Introduction

The Adaptive Immune Response

The adaptive immune response is critical for maintaining host protection against foreign pathogens and for the efficacy of vaccines. Following initial exposure to a pathogen, the innate immune system, comprised largely of monocyte-derived cells and granulocyte populations is activated by recognizing pattern molecules on the surface of the invading pathogen [1]. This rapid detection is instrumental in initial host defense, but often, the innate system is overwhelmed by the infection. Therefore the innate response plays another important role in immune-protection by contributing to the activation of the adaptive immune response. The adaptive immune response is separated into cell-mediated responses and humoral responses which are carried out by two classes of lymphocytes, B cells and T cells [2]. Cell mediated responses are controlled by two classes of T cells, $CD4^+$ and $CD8^+$, which are distinguished by the expression of the CD4 or CD8 glycoprotein on their surface, as well as functional differences. T cells become activated after encountering their cognate antigen (Ag) presented to them on a major histocompatibility molecule by an antigen presenting cells, such as a dendritic cell (DC). The environment of this interaction, which is in part created by the type of pathogen encountered (viral, bacterial, helminth, etc.), largely dictates the functional capacity of the activated T cells [3]. These functions range from direct killing of an infected cell to the production of cytokines that can recruit phagocytic cells, such as macrophages, which can clear infected cells [2].

The humoral response is characterized by the generation of antibodies (Ab) against the invading pathogen. Antibodies are produced by plasma cells (PCs), a subset of terminally differentiated B cells. There are two distinct pathways that lead to the

generation of Ag-specific Abs. T-dependent responses require cognate interaction between an activated Ag-specific $CD4^+$ T cell and an activated B cell, whereas T-independent responses do not require a T cell intermediate. T-dependent responses can be further subdivided into extrafollicular and germinal center [4]. Following activation, both T cells and B cells will migrate from their respective locations towards the border of the T cell zone and B cell zone [5]. Interaction between cognate T and B cells at this border can drive the generation of extrafollicular PCs, which provide an early wave of antibody protection [4]. Alternatively, activated cognate B and T cells can form a structure known as the germinal center (GC). The development and maintenance of this structure is controlled by T follicular helper (T_{FH}) cells, a subset of activated $CD4^+$ T cells which provide survival, maturation and proliferation signals to the cells that will ultimately give rise to the highest affinity antibody-producing PC population (in contrast to the extrafollicular response, which generates Abs of lower affinity to the initiating Ag) [6]. Understanding the biology of T_{FH} cells is therefore critical for dissecting the immune response to microorganisms and designing more effective vaccine strategies.

T_{FH} Cells in the GC Response and the Generation of Plasma Cells

The GC is a microanatomical structure that forms in secondary lymphoid organs following vaccination or infection in a healthy individual (**Figure 1.1**). It is within this structure that the interaction between T_{FH} cells, GC B cells and follicular dendritic cells (fDC) drive substantial B cell proliferation and selection, ultimately leading to cells that have increased affinity to the initiating pathogen [5]. The DNA rearrangement events that occur during this response include the process of somatic hypermutation (SHM).

Mutations that occur in the variable region of the B cell receptor (BCR) alter the affinity of the BCR for its cognate Ag. This provides the opportunity for proliferating B cells to sample greater antigen and present this Ag on its surface in association with MHC class II molecules for recognition by T_{FH} cells. B cells with greater Ag presented are more likely to receive survival signals from T_{FH} cells [7]. Two-photon microscopy has advanced our understanding of germinal center dynamics tremendously. Work from the Nussenzweig lab has demonstrated that GCs are organized into distinct light and dark zones, which confirms previous reports using less sensitive technology [8]. While the light zone appears to be where Ag-driven selection occurs, the dark zone is more closely associated with B cell proliferation and Ag sampling [9]. Furthermore, Nussenzweig's group demonstrated that competition for a small number of T_{FH} cells plays an important role in ensuring high affinity PC selection. Intravital microscopy experiments using full mount lymph nodes (LNs) demonstrate the dynamic nature of T_{FH} cells in the context of the GC response. T_{FH} cells are motile and move from one GC to another within LNs. Newly activated T_{FH} cells can enter already established GCs and contribute to the selection of Ag-specific B cells [10]. This concept is critical as it suggests that Ag non-specific, and potentially autoreactive T_{FH} clones, can alter an ongoing immune response and potentially skew the specificity of the developing PCs.

The maintenance of the GC is critical for achieving a robust protective Ab response. While DCs are required for initiation of T_{FH} development, B cells are dispensable for these early differentiation steps. However, many groups have shown that GC-B cells are also necessary for maintaining the T_{FH} compartment, and hence the GC reaction. Using blocking Abs *in vivo*, Baumjohann et. al. showed that ICOS and CD40

interactions are critical for maintaining the T_{FH} population once the GC response has been established [11]. Presumably, because of the location of T_{FH} cells during the peak of response, ICOS and CD40 engagement could only occur between T_{FH} cells and B cells. As shown in **Figure 1.1**, a robust GC reaction is pivotal for the generation of high affinity memory B cells as well as the terminally differentiated PCs, which are professional Ab secreting cells. The mechanisms that determine whether a GC B cell will become a memory B cell or a PC are not well understood. The interaction between T_{FH} cells and GC-B cells that generate high affinity PCs is mediated through multiple cognate molecules. These molecules include: CD28/B7, SLAM family members/SLAM family members, ICOS/ICOSL, PD-1/PDL1, CD40L/CD40, TCR/major histocompatibility complex II (MHCII).

Figure 1.1

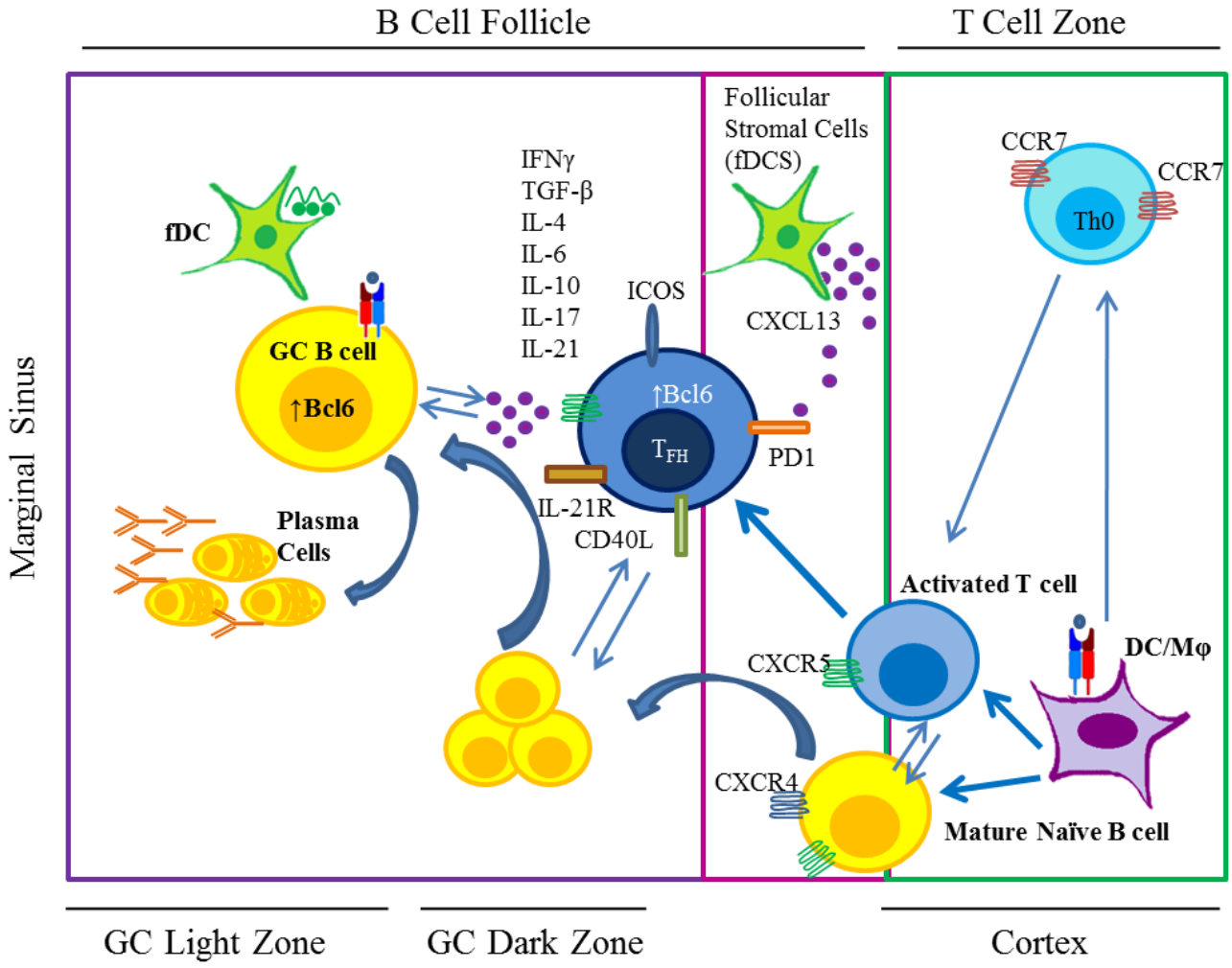
B Cell Follicle
T Cell Zone
Marginal Sinus
fDC
GC B cell
↑Bcl6
Plasma Cells
IFNγ
TGF-β
IL-4
IL-6
IL-10
IL-17
IL-21
ICOS
↑Bcl6
TFH
PD1
IL-21R
CD40L
Follicular Stromal Cells (fDCS)
CXCL13
CCR7
CCR7
Th0
Activated T cell
CXCR5
DC/Mφ
CXCR4
Mature Naïve B cell
GC Light Zone
GC Dark Zone
Cortex

Figure 1.1. The germinal center reaction. Following antigen uptake, DCs home to the secondary lymphoid organs where they come into contact with cognate naïve T and B cells. A subset of activated T cells downregulate the chemokine receptor CCR7 and upregulate the chemokine receptor CXCR5, which allows them to move away from the T cell zone towards the B-T border via the CXCL13 chemokine gradient produced by follicular DCs. Interaction between activated T and B cells at this border may lead to the differentiation of short lived PCs that mature extrafollicularly and provide a wave of low affinity antibodies. Alternatively, sustained interaction at this border drives further proliferation of the B cells and differentiation of the activated T cells into *bona fide* T_{FH} cells. Interaction between fDCs, T_{FH} cells and GC-B cells leads to the formation of a GC. Within the GC, B cells will undergo SHM, class switch recombination (CSR) and affinity maturation. As a consequence, the B cell may undergo apoptosis, or survive and develop into high affinity PCs and/or memory B cells.

Although interactions between surface molecules are important for survival and proliferation of both GC B cells and T_{FH} cells, perhaps the most critical mediator of PC differentiation is the production of IL-21 by T_{FH} cells [12]. Production of the cytokine IL-21 by T_{FH} cells promotes the GC response and generation of PCs by acting in both a paracrine and autocrine fashion [53]. IL-21 overexpression or via delivery of exogenous IL-21 can promote B cell differentiation specifically into IgM and IgG1-producing PCs [13]. In contrast, removing the IL-21 or the IL-21 receptor gene in mice leads to a substantial reduction in the PC response. Although these initial experiments did not restrict IL-21 to T_{FH} cells and IL-21 receptor to GC B cells, subsequent mixed bone marrow chimera experiments demonstrated the necessity of the IL-21/IL-21R axis for optimal PC formation [57]. These findings were verified by other groups and extended to human B cells [58, 59]. Using *in vitro* approaches, Luthje et. al. and Ozaki et. al. identified the mechanism by which IL-21 drives PC differentiation. IL-21 can induce PR domain zinc finger protein 1 (Blimp-1) in splenic B cells and Blimp-1 is critical for generating PCs by targeting and suppressing a host of downstream signaling molecules including MHC, paired box protein 5 (PAX5), class II, major histocompatibility complex and transactivator (CIITA). In addition, Blimp-1 increases the expression of x-box binding protein 1(XBP-1) and IRF-4 that induce PC differentiation [14].

T_{FH} Cells: Identification & Initial Characterization

The first description of T_{FH} cells stems from two seminal papers, published in 2000 [15, 16]. The authors describe a unique subset of $CXCR5^+CD4^+$ T cells found abundantly in human tonsil and to a lesser extent, in circulation. Tonsillar $CXCR5^+$ T

cells localized to GC structures and were able to potently induce B cell responses, including immunoglobulin (Ig) production as well as class switching, when cultured *in vitro* [15, 16]. Further work has distinguished two sub-populations within the tonsil $CXCR5^{+}$ compartment: 1) Inducible T cell co-stimulator $(ICOS)^{low}CXCR5^{low}$ and 2) $ICOS^{high}CXCR5^{high}$. While the $ICOS^{high}CXCR5^{high}$ population is superior at inducing B cell IgG production *in vitro*, this population is prone to apoptosis and does not divide as rapidly as $ICOS^{low}CXCR5^{low}$ $CD4^{+}$ T cells [17]. Work in murine models has begun to elucidate the developmental stages that lead from a naïve $CD4^{+}$ T cell to a germinal center-resident T_{FH} cell (GC-T_{FH}). Although the terminology defining each subset is still heavily debated, naïve $CD4^{+}$ T cells that engage with their cognate Ag presented on DCs will become activated $CD4^{+}$ T cells. The interaction between DCs and a subset of activated T cells can lead to incremental expression of CXCR5 and PD1: from pre-T_{FH} ($CXCR5^{low}PD1^{low}$), to T_{FH} ($CXCR5^{+}PD1^{+}$), and ultimately to a GC-T_{FH} ($CXCR5^{hi}PD1^{hi}GL7^{+}$); the latter two populations requiring interaction with cognate B cells for their differentiation and maintenance [18]. Although pre-T_{FH} and T_{FH} cells have the capacity to provide necessary signals to drive B cell differentiation, proliferation and survival *in vitro*, it is likely only the GC-T_{FH} population localizes to the GC structure and provides these signals *in vivo* [19, 20]. Furthermore, whether a memory T_{FH} compartment may arise from each of the T_{FH} subsets, remains to be elucidated (**Figure 1.2**) [21].

Transcriptional profiles of the T_{FH} population are unique compared to other $CD4^{+}$ T cell subsets and, in addition to ICOS and CXCR5, includes upregulation of molecules such as signaling lymphocyte activation molecule (SLAM) and SLAM associated protein (SAP), B cell lymphoma 6 (Bcl-6), Interleukin (IL)-21, and programmed cell death 1

(PD-1), as well as down-regulation of CCR7 and Blimp-1 [17, 19, 22]. The differential expression of these molecules identify the different phases of T_{FH} differentiation and is important for their function (**Figure 1.2**).

T_{FH} Cells: Opposing Roles of CXCR5& CCR7

Chemokine receptors are critical for the migration of cells into and out of tissues. CCR7, a chemokine receptor necessary for T cell movements as well as the normal architecture of the thymus and secondary lymphoid organs, is abundantly expressed on naïve T cells [23]. CCR7 is required for naïve T cells entry into the spleen or lymph node via the draining lymphatics or high endothelial venules. Once within these structures, expression of its two ligands, CCL19 and CCL21, by reticular stromal cells, dictates the speed and migratory pathway of naïve T cells and restricts their movement to the T cell zone [23]. The confinement of naïve T cells to a limited area increases the likelihood that they will come into contact with a DC bearing their cognate Ag, causing T cell activation. Following activation, $CD4^+$ T cells downregulate CCR7 and upregulate the chemokine receptor CXCR5. Upregulation of CXCR5 allows activated T cells to move towards the B-T border following the CXCL13 gradient produced by follicular dendritic cells residing in the B cell follicle. Continued upregulation of CXCR5 drives developing T_{FH} cells further into the follicle and ultimately the GC [24].

Figure 1.2

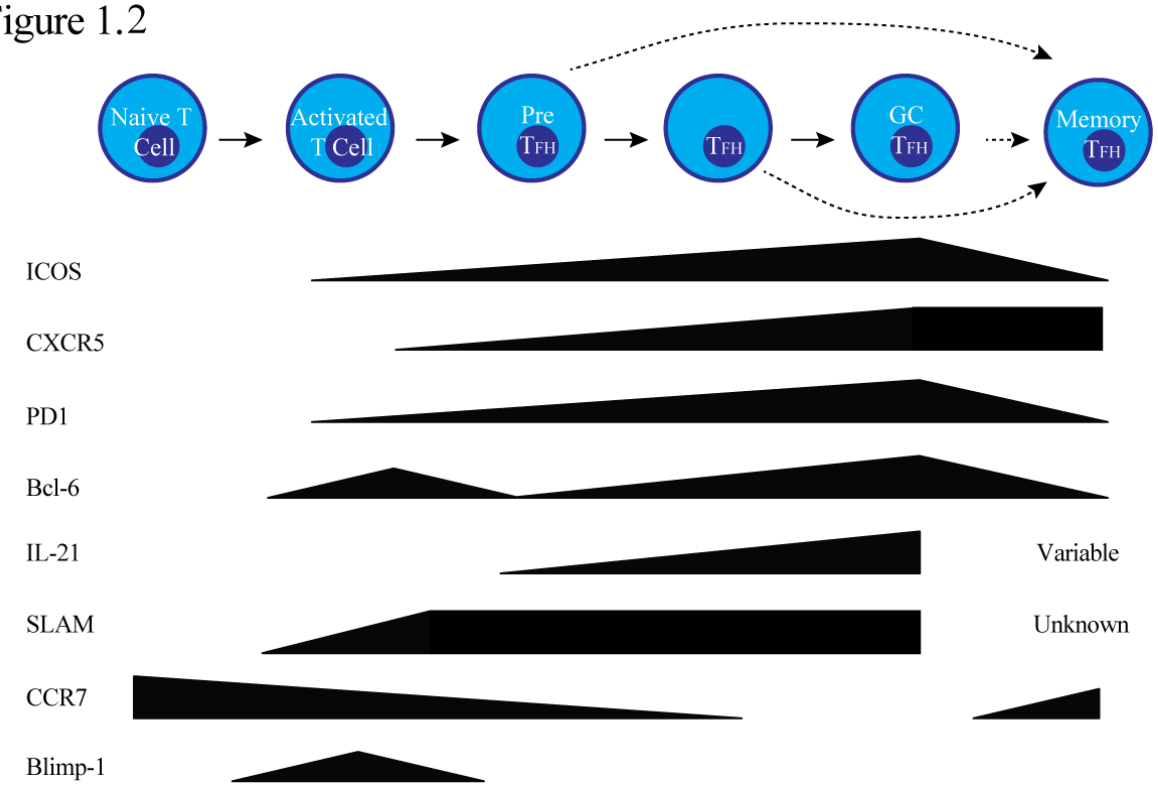

Figure 1.2. Expression of key molecules during T_{FH} differentiation. ICOS, CXCR5, PD1, Bcl-6, SLAM and IL-21 are all upregulated during T_{FH} development. SLAM, PD1 and ICOS upregulation occurs rapidly, along with an initial wave of Bcl-6, followed by CXCR5 and IL-21expression. While CXCR5 is maintained in the memory T_{FH} compartment, the majority of other molecules are downregulated. Although CCR7 is lost during T_{FH} differentiation, it becomes re-expressed as cells enter a memory phase. Blimp-1 is transiently expressed following $CD4^+$ T cell activation, but quickly becomes downregulated as cells commit to the T_{FH} pathway. Solid lines indicate confirmed transitional steps whereas dashed lines indicate a predicted pathway (based on limited experimental evidence).

T_{FH} Cells: The Role of ICOS

ICOS is upregulated rapidly following T cell receptor (TCR) engagement and CD28 co-stimulation (**Figure 1.1**) [25]. Although ICOS expression is not restricted to T_{FH} cells, ICOS controls follicular recruitment of activated T-helper cells in mice by targeting the machinery required for migration. Expression of CXCR5 has been shown to be controlled by ICOS; however, over-expression of CXCR5 using a retroviral vector in $ICOS^{-/-}$ animals can only partially rescue the migratory phenotype, suggesting ICOS may have other targets besides CXCR5 for facilitating T_{FH} migration into B cell follicles [6, 7]. The importance of ICOS in maintaining T_{FH} homeostasis is evident in both murine models as well as humans. Overexpression of ICOS in mice (due to the absence of roquin, a posttranscriptional repressor) leads to aberrant T_{FH} development ultimately leading to autoimmunity [8]. In contrast, ICOS mutations in humans with common variable immunodeficiency (CVID), which prevent the receptor from being expressed, show a substantial reduction in circulating T_{FH} cells and a reduced ability to up-regulate additional T_{FH} markers when stimulated *in vitro* [9, 10]. These finding are similar to the phenotype of T_{FH} cells in mice deficient in ICOS. Together, these data confirm that ICOS is an important regulator of T_{FH} function, localization and balance through both direct and indirect mechanisms.

T_{FH} Cells: The Role of SLAM and SAP

SLAM and its downstream signaling molecule SAP have been shown to be critical for the optimal generation and function of T_{FH} cells. SLAM is expressed on resting and activated T cells and is important for mediating cognate interactions between

T_{FH} cells and B cells as well as DCs [26]. SAP binds to the intracellular portion of SLAM and recruits Fyn kinase that mediates downstream signals to control T helper differentiation [27]. However, more recent evidence has suggested that SLAM may be dispensable for the overall development and function of T_{FH} cells. In support of this, $SLAM^{-/-}$ mice have normal T_{FH} frequency, and are able to mount a robust antibody response. In contrast, $SAP^{-/-}$ animals fail to generate T_{FH} cells that are capable of supporting the differentiation of PCs. Although a population of T_{FH}-like cells does form both *in vivo* and *in vitro in* $SAP^{-/-}$ animals, they are not able to support B cell survival, proliferation or differentiation [26, 28, 29].

T_{FH} Cells: The Role of Transcription Factors

One of the most important regulators for T_{FH} differentiation is the transcriptional repressor, Bcl-6, as mice deficient in Bcl-6, within the $CD4^+$ T cell compartment, fail to form T_{FH} cells. Although earlier reports indicated increased expression of Bcl-6 in the T_{FH} compartment, Johnston et. al. and Nurieva et. al. were the first groups to demonstrate that Bcl-6 is both necessary and sufficient for T_{FH} differentiation *in vivo* [30-33]. Johnston et. al. utilized a T cell receptor (TCR) transgenic system where all $CD4^+$ T cells recognize a single Ag. These SMARTA mice have $CD4^+$ T cells specific to lymphocytic choriomenengitis virus (LCMV). The authors adoptively transferred SMARTA $CD4^+$ T cells, that had been transduce or untransduced with a Bcl-6 over-expressing retrovial vector, into wild-type (WT) mice. The authors observed that Bcl-6 over-expression facilitated a massive increase in T_{FH} differentiation in an Ag-specific manner when the mice were treated with LCMV [30]. Nurieva et. al. expanded on these findings using a

polyclonal system, where the T cell repertoire is not restricted to a single antigen. The authors transferred Bcl-6 deficient or sufficient naïve $CD4^+$ T cells and WT B cells into immuno-deficient $Rag^{-/-}$ mice. Following immunization with the T cell dependent antigen, keyhole limpet protein, the mice that received Bcl-6 deficient T cells failed to form T_{FH} cells or GC-B cells [33]. These findings support a role for Bcl-6 in driving T_{FH} differentiation *in vivo* in a T cell intrinsic manner.

While these findings were compelling, the studies were restricted to a single timepoint and thus failed to establish whether Bcl-6 is important during all phases of T_{FH} development or whether it is confined to the generation of GC-T_{FH} cells. Liu et. al. clarified this question by utilizing a Bcl-6 reporter mouse crossed to a monoclonal TCR system where all T cells recognize the antigen ovalubumin (OVA) (OT-II) [34]. The authors adoptively transferred OT-II-Bcl-6 reporter $CD4^+$ T cells into a WT mouse and then immunized recipient mice with OVA. The authors noted that while CXCR5 and IL-21 were upregulated in the $CD4^+$ compartment as early as 2 days following immunization, Bcl-6 was only detected 3 days post-immunization. Furthermore, Bcl-6 deficient OT-II T cells had an initial generation of T_{FH} cells (day 3) but this had almost completely resolved by day 7 post-immunization [34]. Thus, the experiments by Liu and colleagues clarifies that while Bcl-6 is important in the ultimate generation of fully functional and differentiated T_{FH} cells, as well as their maintenance, it is not critical for the initial steps that lead towards a pre-T_{FH} cell.

More recently, additional transcription factors have been shown to have a profound impact on the optimal formation of the T_{FH} population, either by controlling the frequency of T_{FH} cells or their ability to drive GC-B cell formation. Some of these

molecules include the basic leucine zipper transcription factor, ATF-like (Batf), Proto-oncogene Maf (C-Maf), interferon regulatory factor 4 (IRF4), and achaete-scute homologue 2 (Ascl2). In mice deficient in Batf or its downstream target, C-Maf, T_{FH} cells fail to form appropriately as measured by decreased IL-21 production and a reduction in CD40L expression [35-37]. IRF4-deficient animals also have a reduced frequency of T_{FH} cells. Although this reduction was observed in germline IRF4$^{-/-}$ mice, adoptive transfer of WT CD4^{+} T cells into IRF4$^{-/-}$ animals, restored the loss of T_{FH}, suggesting that IRF4 is a T-cell intrinsic factor necessary for T_{FH} development [38]. In humans Bcl-6 and Maf cooperate to instruct T_{FH} cell differentiation by controlling different aspects of the T_{FH} phenotype. For example, Bcl-6 introduced into human T cells increased CXCR4, CXCR5, CCR7, EBI2, SAP, CD40L, PD-1, and ICOS, whereas Maf transfected into human non-T_{FH} cells resulted in enhanced IL-21 production [39]. Ascl2 has been shown to promote the early stages of T_{FH} development in both mice and humans by upregulating CXCR5 while downregulating CCR7 expression [40].

In contrast to the transcription factors that support T_{FH} development, several molecules are known to be negative regulators of T_{FH} differentiation and include; Blimp-1, forkhead box P1 (Foxp1), and PD-1. Blimp-1 antagonizes Bcl-6 within T_{FH} cells [30]. Using the SMARTA system described above, cells that had been transduced with both Bcl-6 and Blimp-1 failed to generate as many T_{FH} cells following adoptive transfer and infection with LCMV compared to cells that had been transduced with Bcl-6 alone [30]. A very recent studied has identified Foxp1 as a potent negative regulator of T_{FH} cell development. Following TCR stimulation, two isoforms of Foxp1, Foxp1A and Foxp1D, are upregulated and actively suppress the T_{FH} program by inhibiting IL-21 gene

expression as well as dampening expression of ICOS [41]. *In vivo*, adoptively transferred OT-II $CD4^+$ T cells deficient in Foxp1 develop predominantly into GC-T_{FH} cells, as determined by their upregulation of PD-1, CXCR5 and GL-7, following OVA immunization. This expansion of T_{FH} cells resulted in a strong and Ag-specific immune response. PD-1 is another negative regulator of T_{FH} cells. Mice deficient in PD-1 have an increased frequency of T_{FH} cells [42]. Surprisingly, although T_{FH} cells expanded in the absence of PD-1, they fail to produce a robust cytokine response upon stimulation, suggesting that the role of PD-1 within the T_{FH} compartment may be multi-fold [43].

T_{FH} Cell Development

T_{FH} differentiation initiates in the T cell zone immediately following TCR engagement of naïve T cells by peptide MHC-II complexes on DCs (**Figure 1.3**) [44, 45]. Two of the most important predictors of whether a cell will differentiate into a T_{FH} cell following this initial DC interaction are the affinity of the TCR for the Ag and the dose of Ag. T cells with the highest peptide-MCH binding capacity preferentially become T_{FH} cells. Using the B10.BR mice, expressing transgenic TCRs specific for pigeon cytochrome C (PCC) with different levels of affinity towards the Ag, Baujohann et. al. demonstrated that adoptive transfer of equivalent numbers of high and low affinity T cells resulted in the high affinity cells preferentially differentiating into T_{FH} cells. In high affinity cells, but not those of low affinity, increasing doses of antigen led to the upregulation of IL-21 and down-regulation of Blimp-1, which are key features for T_{FH} cells [46]. Following Ag recognition, DCs provide co-stimulation to further activate the $CD4^+$ T cells, via engagement of CD28 by CD80 or CD86. Crosstalk between CD40L on

activated T cells and CD40 on DCs leads to enhanced DC activation which can further support differentiation of $CD4^+$ T cells (**Figure 1.3**) [22, 47]. ICOS-ICOSL interactions between the activated T cell and DCs are critical for inducing expression of Bcl-6 and CXCR5, and thus the early initiation events for becoming a differentiated T_{FH} cell (**Figure 1.3**) [44, 48].

IL-2, IL-6, IL-12, IL-21, and IL-27 are important cytokines for controlling the initiation, differentiation and maintenance of the T_{FH} phenotype by acting in both negative and positive fashions. IL-2 is a negative regulator of T_{FH} differentiation. Normally, in a naïve or recently activated T cell, a T-bet-Bcl-6 complex masks the Bcl-6 deoxyribonucleic acid (DNA) binding domain, which blocks Bcl-6 from repressing its target genes. IL-2 promotes T-bet expression, therefore when IL-2 concentrations are low, Bcl-6 is preferentially increased because there is a skewing of the ratio of T bet to Bcl-6 [31]. IL-6 production by DCs is important for the early generation of T_{FH} cells during LCMV infection by upregulating Bcl-6 in T cells [49]. In humans, IL-12 is very efficient at inducing T_{FH} differentiation *in vitro*, but is not required for murine T_{FH} cell development [50]. Human GC-T_{FH} cells in the tonsil express high levels of the IL-12 receptor, further highlighting the potential significance of IL-12 signaling in human T_{FH} cell generation [33]. Consistent with these *in vitro* findings, patients deficient in a portion of the IL-12 receptor have a lower frequency of circulating T_{FH} cells. T cells from these patients are hypo-responsive to TCR stimuli, failing to produce cytokines necessary to drive optimal B cell responses, such as IL-21 [33].

Figure 1.3

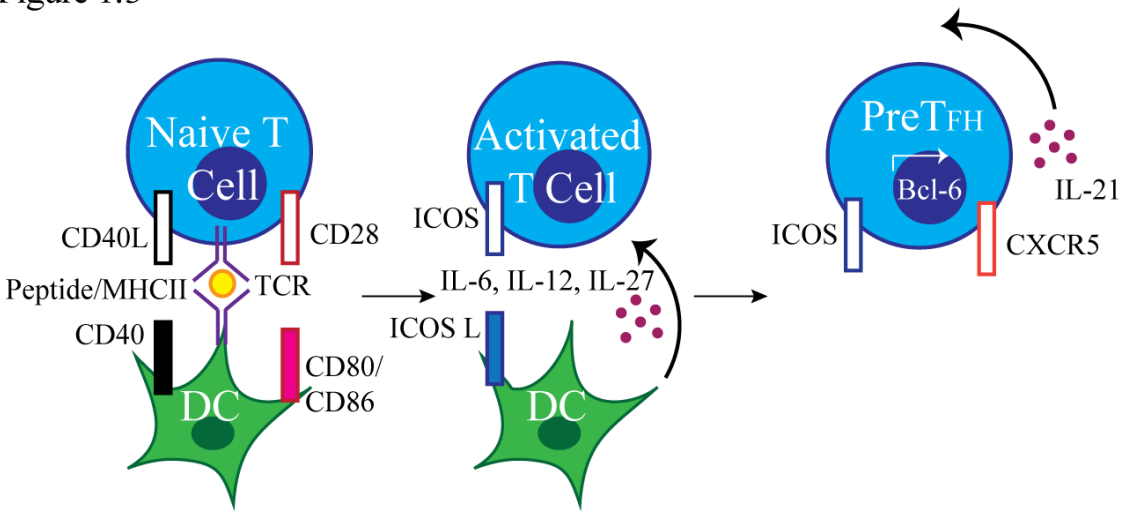

Figure 1.3. Early development of T_{FH} Cells. Initial recognition of Ag presented by MHC-Class II on DCs by the TCR, in combination with co-stimulation mediated by CD40/CD40L and CD28/CD80/CD86, initiates activation of $CD4^+$ T cells. Interaction between ICOS and ICOSL and cytokine production by DCs further drives enhanced ICOS expression, CXCR5 expression, Bcl-6 transcription and the initiation of IL-21 production by the emerging T_{FH} population.

IL-21 is another important cytokine for the generation and maintenance (as well as function) of murine T_{FH} cells but is not critical for human T_{FH} cell differentiation (though still remains important for their function) [51]. IL-21, produced in an autocrine feedback loop by the developing T_{FH} cell, is necessary for optimal CXCR5 expression within the T_{FH} compartment, which is important for maintaining the T_{FH} cell within the B cell follicular [52]. IL-27 supports T_{FH} development by enhancing the autocrine production of IL-21 (**Figure 1.3**) [53]. These findings clearly establish a role for many different cytokines in the differentiation towards a T_{FH} lineage. In addition, type of Ag, immunization route, and timepoints analyzed appear to be critical in the interpretation for the role of specific cytokines.

Cytokine signaling is mediated through the janus kinase (JAK)- signal transducer and activator of transcription (STAT) pathway of proteins. These family members act as both positive and negative regulators of the T_{FH} differentiation program. Consistent with IL-6 and IL-21 playing an important role in the acquisition of a T_{FH}-like phenotype, the target of their signaling, STAT3, is critical for T_{FH} differentiation in both mice and humans [54]. $CD4^+$ T cell helper activity *in vitro* (as measured by Ig production from co-cultured autologous or heterologous B cells), was dependent on STAT3, as shown by a decrease in *in vitro* B helper responses, when STAT3 signaling was blocked with a small interfering (si)RNA [54]. In humans, STAT4 is another important signaling molecule that leads to the development of T_{FH} cells (likely due to the importance of IL-12, which signals through STAT4), but this is not recapitulated in murine T cells [55]. In contrast, STAT5 negatively regulates the development of T_{FH} cells. Overexpression of STAT5 in adoptive transfer of retrovirally-transduced cells reduced T_{FH} cells and GC B cells *in vivo*

following LCMV infection, whereas STAT5 deficiency leads to a massive outgrowth of T_{FH} cells [56]. As a whole, these findings support a model where communication between DCs and T_{FH} cells, within the T cell zone, through cell surface interactions and production of a host of cytokines by the DC drive several transcription pathways that converge to ultimately generate a functional T_{FH} cell.

T_{FH} Cell Memory

The formation of a memory T cell population is paramount to quickly mounting a robust immune response following secondary exposure to a pathogen. Following the resolution of the immune response, less than 10% of the originally generated $CD4^+$ effector population persists as memory [57]. Classically, the $CD4^+$ T cell memory compartment has been divided into central and effector memory. The central memory cells are largely quiescent and circulate through the secondary lymphoid organs. In contrast, effector memory T cells are capable of producing cytokines quickly upon re-engagement of the TCR and homing to sites of tissue inflammation [57]. The concept of a persistent, stable memory population of T_{FH} cells is still being characterized. The importance of this memory T_{FH} population would be to quickly drive new GC responses following re-exposure to a pathogen. Although not yet published, work described by Marc Jenkins at the 2014 Biology of B Cell Responses Keystone meeting suggests that memory T_{FH} can be distinguished from GC-T_{FH} or central memory $CD4^+$ T cells based on the following phenotype; $CXCR5^+Bcl\text{-}6^+Tbet^-CCR7^+$.

Memory T_{FH} cells derive from a $Bcl\text{-}6^+$ precursor and may come from both the pre-T_{FH} compartment as well as the GC-T_{FH} compartment. T_{FH} cells that persist 21 days

after immunization can be transferred into a new recipient and quickly drive a GC response when re-challenged with Ag. Whether T_{FH} cells that remain 21 days post immunization can be classified as a memory-T_{FH} cell is still debated [34]. Adoptive transfer of Th1 (T-bet controlled, IFNγ producing helper cells) or T_{FH} cells, 21 days post infection, into WT mice showed that, following infection, the transferred cells maintained their original phenotype, suggesting a committed lineage a marker of memory. The presence of B cells was necessary to maintain the CXCR5 expression as transfer of memory T_{FH} cells to B cell deficient (uMT) mice led to a 50% reduction in $CXCR5^+$ T_{FH} cells. Interestingly, the injected cells could be identified as late as 45 days post-transfer. However, whether they represent truly memory T_{FH} cells has yet to be determined.

During LCMV infection both Th1 and T_{FH} cells are generated. $CXCR5^+$ T_{FH} cells have a compartment that is $Ly6C^+$ and the $CXCR5^+LY6C^+$ compartment is expanded during the memory phase of LCMV infection. Given that Ly6C has been shown previously to identify the effector/memory subsets, these findings further support their characterization as a memory T_{FH} cell. Putative memory-T_{FH} cells downregulate PD-1, ICOS, GL-7 and Bcl-6, but maintain CXCR5 in the LCMV infection model. In this setting, memory T_{FH} cells (irrespective of Ly6C expression) no longer possess the ability to produce IL-21, but they can produce IFNγ. When transferred into recipient mice, $CXCR5^+$ cells, irrespective of Ly6C expression become more T_{FH}-like after challenge, than their $CXCR5^-$ counterparts [58]. Because these finding are limited to only a handful of reports, the existence of a functional memory T_{FH} compartment is still at an early stage of investigation and requires more work for clarification.

T Follicular Regulatory Cells and Other Mechanisms of T_{FH} Regulation

The resolution of the GC response following generation of PCs and memory B cells is critical for maintaining homeostasis. The necessity of regulating the T_{FH} compartment for ensuring tolerance is one of the fundamental reasons for the restraint of T_{FH} cells both during and following resolution of an infection. Moreover, the outgrowth of the T_{FH} compartment during any phase of the GC reaction would allow for the survival of GC B cells with self-reactive BCRs (a natural phenomenon during the process of SHM where random mutations occur). Therefore, numerous mechanisms may exist to prevent this unwanted immune response and maintain homeostasis.

The factors that control the process that restrains an ongoing GC response are still being explored, but several theories have emerged. One mechanism may stem from a loss of Ag. If Ag is no longer taken up by fDCs, GC-B cells can no longer sample and present Ag to T_{FH} cells and thus GC-B cells would die by neglect from lack of survival signals, leading to GC resolution. Another possibility is that the differentiation of GC-B cells towards a memory B cell or PCs results in GC resolution. Lastly, reduction of T_{FH} cell compartment may remove requisite survival signals that ultimately lead to loss of GC-B cells and hence the GC structure [59].

The mechanisms that control T_{FH} resolution following an immune response are still being elucidated. A potential role for PCs in this process has been reported. *In vitro* co-culture experiments demonstrated that PCs actively decrease IL-21 production and Bcl-6 expression in T_{FH} cells [60]. This finding was further supported by *in vivo* experiments, where a loss of PCs (by depleting Blimp-1 in PCs only), dramatically increase the percentage of T_{FH} cells [60]. An additional mechanism involving $CD8^+$ T

cells has also been proposed. $CD8^+$ T cells can suppress CD4 mediated responses through both lytic (perforin and upregulation of FasL) and non-lytic (IL-10, transforming growth factor beta (TGFβ)) mechanisms [61]. Given that $CD8^+$ T cells can enter B cell follicles during the GC response and $CD8^+$ T cells expressing CXCR5 can be found in human tonsil samples support a model where $CD8^+$ T cells may directly suppress T_{FH} cell responses [62]. However, these mechanisms do not appear to be able to account for all regulation of T_{FH} cells.

T regulatory cells (Treg) are a subset of $CD4^+$ T cells necessary for maintaining immune homeostasis by suppressing the effector function of T helper subsets. Scurfy mice and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients (deficient in Treg cells due to a loss of function mutation in the forkhead box P3 (*foxp3)* gene) have an aberrant immune repertoire and succumb to autoimmune disease very early in life [63]. Mice deficient in *foxp3* develop spontaneous GCs and they have an increased frequency of T_{FH} cells, suggesting an important role for $CD4^+$ T regulatory cells in control of the T_{FH} compartment [62, 64]. This possibility was supported by the discovery of a novel population of regulatory cells in human tonsils identified as: $CD4^+CXCR5^+CXCR6^+CXCR3^+CD25^+CD69^{-/+}$. These cells were located both inside the GC and in the intra-follicular region. To demonstrate their functional ability, Lim et. al. co-cultured autologous T_{FH} cells and B cells in the presence or absence of the tonsillar regulatory cells. These cells potently suppressed T_{FH}-induced B cell responses (specifically *in vitro* IgG production). There was also a reduction in activation induced cytidine deaminase (AID) induced by T_{FH} cells [65]. However, the mechanism

that mediated these suppressive responses and the verification that these cells were in fact FoxP3$^+$ or derived from a FoxP3$^+$ precursor, were not addressed.

Identification of T follicular regulatory (T_{FR}) cells, as they are now known, was performed simultaneously by three research groups. Murine T_{FR} cells are characterized by the following expression pattern: CD4$^+$FOXP3$^+$Blimp-1$^+$Bcl-6^{low}PD1$^+$CXCR5$^+$. T_{FR} cells require T_{FH} cues for their development. For example, Bcl6$^{-/-}$ mice, CD28$^{-/-}$ mice, SAP$^{-/-}$ mice and B cell deficient mice (μMT) fail to generate T_{FR} cells following immunization [59, 66, 67]. Importantly, T_{FR} cells are derived from a FoxP3$^+$ pre-existing Treg and not from a T_{FH} cell that upregulates FoxP3. *In vitro* studies have shown that T_{FR} cells induce suppression of CD4$^+$ T cell proliferation, but whether they differentially suppress conventional CD4$^+$ T cells as compared to T_{FH} cells was not addressed by any of the groups [59, 66, 67] . Although T_{FR} cells have been characterized in human tonsil, whether they share the same function as T_{FR} cells found in the secondary lymphoid organs has not been studied [67]. In addition to T_{FR} cells found in secondary and tertiary lymphoid organs, a recent report in a murine model has identified the presence of circulating T_{FR} cells [68]. Co-transfer of blood-derived murine T_{FR} cells and T_{FH} cells into T-cell deficient hosts substantially reduced the ability of T_{FH} cells to induce Ag-specific B cell responses, compared to recipients who did not receive T_{FR} cells [68].

Although capable of suppressing CD4$^+$ T cell proliferation *in vitro*, the role of T_{FR} cells *in vivo* is more complicated. Using Foxp3-Diptheria Toxin Receptor (DTR) mice, which allows for the ablation of all Foxp3$^+$ cells with a minimal dose of diphtheria toxin [69], one group reported no major changes in the percentage and total number of T_{FH} cells, GC B cells and PCs following ablation of T_{FR} cells, and only observed a reduction

in the number of high affinity Ag-specific PCs [66]. In contrast, another group reported a substantial outgrowth of T_{FH} cells and the Ag-specific B cell response following loss of T_{FR} cells [59, 67] . However, the DTR model depletes all Treg cells and as such is not specific to the T_{FR} population. Therefore, whether T_{FR} cells act to limit the outgrowth of non-Ag specific GC-B cell clones in GCs or are important for limiting the T_{FH} response as a whole is currently unclear.

Conventional regulatory T cells are $CD4^+Foxp3^+$ cells that do not express the T_{FR} markers PD1 or CXCR5. These cells suppress by targeting some aspect of the effector T cell program (cytokine production, proliferation or survival) or by targeting the antigen presenting cells (APCs) that stimulate the effector cells. Treg cells mediate suppression via reduction of cytokines (for example, IL-2) or production of cytokines such as IL-35, IL-10 or TGFβ, which limits the survival of the effector population by acting on co-stimulatory pathways such as CD28, inducing cell-cycle arrest or apoptosis [70-72]. Since most reports demonstrate that transwell experiments are not very effective at changing the suppression ability of regulatory cells, production of soluble mediators must be in combination with important cell surface proteins. Regulatory T cells can also suppress effector populations by reducing the stimulatory capacity of APC via cytotoxic T lymphocyte antigen 4 (CTLA4)-CD80/86 interactions, lymphocyte activation gene 3 (LAG-3)-MHC II, neurolipin 1 (Nrp-1) and CD39-AMP, which serve to decrease costimulation, maintain immaturity status and decrease Ag presentation. Granzyme mediated production can result in apoptosis of conventional T cells (often broadly categorized as $CD4^+CD25^-$) and Galectin-1 expression results in cell cycle arrest [73]. Whether these known mechanisms of suppression by regulatory T cells translate to the

T_{FR} compartment is unknown. Microarray and flow cytometric analyses have demonstrated that T_{FR} cells increase ICOS, glucocorticoid-induced TNFR family related gene (GITR) and CTLA4. T_{FR} cells have reduced expression of CD40L compared to T_{FH} (and similar to normal regulatory T cells). T_{FR} cells do not produce any B-helper cytokines (IL-21, IL-4), but express the regulatory molecule IL-10. Furthermore, while T_{FR} cells don't express granzyme B, they have transcripts for granzyme A and perforin [66]. However, no reports have addressed whether any of these mechanisms are important either *in vitro* or *in vivo* for T_{FR} suppression.

The generation and localization of T_{FH} cells during the immune response requires both spatial as well as temporal regulatory mechanisms. PCs, $CD8^+$ regulatory cells and T_{FR} cells may all contribute to the control of the T_{FH} compartment in a context-dependent manner. Despite the gaps in our understanding of T_{FH} regulation, the importance of T_{FH} control is evidenced by a number of autoimmune diseases that manifest as a result of aberrant increases in this $CD4^+$ compartment. This concept is described in greater detail in the subsequent section.

T_{FH} Cells in Autoimmune Disease – Function and Regulation

The frequency of T_{FH} cells is increased in a number of autoimmune diseases including; myasthenia gravis, Grave's disease and Hashimoto's thyroiditis, juvenile dermatomyositis, SLE, Sjogren's syndrome, rheumatoid arthritis and multiple sclerosis [18]. Despite different etiology and organ involvement, these diseases have one important feature in common; they are characterized by the development of antibodies against self antigens. In multiple sclerosis, an increase in circulating T_{FH} cells correlates with serum

concentration of auto-antibodies [74]. Although this correlation can be observed in most of the autoimmune disease described above, it has been studied extensively in the systemic autoimmune disease, SLE.

SLE patients have increased percentages of $CD4^+CXCR5^+PD1^+$ T_{FH} cells compared to normal controls. Elevated T_{FH} cell levels strongly correlates with SLE Disease Activity Index (SLEDAI), anti-nuclear antibody titers, and PC frequencies. In addition, higher levels of circulating T_{FH} cells are most commonly associated with renal involvement [75]. Lupus nephritis patients generally have a poorer prognosis than SLE patients with non-kidney manifestations. T_{FH} cells can be found throughout the kidney tubulointersitium (45% of samples) of SLE patients [76]. In this study, the severity of kidney inflammation was directly associated with a greater frequency of infiltrating T_{FH}-like cells. T_{FH}-like cells were restricted to tubules and none were found in glomeruli. T_{FH}-like cells in the interstitium formed cognate structures with $CD20^+$ B cells. Using computational tools such as cell distance mapping, the authors demonstrated that the kidney-derived T_{FH}-like cells were forming conjugates similar to those formed in tonsils. Laser microdissection of the T_{FH}-like cells confirmed that cells from the kidney had a phenotype consistent with a T_{FH} cell phenotype, including upregulation of Bcl-6, IL-21 and Batf [77]. These findings suggest that T_{FH} cells may play a role in not only directing B cell response within secondary lymphoid organs, but also operate within the target organ to contribute to end-organ damage *in situ*.

Using Mouse Models to Understand the Role of T_{FH} Cells in Autoimmune Disease

The factors that contribute to the increased frequency of T_{FH} cells in autoimmune disease are still being established. The ability to recapitulate the development of autoimmune disease in the presence of aberrant T_{FH} accumulation using murine models has facilitated our understanding of this pathogenic process. The Sanroque mouse model, which has a spontaneous mutation in the roquin gene, has been instrumental for our understanding of how T_{FH} dysregulation leads to autoimmunity. Sanroque mice have aberrant T_{FH} accumulation ultimately leading to autoimmunity [78]. Roquin contributes to the degradation of ICOS and limiting IFNγ mRNA expression at a posttranscriptional level; thus, null mutations in roquin that prevent ICOS degradation may explain the outgrowth of the T_{FH} population [79]. Upregulation of ICOS leads to an increase in IL-21 production, which promotes the development of GC-B cells. Increased IFNγ receptor (IFNγR) signaling caused Bcl-6 overexpression in T_{FH} cells and suggests that IFNγ supports T_{FH} differentiation [79]. The contribution of ICOS and IFNγ in the autoimmune pathogenesis of the Sanroque model is clearly demonstrated by two observations;1) ablation of ICOS, restores a WT phenotype and 2) IFNγR deficiency prevents lupus development. While additional mechanisms also likely contribute to excessive T_{FH} generation, an important question to consider is why are the regulatory mechanisms that should maintain T_{FH} homeostasis not functioning properly in autoimmune disease?

Although T_{FR} cells have only been recently discovered, there has been a long-standing interest in the function of conventional regulatory T cells in the context of autoimmunity. Although most reports have shown a decrease in the frequency of regulatory T cells in SLE patients, a small number of reports have shown an increase in T

regulatory cells in autoimmune patients [80-83]. In addition, being able to distinguish whether T regulatory cells are unable to suppress, effector T cells are resistant to suppression, or a combination of the two, has been challenging with human SLE patients. However, in a very elegant study by Wehrens et. al., the authors utilized regulatory T cells from two different body locations to demonstrate that in rheumatoid arthritis, resistance to suppression is the cause of aberrant $CD4^+$ T cell accumulation. Regulatory cells taken from the peripheral blood mononuclear cells (PBMCs) were able to suppress effector cells. In contrast, while synovial fluid regulatory cells were able to suppress normal PBMC-derived T cells, they failed to suppress T cell isolated from the synovial fluid [84]. Importantly, none of these studies have distinguished between conventional regulatory T cells and T_{FR} cells. Further work will be required to determine whether T_{FR} cell frequency and/or function is altered in SLE, and if this could be a potential mechanism for the expansion of T_{FH} cells in these patients.

BAFF Signaling Axis

The BAFF signaling axis that regulates the communication among B cells and other cell types, is comprised of five members; the cytokines BAFF and APRIL, and their three shared receptors, BCMA, TACI and BR3 (**Figure 1.4**). BAFF was identified as a member of the TNF ligand superfamily through analysis of a human-granulocyte-derived complementary DNA (cDNA) library [85], and was found to be a potent co-stimulator of B cell activation and antibody production *in vitro* and *in vivo* [86, 87]. The importance of BAFF in normal B cell development and homeostasis is highlighted in BAFF-deficient animals, which have severely reduced peripheral B cells populations [88]. In addition, a

profound biological activity of BAFF on normal B cell effector function was demonstrated by reduced antigen-specific antibody responses and decreased germinal center formation in the spleens of mice treated with soluble TACI after antigenic challenge [89]. In contrast, while APRIL is important for providing proliferation and class switching signals, APRIL-deficient animals have minimal perturbations in peripheral B cell populations [90]. Gross et al. identified BCMA and TACI as receptors for BAFF through analysis of BAFF binding to Cercopithecus aethiops kidney 7 (COS7) cells transfected with a cDNA library prepared from the B lymphoblastoid Roswell park memorial institute (RPMI) 1788 cell line [91]. Rennert et. al. further demonstrated that soluble BCMA could also bind the BAFF homologue, APRIL, whose interaction is of higher affinity compared to BCMA-BAFF interactions. A subsequent report also demonstrated that BCMA has a higher affinity for APRIL than for BAFF, [89, 92] which is due to the tyrosine 13 and arginine 27 residues within the cysteine rich domain of BCMA that are essential for APRIL binding but are dispensable for BAFF binding.

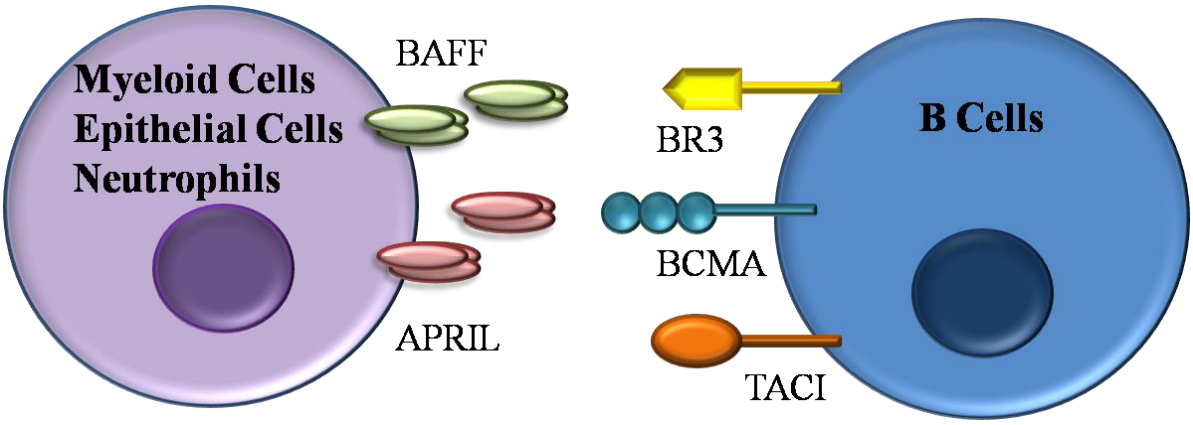

Figure 1.4. The BAFF signaling axis. BAFF and APRIL can be membrane-bound or soluble and are produced by a number of cell types. BAFF binds to BAFF-R (BR3) with extremely high affinity, to TACI with slightly lower affinity and to BCMA with the lowest affinity. APRIL binds to BCMA with high affinity and to TACI with lower affinity but does not bind to BAFF-R (BR3). Additionally, APRIL can bind to heparin sulfate proteoglycans (not shown).

BAFF is a critical factor for B cell maturation, tolerance, and the survival of peripheral B cells through the binding of a third receptor BAFF-R (BR3) [88, 93].

BR3 is first expressed on transitional B cells at the T2 stage and increases its expression throughout B cell maturation, with the highest levels of BR3 expression on B cells in the lymphoid follicle and the marginal zone of the spleen [94, 95]. BR3-deficient mice resemble the phenotype of BAFF-deficient mice, showing impaired B cell maturation at the transitional stage and the loss of mature B cell populations [96, 97]. BCMA is almost exclusively expressed in the PC compartment, but under certain conditions and disease settings, can be found within non-lymphoid tissues as well as other cellular subsets (**Table 1.1**). Because the predominant expression of BCMA is in B lineage cells and BAFF can drive B cell proliferation and antibody production, it was anticipated that a deficiency in the BCMA gene would greatly influence B cell responses. However, analysis of BCMA deficient mice revealed no overt differences in the steady-state frequencies or composition of peripheral B cell subsets (immature, marginal zone, follicular, B1) compared to wild type mice [98]. These observations were consistent with $BCMA^{-/-}$ mice showing normal B cell development in the bone marrow (pre-B, immature) compared to control animals, albeit based on limited analysis. Moreover, there were no differences in basal circulating antibody titers between naïve $BCMA^{-/-}$ and wild type mice. Ag-specific B cell responses to both T cell-independent and T cell-dependent antigens, including the formation of GCs, were also found to be equivalent between $BCMA^{-/-}$ mice and controls [98]. In contrast, the survival of long-lived PCs in the bone marrow is impaired in the absence of BCMA [99, 100].

TACI is primarily expressed on GC B cells and memory B cells. Deficiency in TACI expands T_{FH} cells (and GC B cells) but leads to less PC survival. The increase in T_{FH} frequency in $TACI^{-/-}$ animals is evident within the context of the peyer's patches at steady-state, but in the spleen, only occurs following T-dependent immunization [101]. The increased frequency of T_{FH} cells in $TACI^{-/-}$ mice was found to be due to over-expression of ICOSL on B cells as ablation of one allele of ICOSL leads to normalization of T_{FH}/GC response in $TACI^{-/-}$ animals [101].

The BAFF signaling pathway through BR3 is the best defined among the three BAFF receptors. When BAFF binds to BR3, the adaptor protein TNF receptor associated factor 3 (TRAF3) is recruited to the receptor, preventing the degradation of NF-κB-inducing kinase [102-104]. In turn, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-inducing kinase processes the classical NF-κB pathway substrate, p100, to generate the alternative NF-κB2 substrate, p52. Translocation of p52 into the nucleus promotes cell survival by inducing the anti-apoptotic protein myeloid leukemia cell differentiation protein 1 (Mcl-1) and the serine-threonine kinase proviral integrations of Moloney virus 2 (Pim2) [102, 105]. Mcl-1 and Pim2 promote B cell survival by inhibiting transcription of the pro-apoptotic genes bcl-2 associated agonist of cell death (*Bad)* and Bcl-2 like 11 (*Bim)*, respectively. This B cell survival pathway is both mediated and limited by low amounts of BAFF present in the periphery under normal physiologic conditions and is critical for maintaining non-autoreactive B cells.

Table 1.1. BCMA expression in tissue and cellular sources [106]

Source	Identification Method
Cell Lines:	
Acute Lymphoblastic Leukemia: LBL31[107]	RT-PCR, IP
*Dendritic Cell: D2SC1,[108] CB1DG[108]	RT-PCR
*Epithelial Tumor: 167[107]	RT-PCR
Glioma/Astrocytoma: SKMG12,[109] T98H,[109] SF126,[109] U399MG,[109] U118MG,[109] CB193[109]	RT-PCR
Hodgkin: HD-MyZ,[110] L428,[110] KM-H2,[110] L1236[110]	RT-PCR, WB
Mantle Cell/Burkitt's Lymphoma[111]	RT-PCR, WB, FM
*Myeloma/Carcinoma: U266,[107] HT29,[112] KAS-6/1,[112] DP-6,[112] A20,[108] RPMI8226,[107, 113] KMS11,[114] J558L[108, 115]	*RT-PCR, FC, WB*
Non-Hodgkin's Lymphoma: BL36,[107] Raji,[107] Daudi[107, 116]	NB, RT-PCR, IP
Precursor B Cell: JEA,[107] DLBCL,[117] 207,[118] SUP-B15,[118] BLIN-1,[118] NALM-6[118]	RT-PCR, FC
Primary Cells:	
Adipocytes[119, 120]	IHC, RT-PCR, FC
*Bone Marrow B Cells[121, 122] (Plasma cells, Memory)	FC, RT-PCR
Fibroblast-like Synovial Cells[123]	RT-PCR, IHC
Keratinocytes[124]	FC, IHC
Peripheral Blood Cells[112, 120, 125-135]	FC, MR, RT-PCR, TP
*Peritoneal B Cells[136]	RT-PCR, FC
*Splenic B Cells[112, 122, 126, 137-139] (CD38+, Memory)	FC, RT-PCR
Tonsillar B Cells[110, 111, 121, 126, 140-144] (Germinal center, Naïve, Memory, Centroblasts, Centrocytes)	FC, FM, IHC

Tumor Cells: Waldenstrom Macroglobulinemia,[145] Mantle Cell/Burkitt's Lymphoma,[111] Hibernoma,[119] Liposarcoma,[119] Multiple Myeloma,[112, 146] Chronic Lymphocytic Leukemia,[147] Squamous Cell Carcinoma[124]	RT-PCR, WB, FM, FC, IHC
Tissue:	
Adrenals[107]	RT-PCR
Ilieum/Colon[144]	RT-PCR
Kidney[148]	RT-PCR
Liver[107]	RT-PCR
Lymph Node[107]	RT-PCR
Placenta[149, 150]	SB, RT-PCR, IHC
Spleen[107, 108]	RT-PCR
*Submandibular gland[151, 152]	MR, FM
Thymus[107, 108]	RT-PCR

*Denotes data from both human and mouse

FC – Flow Cytometry, MR –Microarray, FM, Fluorescence Microscopy, IHC – Immunohistochemistry, SB – Southern Blot, NB – Northern Blot, WB – Western Blot, IP – Immunoprecipitation, TP – Taqman Probe

The role for BAFF-BR3 interactions in controlling B cell survival involves additional signals; cross talk between BR3 signaling and B cell receptor signaling pathways is important for regulating B cell homeostasis in the periphery [94]. Since B cells undergo apoptosis in the absence of B cell receptor or BR3 signaling, signals delivered through both receptors appear to be required for the survival of mature B cells[97, 153]. Under normal physiologic conditions, signaling through BR3 alone would eventually exhaust intracellular stores of p100 to generate p52 and curtail B cell survival. Thus, reserves of p100 are needed to support the survival of B cells. Low-level tonic B cell receptor signals trigger the classical NF-κB pathway, leading to the upregulation of p100 transcription that replenishes p100 stores. Interestingly, autoreactive B cells are more dependent than other B cells for BAFF-mediated survival [154, 155]. The increased dependency for BAFF-mediated survival of autoreactive B cells could result from increased occupancy of BR3, or other BAFF receptors. Thus, when the availability of BAFF is limited in normal physiologic conditions, healthy mature B cells preferentially survive while autoreactive B cells die by apoptosis.

The Ashkenazi group was among the first to establish the signaling mechanisms by which BCMA delivers its co-stimulatory activity to B cells for IgM production;[156] interaction of BCMA (as well as TACI) with APRIL or BAFF induces the activation of NF-κB [91, 157-159]. Biochemical analysis of BCMA-mediated activation of the NF-κB pathway further identified multiple downstream signal transducers. Transfection studies using 293 cells that overexpress BCMA and dominant negative mutants of the signaling proteins TNFR-associated factor 2 (TRAF2), TRAF5, TRAF6, NF-κB-inducing kinase, Iκβ kinase (IKKα), and IKKβ inhibited BCMA-mediated activation of the NF-κB

pathway [157]. Consistent with these findings, BCMA was found to physically interact with TRAF2, TRAF5, and TRAF6 as demonstrated from co-immunoprecipitation experiments [157]. TRAF1 and TRAF3 were also determined to physically interact with BCMA, but their functional role in BCMA-mediated NF-κB activation is unknown [159]. Activation of the NF-κB pathway through BCMA signaling was confirmed using an agonistic anti-BCMA antibody to membrane-expressed BCMA on the human macrophage-like cell line THP-1[160], and using the H929 B cell lymphoma line when stimulated with APRIL[161]. However, BAFF stimulation of B cell lymphoma cells demonstrated that BCMA did not interact with TRAF3 in contrast to BR3 and TACI, which both interacted with TRAF3 following stimulation [104]. Analysis of the intracytoplasmic domain of BCMA revealed that a 25 amino acid segment (position 119-143) is critical for its interaction with the TRAFs and the activation of NF-κB [157]. This segment was also found to be important for the activation of ETS domain containing protein (Elk-1), a substrate for c-Jun N-terminal kinase (JNK), p38, and extracellular signal regulated kinase (ERK). Activation of JNK and p38 were also determined in human adipose-derived stem cells when stimulated with BAFF and APRIL; however, since both TACI and BCMA are expressed by these stem cells the relative contribution of TACI and BCMA to MAPK activation is unknown [120].

BAFF & T Cell Responses

Although BAFF is critical for optimal B cell responses, its role within other lymphocyte subsets is less defined. In murine models, BAFF is able to stimulate $CD4^+$ T cell proliferation [142]. BAFF signaling also induces the anti-apoptotic factor Bcl-2 in

$CD4^+$ T cells, which may suggest BAFF plays a role in $CD4^+$ T cell survival. T cells from A/WySnJ mice (which lack BR3) fail to respond to BAFF signaling, suggesting that the majority of responses mediated by BAFF are signaling through BR3 [142]. Consistent with these observations, $CD4^+$ T cells from mice do not express TACI and circulating human T cells do not express BCMA (personal observations). In mice, BR3 expression is highest on effector memory and central memory $CD4^+$ T cells compared to naïve $CD4^+$T cells [162, 163]. In humans, both naïve and memory $CD4^+$ T cells respond to supra-physiological doses of BAFF by proliferation as well as some cytokine production [164]. BAFF can skew previously activated cells towards a Th2 phenotype, and also induce low levels of TNFα, IL-2 and IFNγ. Importantly, BAFF only worked at very high concentration and in conjunction with TCR signaling. This suggests an important regulatory mechanism, that Ag recognition and engagement is required for elevated BAFF levels to exert an effect. BAFF could also induce CD25 expression on T cells. BAFF induced proliferation is IL-2 dependent because blocking CD25 *in vitro* resulted in reduced proliferation [162-164].

Under homeostatic conditions, the levels of circulating BAFF are quite low. However, overproduction of BAFF by hyperactive myeloid cells has been shown to contribute to autoimmune disease by acting directly on T cells to induce the release of IFNγ in murine autoimmune models [165]. Lyn is a member of the src family of protein kinases and is important in cell activation. $Lyn^{-/-}$ mice have overt hyperactivation of multiple myeloid cell populations, increased frequencies of lymphocytes and develop fulminant autoimmune disease [166]. $Lyn^{-/-}$ mice have elevated levels of serum IFNγ and IFNγ-producing $CD4^+CD44^+$ T cells, which increase dramatically as the mice age. Loss

of IFNγ on the $Lyn^{-/-}$ background results in an improved autoimmune phenotype (including reduced BAFF levels). Adoptive transfer of $BR3^{-/-}$ T cells but not WT cells into $lyn^{-/-}$ hosts, led to decreased IFNγ production in $BR3^{-/-}$ T cells compared to WT T cells (from the same host) [165]. These findings suggest that in the context of autoimmunity, BAFF levels can have a profound impact on the $CD4^+$ compartment, leading to proliferation and cytokine production, both of which can be pathogenic. Importantly, all of the studies to date have examined the effect of BAFF on total $CD4^+$ T cells or activated ($CD25^+/CD44^+$) $CD4^+$ T cells, but never specifically at the T_{FH} compartment.

One largely unanswered question is whether or not, in addition to being able to respond to BAFF, T cells produce BAFF. A few reports have shown that murine T cells have BAFF messenger ribonucleic acid (mRNA) expression and produce low levels of BAFF following TCR stimulation [142, 163]. More recently, Cancro's laboratory has shown that T_{FH} cells within the GC have elevated mRNA transcripts for BAFF and can produce BAFF, as measured by immunofluorescence (IF). Importantly, BAFF transcripts were 7-fold lower in T_{FH} cells than $CD11b^+$ cells. However, they showed that while T_{FH}-derived BAFF was dispensable for normal GC cellularity and SHM, it was required for selection of high affinity GC B cell clones following protein immunization [167]. In humans, BAFF has been detected at the mRNA level in peripheral blood T cells. TCR crosslinking appears to increase BAFF at the mRNA level. However, detection at the protein level was not possible (either due to such limited quantities produced or because it was acting back on the T cells so quickly) [162]. In SLE patients, but not healthy controls, BAFF is increased at the protein level (as measured by intracellular flow

cytometry) within circulating $CD4^+$ T cells. In contrast, T_{FH} cells from human tonsil preparations, have no mRNA transcripts for BAFF [17]. Thus, the location of T_{FH} cells as well as the immune status may determine whether BAFF transcription and translation is initiated within $CD4^+$ T cells and, more specifically, within the T_{FH} compartment.

Thesis Rationale

In the past decade, the role of T_{FH} cells in health and disease has become increasingly important as we try to understand the normal immune system, develop new vaccine strategies, as well as treatment options for situations where T_{FH} cells become dysregulated, such as in the context of autoimmune disease. The purpose of this dissertation work is to further characterize the aberrant T_{FH} response that occurs in systemic autoimmune disease, identify novel signaling pathways that T_{FH} cells may employ to become pathogenic in autoimmune disease and extend these findings into the human system in an effort to identify new therapeutic targets. The overall hypothesis of this dissertation work is that BAFF regulates autoimmunity through a T_{FH}-intrinsic pathway. Chapter 2 will focus on the role of the receptor BCMA in controlling T_{FH} cell expansion and function during inflammation and autoimmune susceptibility. Chapter 3 will address the role of dendritic cells in driving aberrant T_{FH} accumulation and function through the production of BAFF. Chapter 4 will highlight a novel role for neutrophils in the pathogenesis of SLE via their direct interaction with $CD4^+$ T cells through both BAFF-dependent and –independent mechanisms. Finally, Chapter 5 will discuss preliminary data related to the dysregulation of the T_{FR} compartment in autoimmunity and in the absence of BCMA.

CHAPTER 2

BCMA Controls T Follicular Helper Cell Expansion & Function During Inflammation and Autoimmune Susceptibility

Peripheral B-cell responses to foreign antigen is a tightly regulated process with multiple checkpoints that generate protective antibodies and prevent the development of autoantibodies [168].The coordinated interplay between antigen-specific B-cells and T_{FH} cells is crucial in this process by establishing germinal centers (GC) that facilitate the selection and differentiation of memory B-cells and plasma cells that produce high-affinity antibodies [169, 170]. It has been shown in mouse models of SLE that accumulation of T_{FH} cells is a significant catalyst of autoantibody production and inhibiting T_{FH} cell formation reduces disease [78]. Therefore, mechanisms must exist that maintain T_{FH} cell homeostasis under normal circumstances to avoid unchecked T_{FH} activity, inhibiting the production of pathogenic autoantibodies that promotes autoimmunity.

Family members belonging to the BAFF cytokine-receptor network (BCMA, BR3, TACI) have been closely linked to B-cell homeostasis and tolerance [171]. Multiple innate immune cell types including dendritic cells and neutrophils produce BAFF [172]. BR3 (but not BCMA) is expressed on mature B-cells, while plasma cells express BCMA (but not BR3). BAFF signaling through BR3 on mature B-cells is critical for their survival [88]. In contrast, BCMA is critically required for survival of bone marrow plasma cells but dispensable for maintaining peripheral B-cell and plasma cell numbers [99, 100]. Increased levels of BAFF have been linked to loss of B-cell tolerance in both autoimmune-prone mice and humans [91, 165, 173, 174]. Given that excess BAFF promotes survival and differentiation of autoreactive B cells that arise in the GC reaction, we initially reasoned that a deficiency in BCMA of lupus-prone mice would deprive autoantibody-producing plasma cells of a key survival factor and therefore reduce

autoantibody production. Paradoxically, BCMA deficiency exacerbates the formation of autoantibody-secreting plasma cells in spleens of autoimmune-prone mice and the reasons for this effect are not understood [139]. Despite evidence that BR3 is expressed on $CD4^+$ T cells, the function in T cells *in vivo* is minimal. Studies in BAFF transgenic mice and a collagen-induced arthritis mouse model demonstrated a role for BAFF in mediating proinflammatory $CD4^+$ T cell responses [175, 176]. However, the potential role for BAFF in T_{FH} cell homeostasis is not known. We therefore sought to address the role of the BAFF signaling axis within the CD4 compartment, in the context of autoimmunity.

Methods

Mice.Tnfrsf17$^{-/-}$, *Nba2*, *Nba2;Tnfrsf17*$^{-/-}$, *lpr*, and *lpr;Tnfrsf17*$^{-/-}$mice, fully backcrossed onto the C57BL/6 (B6) strain, were previously described [139, 177]. Age-matched wild-type (WT) B6 mice carrying the CD45.1 or CD45.2 allele were purchased from NCI. $CD3e^{-/-}$ B6 mice were provided by Taconic. All experiments were performed on individual mice using 4-6 month-old female mice unless indicated. For chimera studies, CD45.1 WT B6 mice were irradiated with 1200 Rad and reconstituted via intravenous (i.v.) injection with 4 x 10^6 bone marrow-derived hematopoietic stem cells from the following CD45.2 donors: 100% WT, 100% *Tnfrsf17*$^{-/-}$, 20:80 ratio of WT:$CD3e^{-/-}$, 20:80 ratio of *Tnfrsf17*$^{-/-}$:$CD3e^{-/-}$. Recipients were maintained on antibiotic water for 4 weeks during reconstitution and used for experiments after 6 weeks of reconstitution. For each experiment, a minimum of 5 mice was used for each strain unless indicated to account for variability in disease phenotype and assess statistical

significance. No mice were excluded from the data analysis and no randomization was performed. Mice were housed in a specific pathogen-free animal facility at the University of Virginia.

Study subjects. Females that met the American College of Rheumatology Revised Criteria for SLE and age-matched healthy females were enrolled in this study during routine clinical care through the Division of Rheumatology at the University of Virginia School of Medicine (Charlottesville, VA). Exclusion criteria included patients who were pregnant or receiving the following treatments: i.v.steroids, cyclophosphamide, >20 mg oral prednisone daily, or B-cell and BAFF-depleting therapies. The SLE Disease Activity Index (SLEDAI) measured disease activity. The SLEDAI is a composite score of 24 symptoms, each with a specific weighted factor, that help to establish the severity of disease. Additional SLE patient blood was purchased from AllCells (Alameda, CA).

Flow cytometry. Mouse spleen single-cell suspensions were prepared and red blood cells were lysed with ammonium chloride–Tris solution. Whole blood was obtained from mice in heparin-treated tubes (Fisher Scientific). Human patient blood was obtained in ACD tubes (BD Bioscience) and was spun down to separate plasma and cellular fractions. Patient sera were prepared with thrombin (Sigma) and peripheral blood mononuclear cells were isolated using a Ficoll gradient (GE Healthcare). For phenotyping, cells were stained at a concentration of 0.5-2 $x10^6$ cells/well in a 100µl volume. Mouse antibodies included the following: BioLegend: BAFF-R (7H22-E16), CD86-PE (GL-1); eBioscience: PD-1-FITC (RMP1-30) or PD-1-biotin (J43), CD25-PE or CD25-APC (PC61.5), orCD25-biotin (eBio7D4), GL-7-Alexa647 or GL-7-EF450 (GL-7), CD4-EF450 or CD4-PerCP (GK1.5), IL-21-PE (mhalx21), ICOS-biotin

(7E.17G9);BD Bioscience: B220-FITC, B220-PE, B220-APC, B220-PECy7 or B220-PerCP/Cy5.5 (RA3-6B2), CD11c-PECy7 or CD11c-biotin (HLS), GL-7-FITC (553666), Bcl-6-PE (K112-91), ICOS-PE (7E.17G9), CD138-APC (281-2), CXCR5-APC or CXCR5-PECy7 (2G8), CD95-PECy7 (Jo2), CD4-PECy7 (RM4-5); R&D Systems: BAFF-R-FITC (FAB1755F), BCMA-FITC (FAB593F), BAFF-R-biotin (MAB1755), TACI (MAB1041). Human antibodies included the following: BioLegend: CXCR5-PerCP/Cy5.5 (TG2), BAFF-R-APCCy7 (11C1);eBioscience: Bcl-6-APC (BCL-UP), PD-1-PE (eBioJ105), PD-1-APC (MIH4), CD4-biotin (OKT4), ICOS-EF450 (ISA-3), TACI-PE (11H3); BD Bioscience: Bcl-6-PE (K112-91);R&D Systems: BCMA-FITC or BCMA-PE (FAB193F). Streptavidin was used on the following fluorophores: FITC, PE, APC, PERCPCy5.5, EF450, and APC-Cy7. Cell viability was determined using live/dead AQUA (Invitrogen) according to manufacturer's instructions. Following staining, cells were sorted, fixed in 1% formaldehyde (Fisher Scientific), or suspended in Fix/Perm Wash Buffer (eBioscience). Samples were acquired on a CyAn ADP LX (Beckman Coulter) or sorted with a Influx Cell Sorter (Becton Dickinson) and analyzed using FlowJo software version 9.3.3 (Treestar Inc.).

Histology and immunofluorescence. Frozen 5μm tissue sections were stained with the following reagents: PNA-FITC or PNA-biotin (Sigma-Aldrich); IgD-EF450 (11-26), CD4-EF450 (GK1.5) from eBioscience; IgD-PE (11-26c.2a) from BioLegend; CD-4 PE (GK1.5) from BD Bioscience; C3-FITC (CL7503F) from Cedar Lane Labs; IgA-biotin or IgG-biotin from Southern Biotech. Streptavidin-EF450 was purchased from eBioscience. Sections were analyzed on an Axio Imager 2 with Apotome (Zeiss). Magnification is provided in the figure legend.

Cytokine and immunoglobulin ELISAs. Murine BAFF (Apotech: capture mAb 5A8; detection mAb 1C9) and human BAFF (eBioscience, Cat# BMS2007INST) levels were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Total IgG, IgA, and antigen-specific antibodies were measured by ELISA as previously described [139].

Co-culture assays. Splenic $PD1^{+}CXCR5^{+}T_{FH}$ cells were sorted to greater than 98% purity. B-cells from spleens of WT mice were purified to greater than 95% purity using Miltenyi MACS® kit. Carboxyfluorescein succinimidyl ester (CFSE)-labeled B-cells (4×10^4) were cultured alone or with T_{FH} cells (4×10^5 or 2.5×10^3) in the presence of anti-CD3(1 μg/ml; 145-2C11, eBioscience), anti-CD28 (1 μg/ml; 37.51, BD Bioscience), IL-2 (10 ng/ml; Peprotech), and anti-IgM (1 μg/ml; B76, ATCC) for 3-6 days. B-cells were analyzed by flow cytometry for B-cell activation and proliferation, and culture supernatants were analyzed for antibody production by ELISA.

Modulation of BAFF in vivo. Mice were given an intra-peritoneal (i.p.) injection of 200μg TACI-mFc or control mFcIgG4 (Merck-Serono) every other day for 12 days. On day 13, spleens were removed for analysis. Alternatively, mice were given an i.p. injection of 100μg murine BAFF (BioExpress) for 3 consecutive days. On day 4, spleens were removed for analysis.

Polymerase Chain Reaction (PCR) and real-time quantitative PCR (RT-PCR). For PCR, cells were isolated and suspend in the following buffer; 1 molar (M) Tris-HCL, 0.5M EDTA, 5M NaCl, 10% SDS in water. Following incubation, samples were washed with 75% ethanol. The pellet of DNA was then re-suspended in deionized water for use in the PCR reaction. DNA was added to a cocktail of di-nucleotide triphosphates

(Promega), Taq polymerase (Invitrogen) and magnesium chloride (Invitrogen). PCR was performed using the following primer sequences (IDT Technologies): Neo Forward (Fwd)-5'-CTT GGG TGG AGA GGC TAT TC-3', Neo Reverse (Rev)-5'-AGG TGA GAT GAC AGG AGA TC-3', *β-actin* Fwd-5-AGG CCA ACC GCG AGA AGA TGA-3', *β-actin* Rev-5'-GAA GTC CAG GGC GAC GTA GCA-3'. For RT-PCR, cells were re-suspended in RLT/2-ME buffer and total RNA was isolated using the RNAqueous® Micro Kit (Ambion). cDNA was generated using the iScript™ cDNA synthesis kit (Bio-Rad), according to manufacturer's instructions. Real-time PCR was performed using the following primer sequences (IDT Technologies):*BCMA* Fwd-5'-GGC GCA ACA GTG TTT CCA CA-3', *BCMA* Rev-5'-CTC GGT GTC GGC CTT GTC CA-3', *GAPDH* Fwd-5'-AAC GAC CCC TTC ATT GAC-3',*GAPDH* Rev-5'-TTC ACG ACA TAC TCA GCA C-3'. Samples were run on a BioRadMyiQ™ System.

BAFF binding assays. Splenocytes were incubated with 1 μg/ml Flag®-tagged BAFF (BioExpress) for 30 minutes on ice. After 3 washes, cells were stained with anti-DYKDDDK Flag-specific monoclonal antibody (mAb) (BioLegend) and T_{FH} cell surface markers, and analyzed by flow cytometry. Data are presented as corrected mean fluorescence intensity (MFI), which is calculated as the MFI of the stained samples minus the MFI of the fluorescence minus one (FMO) control stains.

Pristane treatment. Mice were given a single i.p. injection (0.5mL) of PBS (Gibco) or TMPD (2,6,10,14-tetramethylpentadecane), commonly known as pristane (Sigma-Aldrich), as previously described [178]. After one month, kidneys, spleens, and sera were removed and prepared for immunofluorescence to measure complement 3 (C3) and IgG deposition, flow cytometric analysis of T and B-cell populations, and ELISA to

measure BAFF, and autoantibody serum titers. A scoring system was developed to determine the severity of symptoms (**Table 2.1**). For adoptive transfer studies, splenic naïve $CD4^+$ T cells (5×10^6) from CD45.2 WT and *Tnfrsf17*$^{-/-}$ mice were i.v. injected into naïve WT CD45.1 recipients followed by i.p. injection with pristane. After one month, autoimmune traits were measured.

Statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Multiple group comparisons were performed by one-way analysis of variance (ANOVA) and two-way ANOVA was used for analysis of more than two groups when repeated measures were being compared. Two-tailed Student's t test was used for the analysis of two independent groups. Pearson's correlation coefficient was used to determine the strength of association between two variables. All data were normally distributed with similar variance between the groups. For all tests, a p value less than 0.05 was considered statistically significant and stated in the figure legend (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Prism software v5.0 (GraphPad Software) was used for statistical analyses.

Study approval. All animal procedures were conducted in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Virginia (protocol no. 3506). Female healthy donor and SLE patient blood for this study were obtained with written informed consent from the protocol, Plasma Cells in Systemic Lupus Erythematosus Pathogenesis, which was approved by the University of Virginia Institutional Review Board (protocol no. 16244).

Results

Deficiency in BCMA promotes accumulation of T_{FH} cells and abnormal GC B-cell responses in autoimmune-prone mice. To evaluate the role of BCMA on the GC reaction in autoimmunity, we studied the steady-state GC responses in spleens of naïve healthy C57BL/6 (WT) mice and congenic autoimmune-prone mice that express the murine lupus susceptibility locus *Nba2*on the C57BL/6 background [177], and compared mice sufficient or deficient in BCMA expression.C57BL/6 mice deficient in the gene *Tnfrsf17* that encodes BCMA (*Tnfrsf17*$^{-/-}$) [98] were intercrossed with *Nba2* mice to generate congenic *Nba2;Tnfrsf17*$^{-/-}$ mice [139]. *Tnfrsf17*$^{-/-}$ mice and*Nba2;Tnfrsf17*$^{-/-}$ mice showed significantly higher frequencies of GC structures, GC B-cell numbers and percentages, which arose at an earlier age compared to WT and*Nba2* controls, respectively (**Figure 2.1, Figure 2.2A**).The heightened expansion of GC B-cells in *Nba2;Tnfrsf17*$^{-/-}$ mice correlated with the development of serum autoantibody titers (**Figure 2.2B**); this was dependent on *Nba2* predisposition since *Tnfrsf17*$^{-/-}$ mice produced minimal autoantibodies. Histological examination of GCs showed infiltrating $CD4^+$ T cells in *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$mice (**Figure 2.2C**), and significantly increased $CD4^+$ T cells in aging *Nba2;Tnfrsf17*$^{-/-}$ mice. These $CD4^+$ T cells expressed PD1 andCXCR5, ICOS, Bcl-6, as well as IL-21, confirming their T_{FH} cell identity (**Figure 2.2D and data not shown**).These molecules promote the activation and differentiation of $CD4^+$ T cells into T_{FH} cells [22, 32, 39, 51]. The T_{FH} cell population (defined as $CD4^+PD1^+CXCR5^+$) contains at least two phenotypically distinct populations of T_{FH} cells; T_{FH} and GC T_{FH} cells, with the latter expressing the highest levels of PD1 and CXCR5 and localized in GCs.

Figure 2.1

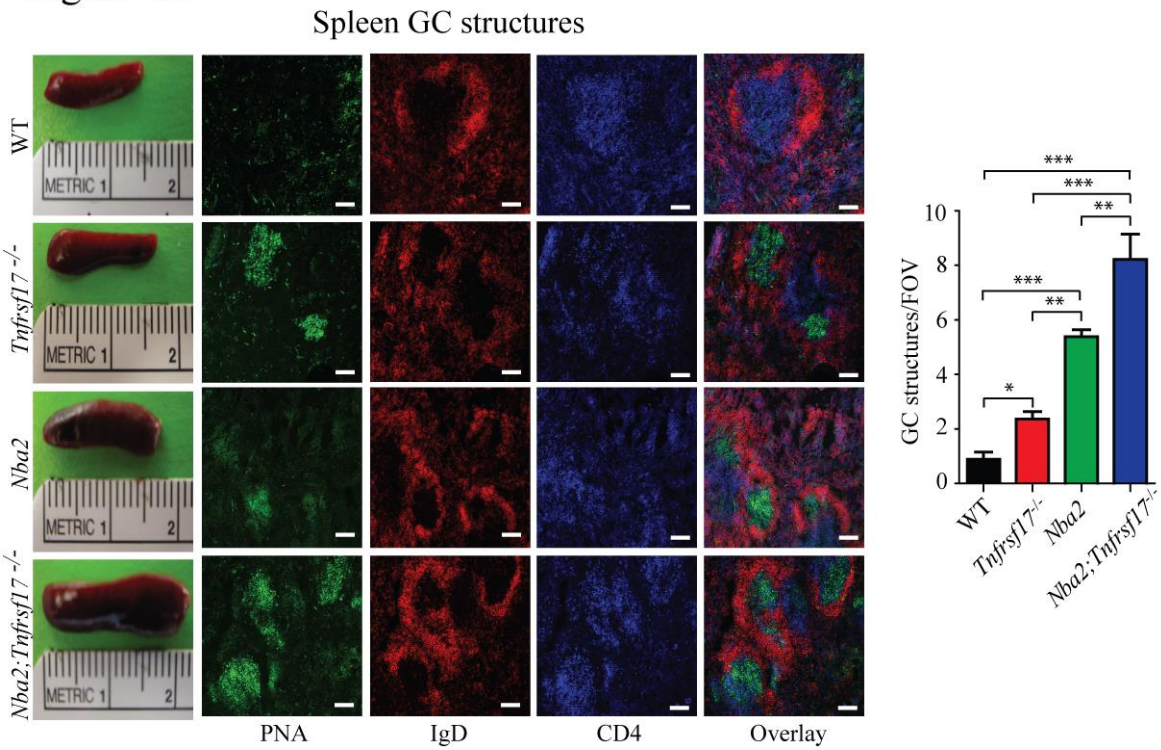

Figure 2.1. Increased spleen size and GC frequency in BCMA-deficient animals. Left panel: Spleens were isolated from 25-week-old mice and measured in cm. IF staining of GCs ($PNA^{+}IgD^{-}$) in spleen sections. PNA – green, IgD – red, CD4 – blue. Scale bars represent 100μm. Data are representative of > 4 mice per strain. Right panel: The number of GC structures was quantified in spleen sections from 4-5 month old female mice by IF. FOV – field of view. Combined data from > 4 mice per strain. Data are presented as mean ± SEM. Statistics determined with a one-way ANOVA and denoted as follows: * P<0.05, ** P<0.01, *** P<0.001.

Figure 2.2

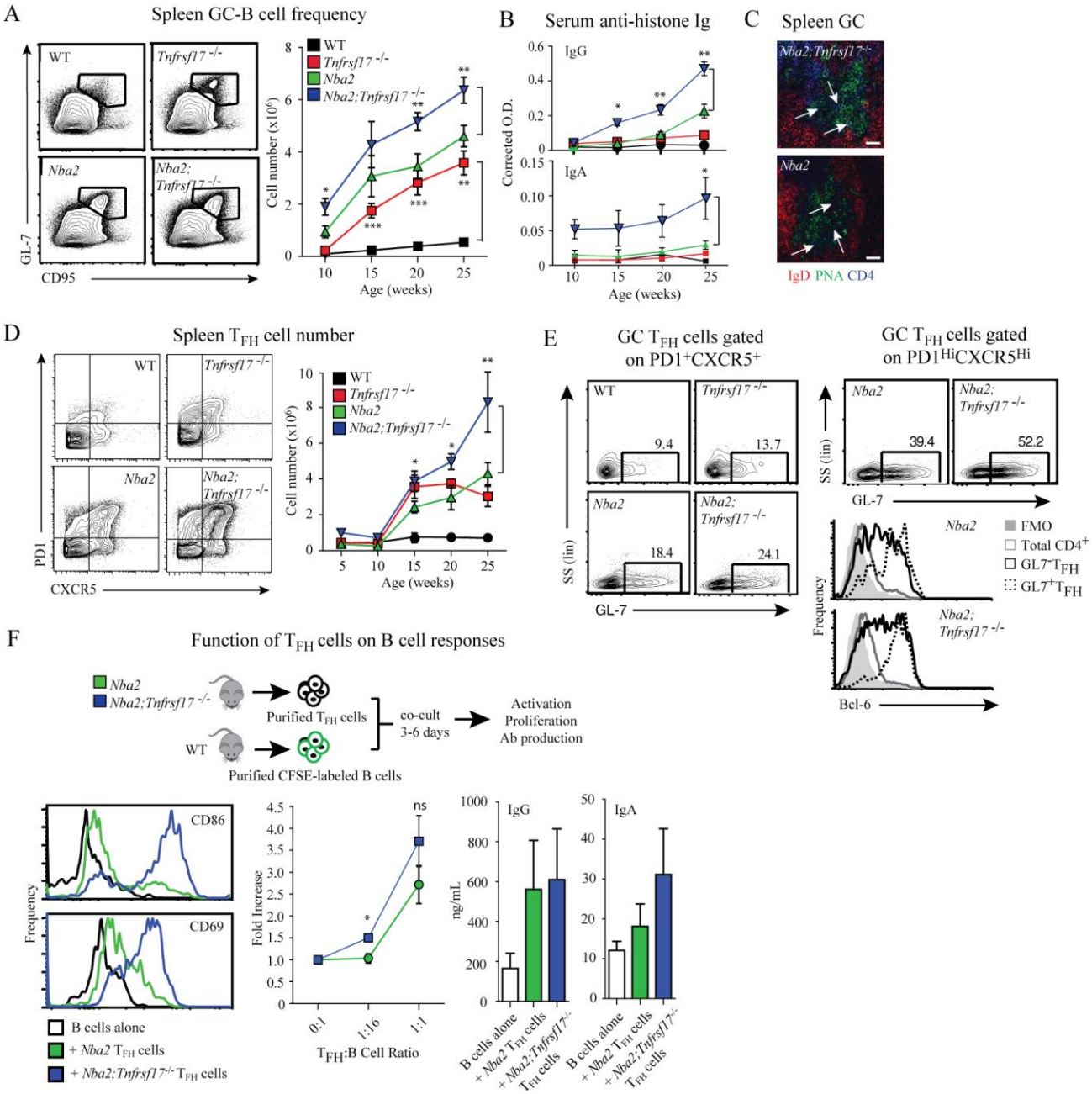
A
Spleen GC-B cell frequency
WT
Tnfrsf17 -/-
Nba2
Nba2; Tnfrsf17 -/-
GL-7
CD95
Cell number (x10⁶)
Age (weeks)
B
Serum anti-histone Ig
IgG
IgA
Corrected O.D.
Age (weeks)
C
Spleen GC
Nba2;Tnfrsf17-/-
Nba2
IgD PNA CD4
D
Spleen T_FH cell number
PD1
CXCR5
E
GC T_FH cells gated on PD1+CXCR5+
GC T_FH cells gated on PD1HiCXCR5Hi
9.4
13.7
18.4
24.1
39.4
52.2
SS (lin)
GL-7
FMO
Total CD4+
GL7-T_FH
GL7+T_FH
Frequency
Bcl-6
F
Function of T_FH cells on B cell responses
Purified T_FH cells
co-cult 3-6 days
Activation
Proliferation
Ab production
Purified CFSE-labeled B cells
CD86
CD69
Fold Increase
ns
T_FH:B Cell Ratio
IgG
IgA
ng/mL
B cells alone
+ Nba2 T_FH cells
+ Nba2;Tnfrsf17-/- T_FH cells

Figure 2.2. BCMA controls the frequency and effector function of T_{FH} cells in autoimmune-prone mice. (**A**) The total numbers of GC B-cells ($B220^+CD95^+GL\text{-}7^+$) were quantified from spleens of 10- to 25-week-old mice by flow cytometry.1 representative contour plot and combined data from WT (n=5), *Tnfrsf17*$^{-/-}$ (n=5), *Nba2* (n=6), *Nba2;Tnfrsf17*$^{-/-}$ (n=7) mice. (**B**) Serum anti-histone IgG and IgA antibodies. Combined data from WT (n=5), *Tnfrsf17*$^{-/-}$ (n=7), *Nba2* (n=7), *Nba2;Tnfrsf17*$^{-/-}$ (n=9) mice. (**C**) Representative confocal microscopy images showing $CD4^+$ T cells within GCs ($CD4^+PNA^+IgD^-$; white arrows) of spleens from 20-week-old mice. Scale bar represents 50μm. (**D**) Total numbers of T_{FH} cells ($CD4^+PD1^+CXCR5^+$) in spleens of mice at the indicated ages were quantified by flow cytometry. One representative contour plot and combined data from WT (n=7), *Tnfrsf17*$^{-/-}$ (n=5), *Nba2* (n=8), *Nba2;Tnfrsf17*$^{-/-}$ (n=9). (**E**) Left: representative flow cytometry plots showing the frequencies of GC T_{FH} cells ($CD4^+PD1^+CXCR5^+GL\text{-}7^+$) in 20-week-old mice of the indicated strains. Right: representative flow cytometry plots showing the frequencies of GC T_{FH} cells gated on the$PD1^{hi}CXCR5^{hi}$population and Bcl-6 expression levels in the indicated T cell subsets of the same mice. One plot from at least 5 individual mice/genotype analyzed. (**F**) Functional analysis of T_{FH} cells to support B-cell responses. Representative histograms showing CD86 and CD69 expression levels on cultured B-cells, fold increase of B-cell proliferation, and IgG/IgA concentrations in culture supernatant of the indicated groups. For each assay, one of three independent experiments with similar results is shown. All graphed data are presented as mean ± SEM. Statistics determined with a one-way ANOVA (**A**) or a two-way ANOVA (**A,B,D,F**) and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The WT and *Tnfrsf17*$^{-/-}$ mice had predominantly T_{FH} cells, while the *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice had both T_{FH} and GC T_{FH} cells at steady state (**Figure 2.2D**). The data suggest that BCMA expression is critical for restraining T_{FH} cell numbers in autoimmunity. Since the increased numbers of T_{FH} cells would be expected to act on autoantibody producing B-cells within the germinal centers, we addressed this further. GC T_{FH} cells express the highest levels of Bcl-6 [26, 170, 179, 180], and the expression of GL-7 has been reported to also identify GC T_{FH} cells [181]. The total PD1^{+}CXCR5^{+}T_{FH} compartment and PD1^{hi}CXCR5^{hi}GC T_{FH} cells showed a substantial increase in those expressing GL-7 in *Nba2* mice and even more in *Nba2;Tnfrsf17*$^{-/-}$ mice compared to controls (**Figure 2.2E**). Expression of the transcription factor Bcl-6 is critical for the differentiation of CD4^{+} T cells into T_{FH} cells [30, 33]. GL-7^{+}GC T_{FH} cells had the highest levels of Bcl-6 expression compared to GL-7^{-}T_{FH} cells, suggesting that GC T_{FH} cells accumulate to a greater extent with BCMA deficiency. To directly test whether the intrinsic helper activity of T_{FH} cells is influenced by BCMA, we developed a co-culture system to measure T_{FH} cell-mediated B-cell responses *in vitro*.

Sorted PD1^{+}CXCR5^{+}T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice were co-cultured with mature splenic B-cells from WT mice and B-cell activation, proliferation, and antibody production in the culture supernatants were measured. Using this assay, we found that co-cultures with T_{FH} cells from *Nba2;Tnfrsf17*$^{-/-}$but not *Nba2* mice significantly induced B-cell activation, as determined by increased levels of CD86 and CD69 on B-cells (**Figure 2.2F**). However, T_{FH} cells from both mouse strains augmented B-cell proliferation and promoted secretion of IgG and IgA. These data indicate that BCMA deficiency moderately affects T_{FH} cell helper activity of B-cells when evaluated

on a per cell basis *in vitro*. We validated our findings in a second model, and showed that the loss of BCMA leads to an expansion of T_{FH} cells (**Figure 2.3A,B**). Collectively, these results demonstrate that BCMA is critical for maintaining T_{FH} cell homeostasis in autoimmune-prone mice that in turn restrains germinal center responses and autoantibody production.

BCMA reduces T_{FH} cell expansion and development of autoimmunity caused by pristane exposure. To confirm that these findings were not limited to murine autoimmune susceptibility genes, we examined environmentally-induced autoimmune responses in WT and *Tnfrsf17*$^{-/-}$ mice after treatment with the inflammatory agent pristine [178]. We generated a clinical scoring system based on the degree of proteinuria, kidney immune complexes, and serum autoantibody titers (**Table 2.1**). *Tnfrsf17*$^{-/-}$animals had significantly higher clinical scores compared to WT mice following pristane treatment and mock-treated controls (**Figure 2.4A**). Furthermore, *Tnfrsf17*$^{-/-}$ mice had increased circulating levels of BAFF compared to WT mice after pristane treatment and control animals (**Figure 2.4B and data not shown**), a feature associated with systemic autoimmunity [91, 165, 173]. *Tnfrsf17*$^{-/-}$mice treated with pristane showed increased frequencies of T_{FH} cells, which correlated with serum BAFF levels and increased frequencies of GC B-cells and plasma cells (**Figure 2.4B,C**). These data indicate that BCMA is a key regulator of T_{FH} cell accumulation and humoral autoimmunity during inflammation.

Figure 2.3

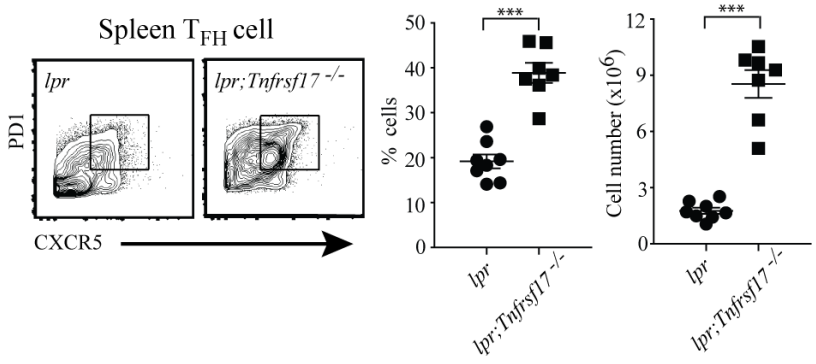

Figure 2.3. Spontaneous expansion of T_{FH} cells in spleens of multiple murine autoimmune models. Representative flow cytometry plots showing percentages of T_{FH} cells ($CD4^+PD1^+CXCR5^+$) in spleens of 20-week-old female *lpr* and *lpr;Tnfrsf17*$^{-/-}$ mice. Percentages and total numbers of T_{FH} cells were quantified from n=8 (*lpr*) and n=7 (*lpr;Tnfrsf17*$^{-/-}$) mice. Each symbol represents an individual mouse and error bars indicate mean± SEM. Statistics determined with a Student's t-test and denoted as follows: ***p <0.001.

Table 2.1. Scoring of serum autoantibody and kidney changes in pristane-treated mice.

Score	Proteinuria[¶]	Kidney ICs*		Autoantibody Titers[*§]	
		IgG	IgA	IgG	IgA
1	<30mg/dL	1-2 complexes	1-2 complexes	<2 fold increase	<2 fold increase
2	30-100mg/dL	3-4 complexes	3-4 complexes	2-2.5 fold increase	2-2.5 fold increase
3	100-300mg/dL	5-6 complexes	5-6 complexes	2.5-3 fold increase	2.5-3 fold increase
4	300-2000mg/dL	7-8 complexes	7-8 complexes	3-3.5 fold increase	3-3.5 fold increase
5	>2000mg/dL	>8 complexes	>8 complexes	>3.5 fold increase	>3.5 fold increase

¶ Protein concentration was determined by colorimetric change using an Uristix® (Bayer) strip, by two separate individuals, who were blinded to the animal genotype and treatment condition

* Scores from IgG and IgA are combined for a total score

§ The score from each Ig isotype consists of an average of the fold change in O.D. values for anti-single stranded DNA (α-ssDNA), anti-double stranded DNA (α-dsDNA), α-histone, and α-chromatin antibodies between the pre-treatment bleed and the post treatment collection

Figure 2.4

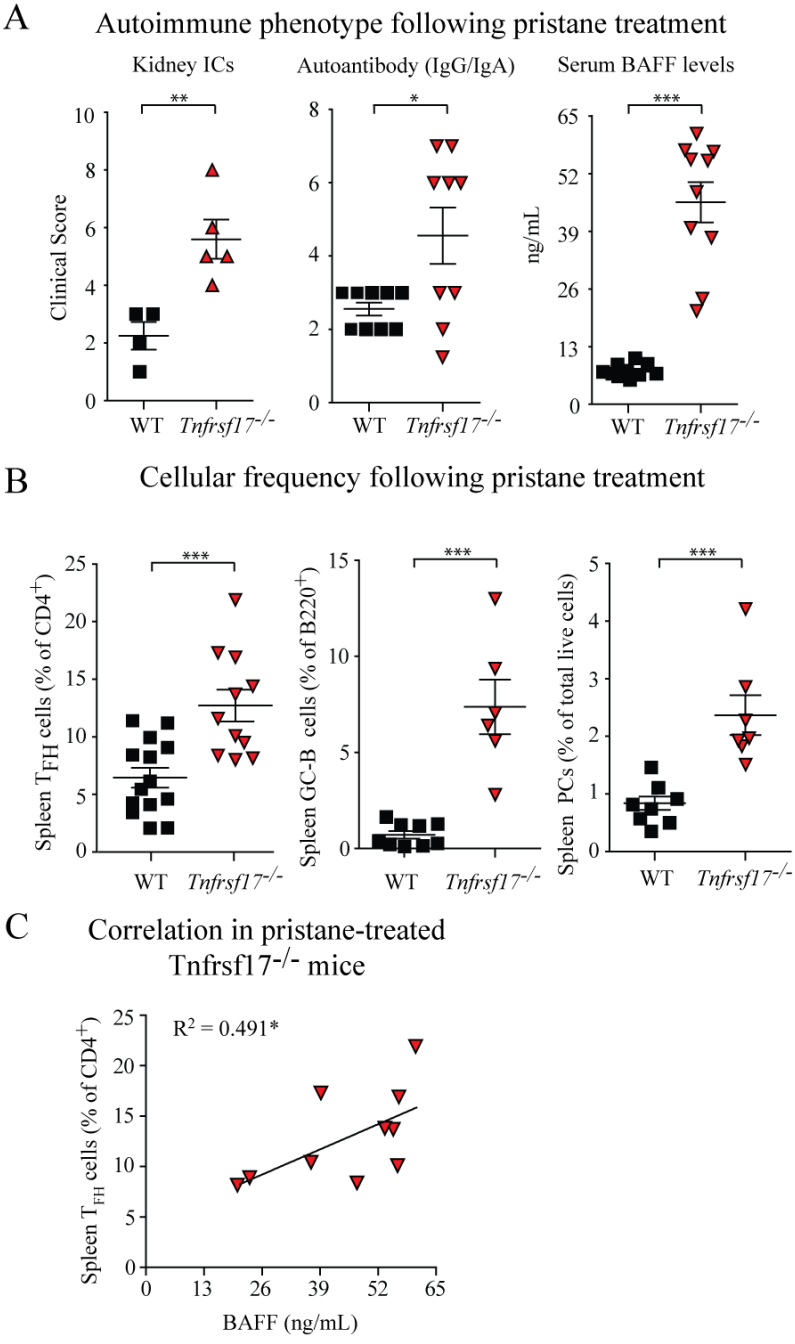
A
Autoimmune phenotype following pristane treatment
Kidney ICs
Autoantibody (IgG/IgA)
Serum BAFF levels
Clinical Score
ng/mL
WT
Tnfrsf17-/-
B
Cellular frequency following pristane treatment
Spleen TFH cells (% of CD4+)
Spleen GC-B cells (% of B220+)
Spleen PCs (% of total live cells)
C
Correlation in pristane-treated Tnfrsf17-/- mice
R2 = 0.491*
Spleen TFH cells (% of CD4+)
BAFF (ng/mL)

Figure 2.4. BCMA mediates tolerance under inflammatory conditions. (**A-C**) 12-week-old WT and*Tnfrsf17*$^{-/-}$ mice were given a single i.p. injection of pristane. After 4 weeks, mice were analyzed for the development of autoimmune traits. (**A**) Clinical scores shown were determined from disease activity as described in the Table 2.1. IC, immune complexes. (**B**) Percentages of T_{FH} cells ($CD4^+PD1^+CXCR5^+$), GC B cells ($B220^+GL\text{-}7^+CD95^+$) and PCs ($B220^{low/-}CD138^+$) in spleens of pristane-treated mice were quantified by flow cytometry. (**C**) Correlation between the percentage of T_{FH} cells and serum BAFF levels of*Tnfrsf17*$^{-/-}$ mice following pristane treatment. (**A-C**) Each symbol represents an individual mouse and error bars represent the mean ±SEM. Combined data from 2-3 experiments. Statistics determined with a Student's t-test (**A,B**) or Pearson's correlation (**C**) and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

BCMA deficiency in CD4$^+$ T cells is sufficient to drive abnormal T_{FH} cell and GC B-cell responses. We next tested whether the increased frequency and cumulative B-cell helper activity of T_{FH} cells from *Tnfrsf17$^{-/-}$*mice were features intrinsic to T cells following pristane treatment. Sorted resting CD4$^+$ T cells from WT and*Tnfrsf17$^{-/-}$*mice were adoptively transferred into CD45.1 WT recipients, which could be distinguished by expression of the congenic marker CD45.2, and were detected at equivalent frequencies (**Figure 2.5A,B**). Recipients were given phosphate buffered saline (PBS) or pristane and spleen cellular responses were evaluated. Mice that received *Tnfrsf17$^{-/-}$* CD4$^+$ T cells had significantly increased numbers of donor T_{FH} cells, endogenous GC B-cells, and increased autoantibody titers following pristane treatment compared to mice that received WT CD4$^+$ T cells (**Figure 2.6A,B**). Moreover, we observed no differences in the frequencies of T_{FH} cells and GC B-cells, or autoantibody titers, in pristane-treated mice that received WT CD4$^+$ T cells compared to control WT mice. These data indicate that BCMA deficient CD4$^+$ T cells themselves promote abnormal T_{FH} cell accumulation, spontaneous GC formation, and autoantibody production. Finally, we observed that the numbers of T_{FH} cells and autoantibody titers were higher in pristane-treated *Tnfrsf17$^{-/-}$* mice compared to WT mice that received *Tnfrsf17$^{-/-}$* CD4$^+$ T cells. These findings likely reflect differences in the total numbers of *Tnfrs17$^{-/-}$* CD4$^+$ T cells responding to pristane-induced inflammatory stimuli and the contribution of additional immune cell types.

Figure 2.5

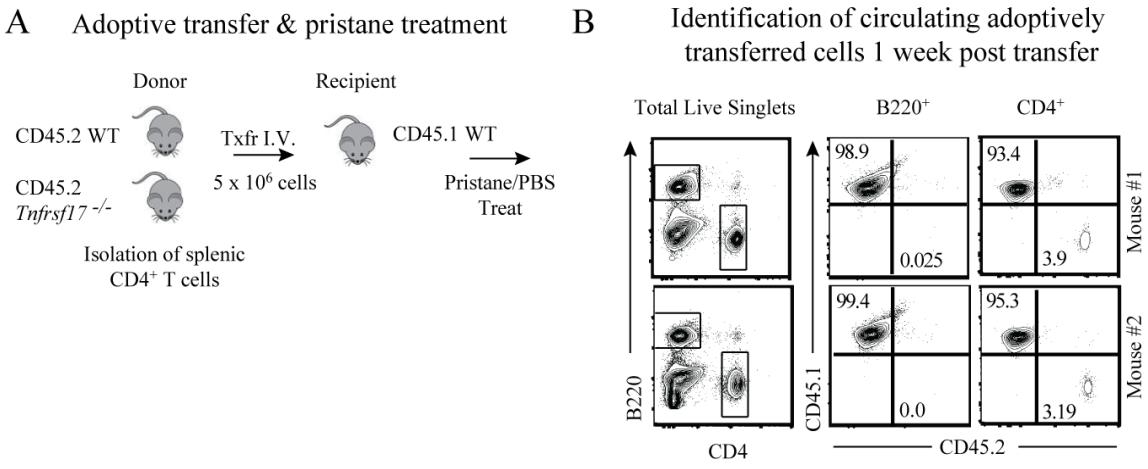

Figure 2.5. Pristane treatment of recipients following adoptive transfer of CD4$^+$ T cells. Sorted naive CD4$^+$ T cells (CD45.2) were isolated from spleens of WT and *Tnfrsf17*$^{-/-}$mice and adoptively transferred into CD45.1 WT recipients. Mice were then treated with either PBS or pristane. (**A**) Schematic of transfer and treatment strategy. (**B**) One week after transfer, percentages of donor CD45.2 CD4$^+$ T cells were measured in peripheral blood of recipient mice. Data shown represent 2 of 21 recipients.

Figure 2.6

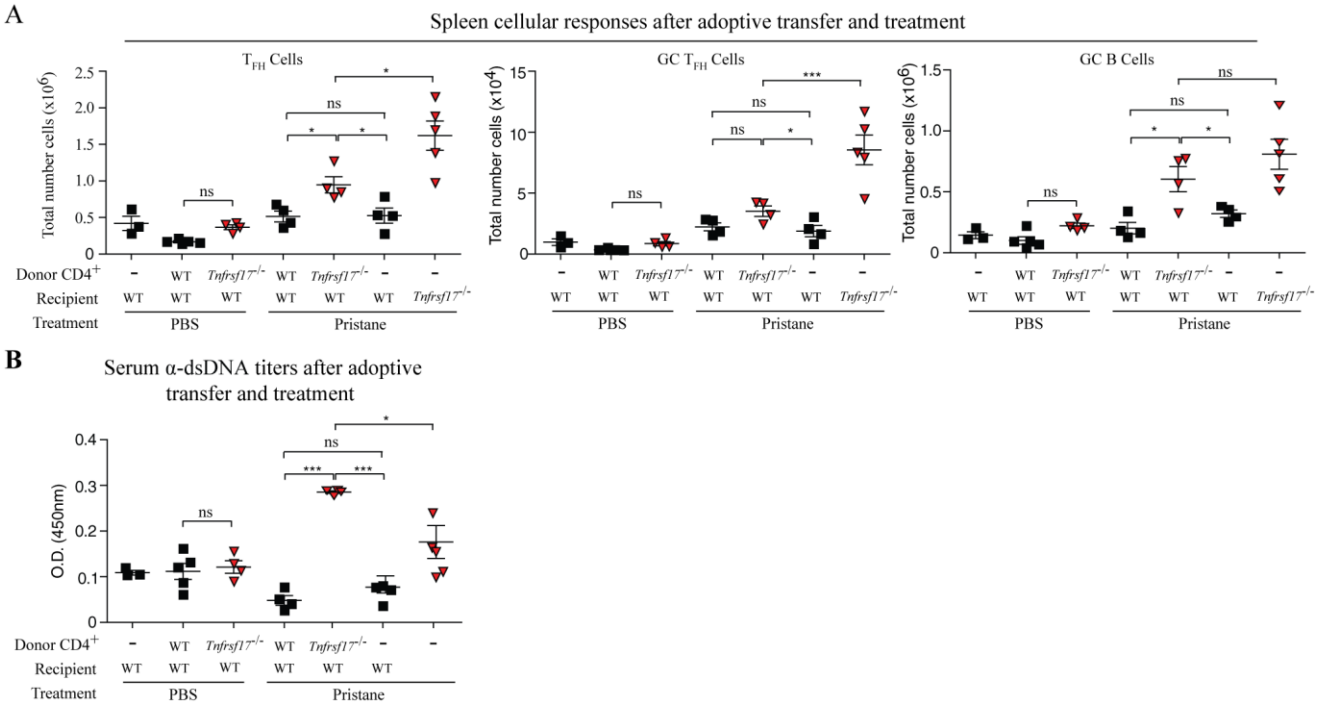
A
Spleen cellular responses after adoptive transfer and treatment
T$_{FH}$ Cells
GC T$_{FH}$ Cells
GC B Cells
Total number cells (x10^6)
Total number cells (x10^4)
Donor CD4$^+$
Recipient
Treatment
PBS
Pristane
B
Serum α-dsDNA titers after adoptive transfer and treatment
O.D. (450nm)

Figure 2.6. BCMA deficient T cells are sufficient to break tolerance under inflammatory conditions. (**A,B**) Sorted naïve $CD4^+$ T cells (5×10^6) from spleens of CD45.2 WT and *Tnfrsf17*$^{-/-}$ mice were adoptively transferred into 12-week-old CD45.1 WT mice. Recipient mice, as well as WT and *Tnfrsf17*$^{-/-}$ animals serving as controls, were injected with either PBS or pristane. (**A**) After 4 weeks, total numbers of donor T_{FH} cells ($CD4^+CD45.2^+PD1^+CXCR5^+$), GC-$T_{FH}$ cells ($CD4^+CD45.2^+PD1^+CXCR5^+$) and GC B-cells ($B220^+GL\text{-}7^+CD95^+$) in spleens of the indicated mice were quantified. (**B**) Serum anti-dsDNA titers of the same animals were measured and shown as optical density (O.D.). (**A,B**) Each symbol represents an individual animal. All data are presented as mean ±SEM. Statistics determined with a one-way ANOVA (**A,B**) and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns – not significant.

To investigate further whether BCMA was functioning predominantly in $CD4^+$ T cells to blunt abnormal T_{FH} cell and GC B-cell responses, we generated chimeric mice in which only T cells would be deficient in BCMA. Lethally irradiated CD45.1 WT recipient mice were reconstituted with a 80%:20% mix of *CD3e*$^{-/-}$ and *Tnfrsf17*$^{-/-}$ bone marrow cells, respectively (**Figure 2.7A**). Chimeras with T cells sufficient in BCMA expression were generated with a 80%:20% mix of *CD3e*$^{-/-}$ and WT bone marrow cells. Cohorts of 100% chimeras with either *Tnfrsf17*$^{-/-}$ or WT bone marrow cells were also generated and used as controls. Reconstituted animals were treated with pristane and analyzed one month later. To validate BCMA deficiency within the $CD4^+$ T cell compartment, we purified $CD4^+$ T cells from the spleen of the chimeras, as well as controls and ran PCR on the isolated DNA. The mice generated using the 20:80 mixture of WT and *CD3e*$^{-/-}$ bone marrow generated mice that did not contain the BCMA neo-cassette, whereas the mice generated with a 20:80 mixture of *Tnfrsf17*$^{-/-}$ and *CD3e*$^{-/-}$ bone marrow clearly contained the BCMA neo-cassette (**Figure 2.7B**) [139]. BCMA deficiency in T cells increased the numbers of T_{FH} cells and GC B-cells to the levels measured in 100% *Tnfrsf17*$^{-/-}$ mice, indicating that the absence of BCMA in T cells leads to T_{FH} cell accumulation and germinal centers (**Figure 2.7C**). Selective deficiency of BCMA in T cells also contributed to increased levels of serum autoantibodies (**Figure 2.7D**). Together, these results highlight a new role for BCMA in controlling the development of T_{FH} cells in response to inflammatory stimuli and suggest that dysregulation of T_{FH} cells is BAFF-dependent.

Figure 2.7

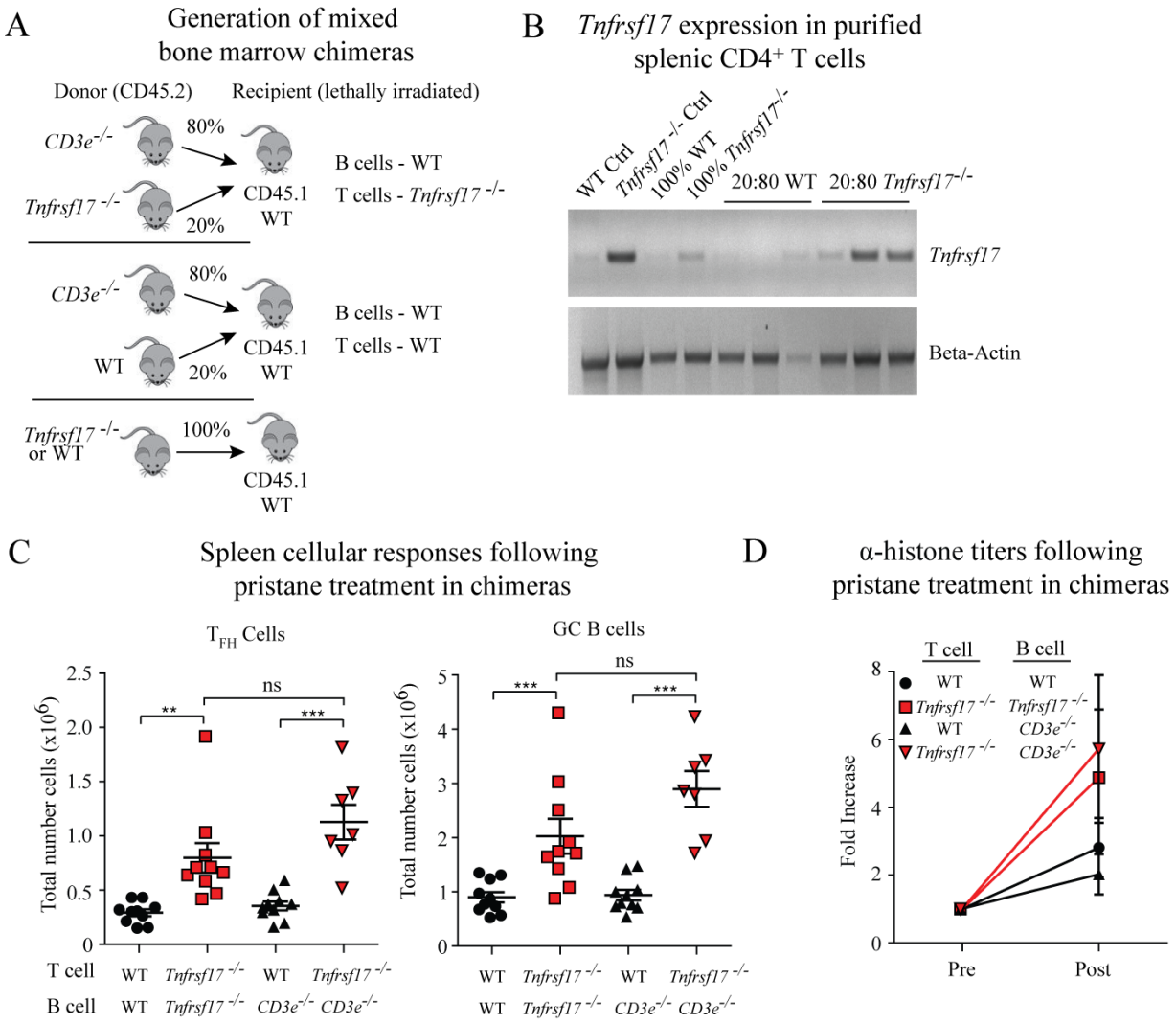
A
Generation of mixed bone marrow chimeras
Donor (CD45.2)
Recipient (lethally irradiated)
CD3e-/-
80%
Tnfrsf17 -/-
20%
CD45.1 WT
B cells - WT
T cells - Tnfrsf17 -/-
CD3e-/-
80%
WT
20%
CD45.1 WT
B cells - WT
T cells - WT
Tnfrsf17 -/- or WT
100%
CD45.1 WT
B
Tnfrsf17 expression in purified splenic CD4+ T cells
WT Ctrl
Tnfrsf17 -/- Ctrl
100% WT
100% Tnfrsf17-/-
20:80 WT
20:80 Tnfrsf17-/-
Tnfrsf17
Beta-Actin
C
Spleen cellular responses following pristane treatment in chimeras
TFH Cells
GC B cells
Total number cells (x10^6)
ns
**
***
T cell
B cell
WT
Tnfrsf17 -/-
CD3e-/-
D
α-histone titers following pristane treatment in chimeras
T cell
B cell
Fold Increase
Pre
Post

Figure 2.7. Generation of T cell restricted BCMA deficient bone marrow chimeras. (**A-D**) Mixed bone marrow chimeras were generated to test BCMA deficiency when restricted to the T cell compartment. (**A**) Strategy for the generation of mixed bone marrow chimeras. Chimeras were reconstituted for 6 weeks and then injected with pristane. (**B**) Identification of the BCMA neo-cassette in purified splenic $CD4^+$ T cells. Beta-actin served as an expression control. Non-chimeric B6 and *Tnfrsf17*$^{-/-}$ mice served as controls. One representative animal for the 100% chimeras from ten animals generated and three representative animals from the 20:80 mixed chimeras from 7-10 animals generated. (**C**) Four weeks post pristine treatment, the total number of splenic T_{FH} cells and GC B-cells were quantified. Each symbol represents an individual animal. (**D**) Serum anti-histone IgG titers of the same animals were measured before pristane treatment and four weeks after pristane injection. Combined data from 10 animals per group (WT-WT, *Tnfrsf17*$^{-/-}$- *Tnfrsf17*$^{-/-}$, WT-CD3e$^{-/-}$) or 7 animals per group (*Tnfrsf17*$^{-/-}$- CD3e$^{-/-}$). All data are presented as mean ±SEM. Statistics determined with a one-way ANOVA (**C**) and denoted as follows: **p < 0.01, ***p <0.001, ns – not significant.

Modulation of BAFF levels controls the frequency of T_{FH} cells. BAFF blockade in autoimmune diabetes and arthritis mouse models reduces the proliferation and production of cytokines in $CD4^+$ T cells [163, 172], suggesting that BAFF signaling pathways control T cell responses. Given that *Nba2;Tnfrsf17*$^{-/-}$ mice had increased levels of circulating BAFF at 25 weeks of age (**Figure 2.8A**), we examined whether blocking BAFF influences the frequencies of T_{FH} cells during an established GC response in these mice since GC B-cells have been shown to be required for the maintenance of T_{FH} cells [27]. Recombinant murine TACI-Fc fusion protein given at two-day intervals for two weeks resulted in markedly reduced numbers of T_{FH} cells, GC B-cells, and plasma cells in the spleen (**Figure 2.8B**). Splenic B-cells were reduced to approximately 34% of total cells in mice treated with TACI-Fc compared to 55% in mice treated with control Fc, indicating that TACI-Fc was effective in decreasing peripheral B-cells as expected, but did not result in substantial B-cell lymphopenia (**data not shown**). These data indicate an important role for excess BAFF levels in the maintenance of the T_{FH} cell phenotype during established GC responses. As a corollary, we tested whether artificially raising BAFF levels in naïve *Tnfrsf17*$^{-/-}$ mice, which have low physiologic BAFF levels (**Figure 2.8A**), could drive T_{FH} cell-mediated B-cell responses. Recombinant BAFF given daily for three days resulted in significantly increased numbers of T_{FH} cells, GC B-cells, and plasma cells in spleens of *Tnfrsf17*$^{-/-}$ mice but not controls (**Figure 2.8C**). These findings demonstrate that T_{FH} cell homeostasis in autoimmune-prone mice is regulated in a BCMA-dependent manner and by the availability of BAFF.

Figure 2.8

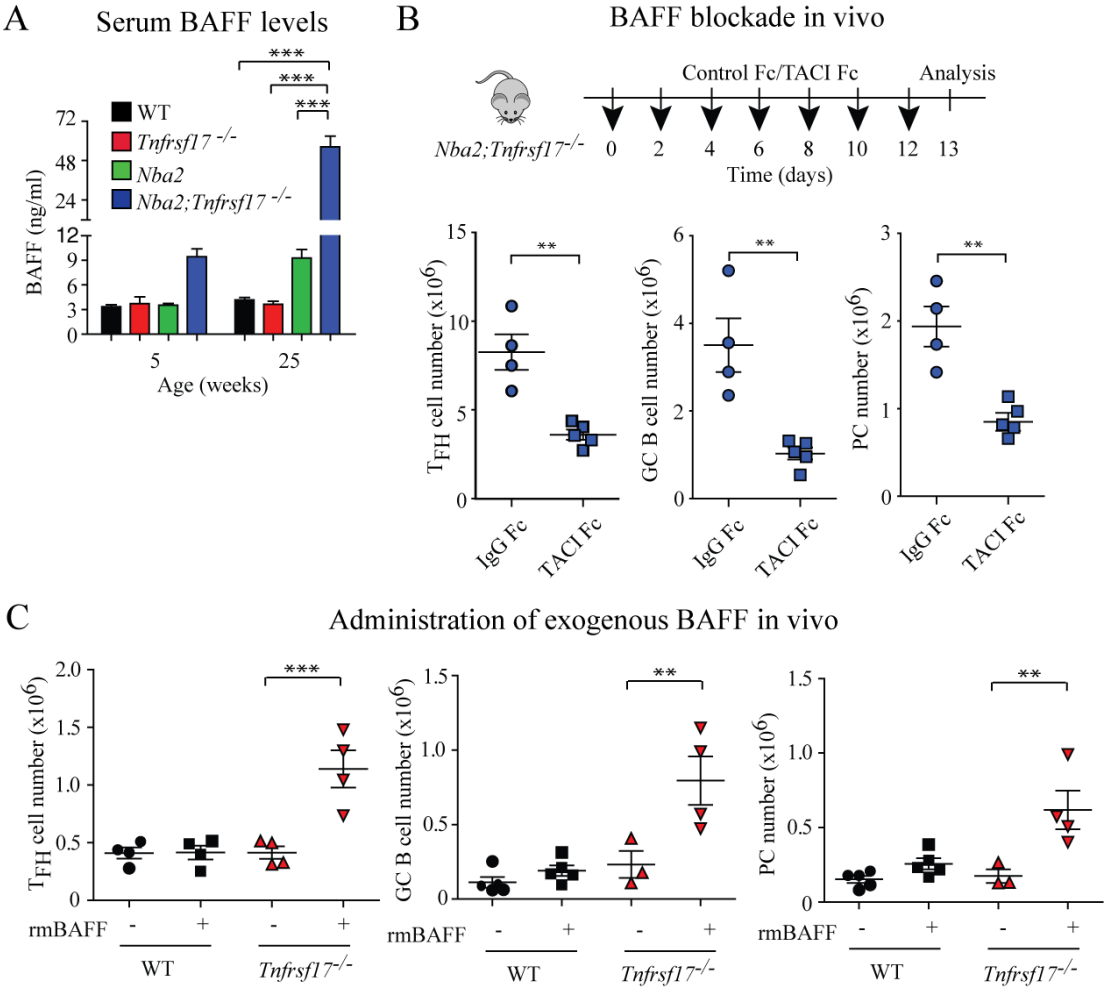
A
Serum BAFF levels
***
***
***
WT
Tnfrsf17 -/-
Nba2
Nba2;Tnfrsf17 -/-
BAFF (ng/ml)
72
48
24
12
9
6
3
0
5
25
Age (weeks)
B
BAFF blockade in vivo
Control Fc/TACI Fc
Analysis
Nba2;Tnfrsf17-/-
0 2 4 6 8 10 12 13
Time (days)
TFH cell number (x10^6)
GC B cell number (x10^6)
PC number (x10^6)
**
IgG Fc
TACI Fc
C
Administration of exogenous BAFF in vivo
TFH cell number (x10^6)
GC B cell number (x10^6)
PC number (x10^6)
***
**
rmBAFF
WT
Tnfrsf17-/-

Figure 2.8.BAFF levels affect T_{FH} cell homeostasis. (**A**) BAFF concentrations in serum of 5- and 25-week-old mice were measured by ELISA. Combined data from WT (n=5), *Tnfrsf17*$^{-/-}$ (n=5), *Nba2* (n=8), and*Nba2;Tnfrsf17*$^{-/-}$ (n=8) for each group. (**B**) Twenty-five-week-old*Nba2*;*Tnfrsf17*$^{-/-}$ mice were given i.p. injections of TACI Fc or control IgG Fc fusion protein. The total numbers of T_{FH} cells ($CD4^+PD1^+CXCR5^+$), GC B-cells ($B220^+GL\text{-}7^+CD95^+$) and plasma cells (PC; $B220^{low/-}CD138^+$) in spleens of mice were quantified. (**C**) WT and*Tnfrsf17*$^{-/-}$ mice were given daily injections of recombinant BAFF or PBS. After 4 days, the numbers of indicated cell types in spleens of mice were quantified. (**B,C**) Each symbol represents an individual animal. (**A,B**) One of two experiments with similar results is shown. Data are represented as mean ± SEM. Statistics determined using a one-way ANOVA (**A,C**) or Student's t-test (**B**) and denoted as follows: *p < 0.05, **p <0.01, ***p <0.001.

T_{FH} cells constitutively express BR3 and upregulate BCMA in autoimmunity. To better understand the effect of BAFF on T_{FH} cell function, we examined whether BAFF could directly bind to T_{FH} cells and through which receptor. Treatment of T_{FH} cells with Flag®-tagged recombinant BAFF resulted in equivalent positive labeling of T_{FH} cells, with a trend for higher labeling on GC T_{FH} cells of autoimmune-prone mice compared to control mice (**Figure 2.9A,B**). Interestingly, we found undetectable levels of the BAFF homologue, APRIL, bound to $CD4^+$ T cell subsets, suggesting that BAFF was the most likely biologically relevant cytokine in regulating T_{FH} cell homeostasis (**data not shown**).

Little is known about how BCMA expression is regulated. Several cytokines and B cell co-stimulatory molecules have been identified that lead to BCMA transcription. Stimulation of primary murine B cells with IL-4 and IL-6 upregulates the expression of BCMA mRNA transcript levels [158], which are further increased when cells are exposed to BAFF and an agonistic anti-CD40 antibody [138]. Finally, post-translational modification of BCMA could also regulate the expression and activity of BCMA protein. BCMA contains several post-translational modification sites, with only 4 shared between mouse and human [108]. These include a phosphorylation site in the N-terminus, and several phosphorylation sites in the C-terminus. The impact of these modification sites on both the expression and function of BCMA remains to be determined.

To address whether any of the BAFF signaling axis receptors were expressed within T_{FH} cells, we measured expression of all three BAFF receptors (BR3, BCMA, TACI) from WT and *Nba2* mice. Detection of BCMA on the cell surface of murine cells compared to human cells has been unreliable due to poor reagents [106]; thus, we

analyzed mouse BCMA expression by measuring mRNA transcript levels. BCMA in T_{FH} cells was significantly increased in the *Nba2* mice compared to WT mice, which did not express BCMA at steady state as indicated by analysis of T_{FH} cells of *Tnfrsf17*$^{-/-}$ controls (**Figure 2.9C**). Furthermore, BCMA was upregulated in T_{FH} cells of pristane-treated WT mice. Analysis of BCMA in $CD4^+$ naïve, regulatory, and memory T cells of *Nba2* mice and WT mice treated with pristane demonstrated that BCMA was not induced. Plasma cells served as a positive control, whereas naïve B-cells and GC B-cells from WT mice that do not express BCMA served as negative controls (**Figure 2.9D).** The frequencies of naïve, memory, and regulatory $CD4^+$ T cell subsets were not significantly altered by BCMA deficiency **(**data not shown).These data suggest that the expression of BCMA in the $CD4^+$ T cell compartment of autoimmune-prone mice is regulated during the development of T_{FH} cells, possibly primed $CD4^+$ T cells that are fated to a T_{FH} cell differentiation pathway, and therefore involved in the development and function of T_{FH} cells. In contrast, BR3 was expressed on T_{FH} cells of all of the commonly used mouse strains at equivalent levels, which was comparable to splenic B-cells that constitutively express BR3 (**Figure 2.9E**). BR3 was not expressed at detectable levels on $CD4^+$ naïve, regulatory, and memory T cell subsets and no detectable levels of TACI were found on T_{FH} cells or other $CD4^+$ T cells (**data not shown**). Together, these data demonstrate that T_{FH} cells of autoimmune-prone mice express BCMA and BR3, and suggest that excess BAFF promotes T_{FH} cell accumulation by signals transduced through BR3 in the absence of BCMA.

Figure 2.9

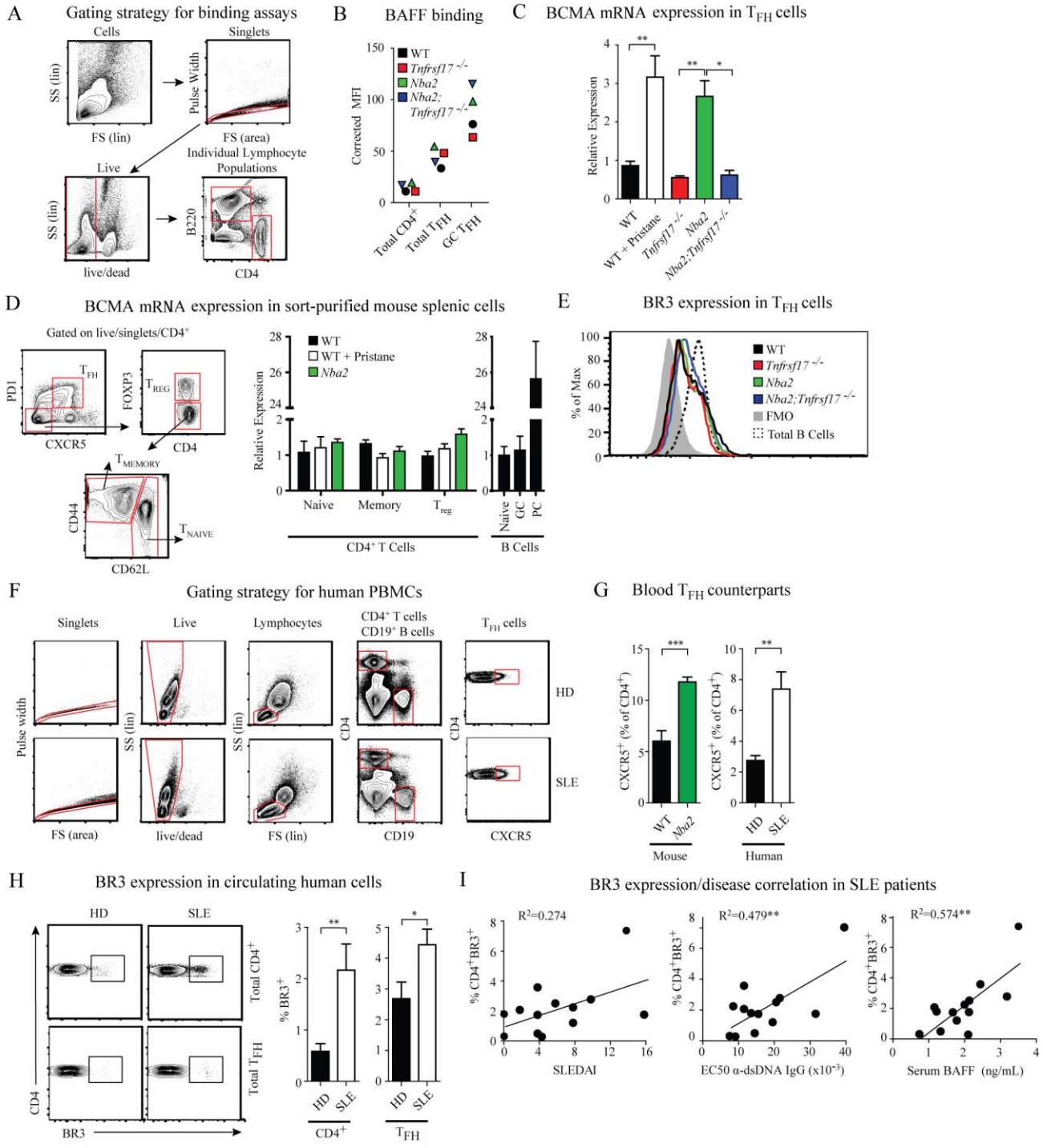
A
Gating strategy for binding assays
Cells
Singlets
SS (lin)
FS (lin)
Pulse Width
FS (area)
Live
Individual Lymphocyte Populations
SS (lin)
live/dead
B220
CD4
B
BAFF binding
WT
Tnfrsf17 -/-
Nba2
Nba2; Tnfrsf17 -/-
Corrected MFI
Total CD4+
Total TFH
GC TFH
C
BCMA mRNA expression in TFH cells
Relative Expression
WT
WT + Pristane
Tnfrsf17 -/-
Nba2
Nba2;Tnfrsf17 -/-
D
BCMA mRNA expression in sort-purified mouse splenic cells
Gated on live/singlets/CD4+
PD1
TFH
CXCR5
FOXP3
TREG
CD4
TMEMORY
CD44
TNAIVE
CD62L
WT
WT + Pristane
Nba2
Relative Expression
Naive
Memory
Treg
CD4+ T Cells
Naive
GC
PC
B Cells
E
BR3 expression in TFH cells
% of Max
WT
Tnfrsf17 -/-
Nba2
Nba2;Tnfrsf17 -/-
FMO
Total B Cells
F
Gating strategy for human PBMCs
Singlets
Live
Lymphocytes
CD4+ T cells
CD19+ B cells
TFH cells
Pulse width
FS (area)
SS (lin)
live/dead
FS (lin)
CD4
CD19
CXCR5
HD
SLE
G
Blood TFH counterparts
CXCR5+ (% of CD4+)
WT
Nba2
Mouse
HD
SLE
Human
H
BR3 expression in circulating human cells
HD
SLE
Total CD4+
Total TFH
CD4
BR3
% BR3+
HD
SLE
CD4+
TFH
I
BR3 expression/disease correlation in SLE patients
R2=0.274
% CD4+BR3+
SLEDAI
R2=0.479**
EC50 α-dsDNA IgG (x10-3)
R2=0.574**
Serum BAFF (ng/mL)

Figure 2.9. Differential expression of BCMA and BR3 in T_{FH} cells of autoimmune-prone mice and SLE patients. (**A**) Gating strategy for BAFF binding assays. (**B**) Flow cytometric values measuring BAFF binding to the indicated $CD4^+$ T cell subsets in spleens of 25-week-old mice. Data are representative of three independent experiments with n=6 mice per group. (**C**) Gene expression values of*Tnfrsf17* relative to the house-keeping gene glyceraldehyde-3 phosphate dehydrogenase (*Gapdh)* in sorted T_{FH} cells of the indicated mice were determined by real-time PCR on total RNA. The expression of *Tnfrsf17* in WT T_{FH} cells was set to one. Combined data from three independent mice per group. (**D**) Gating strategy and gene expression values of *Tnfrsf17* relative to *Gapdh* in sorted cell populations of the indicated mice as determined by real-time PCR on total RNA: naïve T cells ($CXCR5^-PD1^-CD62L^+CD44^-Foxp3^-$), memory T cells ($CXCR5^-PD1^-CD62L^-CD44^+Foxp3^-$) and regulatory T cells ($CXCR5^-PD1^-CD62L^{+/-}CD44^{+/-}Foxp3^+$). Naïve B-cells ($B220^+CD138^-GL7^-CD95^-IgD^+$) and GC B-cells ($B220^+$ $GL7^+CD95^+$) from WT mice served as negative controls, whereas plasma cells ($B220low/^-CD138^+$) served as a positive control. The expression of *Tnfrsf17* in WT naïve $CD4^+$ T cells was set to one. Combined data from three independent mice per group. (**E**) Representative histograms showing BR3 expression on T_{FH} cells from spleens of the indicated mouse strains. Total B-cells and a FMO stain serve as controls. n=3 mice per group. (**F**) Gating strategy for human PBMC samples. HD – healthy donor, SLE – systemic lupus erythematosis. (**G**) Percentages of circulating T_{FH} cell counterparts ($CD4^+CXCR5^+$) were measured in peripheral blood samples from WT and *Nba2* mice or HD and SLE patients. Combined data from WT (n=5) and*Nba2* (n=9) mice, or HD (n=10) and SLE (n=13) subjects. (**H**) Percentages of total $CD4^+$ T cells and T_{FH}-like cells that express BR3 were

measured in the same human subjects as in panel (**G**). (**I**) Pearson's correlation between the percentages of $CD4^+BR3^+$ T cells and SLEDAI score (left), serum α-dsDNA IgG titers (middle), and serum BAFF levels (right) in SLE patients. Each symbol represents an individual patient. Error bars shown are mean ± SEM. Statistics determined using a Student's t-test (**G,H**), one-way ANOVA (**C**), or linear regression of Pearson's correlation (**I**) and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A population of $CXCR5^+CD4^+$ T cells has been identified in human peripheral blood and shares phenotypic features with T_{FH}-like cells [15, 16, 39]. To determine whether BR3 and BCMA were expressed on blood $CXCR5^+CD4^+$ T cells and were linked to autoimmunity in humans, we first measured the frequencies of circulating T_{FH} cell counterparts in the peripheral blood of SLE patients compared to healthy donors (**Table 2.2**). Increased frequencies of $CXCR5^+$ cells within the $CD4^+$ T cell population were found in SLE patients compared to healthy controls, consistent with previous reports[75, 182], and paralleled increased frequencies of $CXCR5^+CD4^+$ T cells in the peripheral blood of *Nba2* mice (**Figure 2.9F,G**). Within total $CD4^+$ T cells and the T_{FH}-like population, the percentages that express BR3 were significantly increased in SLE patients compared to healthy donors (**Figure 2.9H**). BCMA was not detected on circulating $CD4^+$ T cells or T_{FH}-like cells of healthy subjects nor SLE patients (**data not shown**). We next examined whether the frequency of $BR3^+CD4^+$ T cells correlated with disease activity of SLE patients. Although the percentages of circulating $BR3^+CD4^+$ T cells of SLE patients did not significantly correlate with the severity of SLEDAI score, the frequency of these T cells, and by extension T_{FH}-like cells, increased proportionally to the amount of serum anti-dsDNA IgG and BAFF (**Figure 2.9I**). These data indicate a link between excess BAFF levels and dysregulation of circulating $BR3^+CD4^+$ T cells including T_{FH}-like cells among a subset of SLE patients.

Supplementary Table 2.2. SLE patient clinical information

Patient ID	Age	Gender	Drug Regimen	SLEDAI
SLE 12-1	39	F	Hydroxychloroquine	10
SLE 12-2	53	F	Hydroxychloroquine	0
SLE 12-3	33	F	Mycophenolate, hydroxychloroquine, prednisone	6
SLE 12-4	44	F	Azathioprine, hydroxychloroquine, prednisone	2
SLE 12-6	30	F	Hydroxychloroquine, etanercept	8
SLE 12-7	30	F	Azathioprine, hydroxychloroquine, prednisone	8
SLE 12-8	59	F	Azathioprine, prednisone	4
SLE 12-9	61	F	Hydroxychloroquine	0
SLE130801	65	F	Methotrexate, folic acid, tramadol, prednisone, zoloft	4
SLE130718A	51	F	Modafinal, tylenol, metoprolol, prilosec	4
SLE130718B	53	F	Prednisone, plaquenil, norvasc, tretin-x, tramadol	4
SLEP131023B	40	F	Prednisone, plaquenil, zoloft, trazodone	16
SLEP13025	37	F	Seroquel, zoloft, prednisone, lortab	16

Discussion

Although autoimmunity has been linked to the abnormal production of BAFF and uncontrolled T_{FH} cell expansion that promote activation of autoreactive B-cells, the role of BAFF and its receptors in controlling T_{FH} cell homeostasis in this process has not been addressed. Here we have discovered that both the amount of BAFF and the expression of BCMA and BR3 on T_{FH} cells critically influence immune tolerance. Thus, apart from BAFF directly affecting B-cell activation and survival, the data presented here suggest that BAFF-dependent regulation of T_{FH} cell responses is a checkpoint of tolerance to control the germinal center reaction. Our work newly identify that both BCMA and BR3 are expressed on T_{FH} cells of autoimmune-prone mice. Our studies suggest that BCMA on T_{FH} cells restrains their expansion and thus B-cell helper activity in inflammation and autoimmunity, and that if BCMA is lacking in the context of excess BAFF, then signals transduced through BR3 leads to uncontrolled T_{FH} cell differentiation and accumulation. These findings have clinical relevance since increased frequencies of circulating T_{FH}-like cells are associated with certain SLE patients and other autoimmune disorders [75, 182, 183]. We propose that SLE patients with increased $BR3^{+}$ T_{FH}-like cells and heightened BAFF levels may respond better to anti-BAFF therapy compared to patients that do not show this correlation. Our data also reveals for the first time that BR3 but not BCMA is expressed on T_{FH} cells of healthy hosts. This, together with the demonstration that BAFF levels influence both the frequencies of T_{FH} cells and GC T_{FH} cells that express high levels of Bcl-6, suggests an important role for BR3 to regulate T_{FH} cell differentiation and/or survival in T cell-dependent immune responses. This process may be tightly controlled by the limited availability of BAFF to bind BR3 in healthy state.

Summary Model

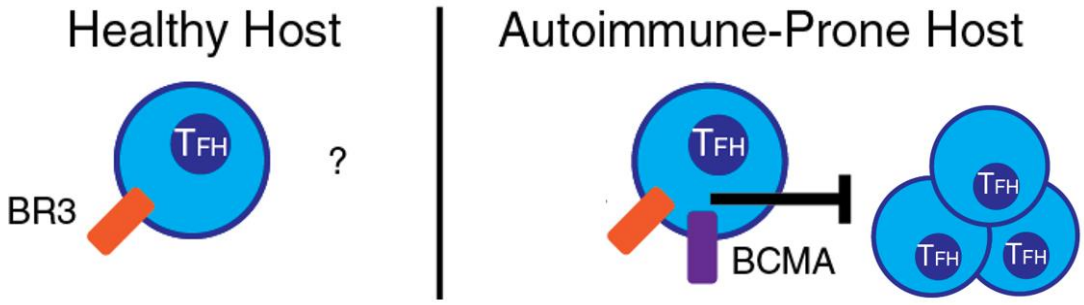

Figure 2.10. BCMA controls T follicular helper cell expansion & function during inflammation and autoimmune susceptibility. In WT mice as well as healthy donors, BR3 is expressed on T_{FH} cells. In autoimmune-prone mice, mice injected with the inflammatory reagent pristine, or SLE patients, BR3 is also expressed on T_{FH} cells. In the murine models, BCMA expression by T_{FH} cells is specific to autoimmune susceptibility or inflammation and acts to restrict the outgrowth of the T_{FH} population. Whether this process occurs in the human population is unknown. The role of signaling through BR3 under homeostatic conditions or autoimmune susceptibility is unclear.

CHAPTER 3

Dendritic Cells and T Follicular Helper Cells Participate in a Feedback Loop Driven by BAFF and IFNγ Production

Interferons (IFNs) are important contributors to autoimmune pathogenesis, particularly in SLE. Although IFNs likely act on many levels, one of the major mechanisms by which they contribute to disease is through their ability to potently activate myeloid cells that in turn activate lymphocyte populations. IFNs are divided into three distinct subclasses (type I – III) with the type I IFN, IFNα, strongly associated with SLE [184, 185]. More recently, increasing evidence has supported a role for the type II IFN, IFNγ, in SLE pathology. High levels of IFNγ have been associated with late stage disease in both mouse models and human SLE. Excess IFNγ can induce lupus-like symptoms in mice as well as humans (who were treated with recombinant IFNγ for other diseases) [186]. Furthermore, multiple lupus-prone mouse models (CBAxC57/BL10 F1, NZB/NZW, MRL-Faslpr, NOD/ShiLtJ – as well as the induced models, pristane) show exacerbated disease when administered exogenous IFNγ whereas disease is ameliorated when endogenous IFNγ is blocked or the IFNγ receptor is deleted [187]. These findings indicate that IFNγ is pathogenic in the context of autoimmunity, but the source of the cytokine as well as the cellular subsets being affected by IFNγ are still being elucidated. In the context of autoimmune disease, IFNγ may have several important functions. IFNγ promotes class switching to IgG2a, IgG2b and IgG3 and in this capacity may lead to the generation of Ig subclasses considered more pathogenic. Furthermore, IFNγ may play a role in end organ damage. Production of IFNγ in key tissues promotes macrophage recruitment and activation, leading to inflammation. Observation of high levels if $IFN\gamma^{+}$ cells in the kidney interstitium of SLE patients is consistent with these predictions. IFNγ expression correlates with serum creatinine and is highest in patients with Class IV (most severe) kidney pathology [188].

A number of cell types including T cells, NK cells, NK T cells, B cells as well as other APCs, can produce IFNγ in a context-dependent manner [189]. Numerous reports have demonstrated that IFNγ produced by $CD4^+$ T cells may be one of the major contributors to the overall elevated levels of IFNγ in autoimmunity. Peripheral blood T cells from SLE patients produce larger amounts of IFNγ following TCR crosslinking [190]. Furthermore, excess IFNγ correlates with patients with more severe disease. ICOS co-stimulation of $CD4^+$ T cell led to significant proliferation and production of IFNγ in active SLE patients and to a lesser extent with patients that were not experiencing a flare [191]. Moreover, supernatants from SLE patient-derived T cells stimulated with anti-CD28 had the ability to induce BAFF from monocytes, which was completely abrogated in the presence of an anti-IFNγ Ab [190]. This last piece of data suggests a link between aberrant IFNγ production and elevated BAFF levels in autoimmune patients.

An amplification loop between BAFF and IFNγ in autoimmunity is a concept that has been proposed by a number of groups. IFNγ can upregulate BAFF by macrophages, monocytes, DCs and neutrophils in a c-AMP response element binding protein (CREB) and protein kinase A-dependent manner [180]. Additionally, transgenic mice that overexpress BAFF have an enhanced delayed-type hypersensitivity response to injection of murine bovine serum albumin in complete Freund's adjuvant which is shown by increased T cell proliferation and more IFNγ production [176]. In autoimmune disease, work from the Lowell laboratory has described the development of autoimmunity in $lyn^{-/-}$ mice as a consequence of myeloid cells overproducing BAFF which acts directly on T cells to induce IFNγ. After adoptive transfer of both WT and $BR3^{-/-}$ T cells into $lyn^{-/-}$ mice, WT transferred cells had similar activation phenotype and IFNγ production as

endogenous cells, but BR3$^{-/-}$ T cells had reduced CD44 expression and IFNγ production [165]. Overall their data support a model where reciprocal production of BAFF by myeloid cells and IFNγ by T cells, establishes and maintains a pathogenic feedback loop in autoimmunity. In another autoimmune model, the Vinuesa group showed that in the sanroque mice, IFNγ acts directly on the T_{FH} compartment to increase Bcl-6 expression, and thus supports the development and maintenance of the T_{FH} population [79]. These findings suggest an important crosstalk between IFNγ-producing CD4^{+} T cells and BAFF-producing myeloid cells in the generation and maintenance of the T_{FH} compartment. Whether this link is dependent on one or more of the BAFF receptors in the context of autoimmunity, is unknown. Therefore, we examined the role of BAFF on T_{FH} cells in the presence or absence of BCMA.

Methods

Mice.Tnfrsf17$^{-/-}$, *Nba2*, *Nba2;Tnfrsf17*$^{-/-}$, mice, fully backcrossed onto the C57BL/6 (B6) strain, were previously described[139, 177]. Age-matched WT B6 mice were purchased from NCI. IFNγR1$^{-/-}$ mice were purchased from the Jackson Laboratory. All experiments were performed on individual mice using 4-6 month-old female mice unless indicated. For each experiment, a minimum of 5 mice was used for each strain unless indicated to account for variability in disease phenotype and assess statistical significance. No mice were excluded from the data analysis and no randomization was performed. Investigators were not blinded to experimental conditions. Mice were housed in a specific pathogen-free animal facility at the University of Virginia.

Study subjects. Females that met the American College of Rheumatology Revised Criteria for SLE and age-matched healthy females were enrolled in this study during routine clinical care through the Division of Rheumatology at the University of Virginia School of Medicine (Charlottesville, VA). Exclusion criteria included patients who were pregnant or receiving the following treatments: i.v.steroids, cyclophosphamide, >20 mg oral prednisone daily, or B-cell and BAFF-depleting therapies. The SLE Disease Activity Index (SLEDAI) measured disease activity. Additional SLE patient blood was purchased from AllCells (Alameda, CA). No blood samples were excluded from the data analysis.

Flow cytometry. Mouse spleen single-cell suspensions were prepared and red blood cells were lysed with ammonium chloride–Tris solution. Whole blood was obtained from mice in heparin-treated tubes (Fisher Scientific). Human patient blood was obtained in ACD tubes (BD Bioscience) and was spun down to separate plasma and cellular fractions. Patient sera were prepared with thrombin (Sigma) and peripheral blood mononuclear cells were isolated using a Ficollgradient (GE Healthcare). For phenotyping, cells were stained at a concentration of 0.5-2 $x10^6$ cells/well in a 100μl volume. Mouse antibodies included the following: eBioscience: PD-1-FITC (RMP1-30) or PD-1-biotin (J43), CD4-EF450 or CD4-PerCP (GK1.5), IFNγ-APC (XMG1.2);BD Bioscience: CD11c-PECy7 or CD11c-biotin (HLS), CXCR5-APC or CXCR5-PECy7 (2G8), CD4-PECy7 (RM4-5); Human antibodies included the following: BioLegend: CXCR5-PerCP/Cy5.5 (TG2), BAFF-R-APCCy7 (11C1);eBioscience: PD-1-PE (eBioJ105), PD-1-APC (MIH4), CD4-biotin (OKT4), ICOS-EF450 (ISA-3); Streptavidin was used on the following fluorophores: FITC, PE, APC, PERCPCy5.5, EF450, and APC-Cy7. Cell viability was determined using live/dead AQUA (Invitrogen) according

to manufacturer's instructions. Following staining, cells were sorted, fixed in 1% formaldehyde (Fisher Scientific), or suspended in Fix/Perm Wash Buffer (eBioscience). Samples were acquired on a CyAn ADP LX (Beckman Coulter) or sorted with an Influx Cell Sorter (Becton Dickinson) and analyzed using FlowJo software version 9.3.3 (Treestar Inc.).

Cytokine & Immunoglobulin ELISAs. To determine cytokine production, 1 x 10^6 purified $CD4^+$ T cells were stimulated with 1μg/mL anti-CD3 and 2μg/mL anti-CD28 for three days. T helper 1(Th1) and Th2 cytokines in culture supernatants were measured by Luminex® analysis. Murine IFNγ was measured by ELISA (R&D, Cat# MIF00). Human IFNγ levels were determined by generating a sandwich ELISA using the following reagents: purified anti-human IFNγ (BioLegend, B27), biotinylated anti-human IFNγ (BioLegend, 4S.B3), and recombinant human IFNγ (Peprotech). Total IgG, IgA, and antigen-specific antibodies were measured by ELISA as previously described [139].

Co-culture assays. To evaluate BAFF expression in DCs, purified DCs from WT and IFNγR1$^{-/-}$ mice were cultured ± recombinant murine IFNγ (100 ng/ml; Peprotech) for 24 hours. To evaluate T_{FH} cell-derived IFNγ to induce BAFF expression in DCs, T_{FH} cells were stimulated with anti-CD3 and IL-2 ± BAFF. After 48 hours, culture supernatants were removed and added to WT and IFNγR1$^{-/-}$ DCs (5 x 10^5) ± anti-IFNγ blocking antibody (AN-18, BD Bioscience). After 24 hours, DCs were then collected for RNA.

Real-time quantitative PCR. Cells were suspended in RLT/2-ME buffer and total RNA was isolated using the RNAqueous® Micro Kit (Ambion). cDNA was generated using the iScript™ cDNA synthesis kit (Bio-Rad), according to manufacturer's

instructions. Real-time PCR was performed using the following primer sequences (IDT Technologies):*Tnfrsf17* Fwd-5'-GGC GCA ACA GTG TTT CCA CA-3', *Tnfrsf17* Rev-5'-CTC GGT GTC GGC CTT GTC CA-3', *GAPDH* Fwd-5'-AAC GAC CCC TTC ATT GAC-3',*GAPDH* Rev-5'-TTC ACG ACA TAC TCA GCA C-3', *BAFF* Fwd-5'-GGC AGG TAC TAC GAC CAT CTC-3', *BAFF* Rev-5'-TGG GCC TTT TCT CAC AGA AGT-3', *IFNγ* Fwd-5'-ATG AAG GCT ACA CAC TGC ATC-3', *IFNγ* Rev-5'-CCA TCC TTT TGC CAG TTC CTC-3', *Tnfrsf13c* Fwd-5'-GCC CAG ACT CGG AAC TGT CCC A-3', *Tnfrsf13c* Rev-5'-GCC CAG TAG AGA TCC CTG GGT TCC-3'. Samples were run on a BioRadMyiQ™ System.

Study approval. All animal procedures were conducted in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Virginia (protocol no. 3506). Female healthy donor and SLE patient blood for this study were obtained with written informed consent from the protocol, Plasma Cells in Systemic Lupus Erythematosus Pathogenesis, which was approved by the University of Virginia Institutional Review Board (protocol no. 16244).

Statistical analysis. Data are presented as mean ± SEM. Multiple group comparisons were performed by one-way ANOVA and two-way ANOVA was used for analysis of more than two groups when repeated measures were being compared. Two-tailed Student's t test was used for the analysis of two independent groups. Pearson's correlation coefficient was used to determine the strength of association between two variables. All data were normally distributed with similar variance between the groups. For all tests, a p value less than 0.05 was considered statistically significant and stated in

the figure legend (*p< 0.05, **p< 0.01, ***p < 0.001). Prism software v5.0 (GraphPad Software) was used for statistical analyses.

Results

BR3 signaling in T_{FH} cells enhances IFNγ production. To determine whether BAFF may be involved in cytokine production by T_{FH} cells, we stimulated purified $CD4^+$ T cells from BCMA-sufficient and –deficient autoimmune prone mice and assayed for cytokine production in the culture supernatant. We detected IFNγ and IL-4 production by both strains, however only in the case of IFNγ did we measure a statistically significant difference between the BCMA-sufficient and –deficient $CD4^+$ T cells (**Figure 3.1A**). Abnormal production of IFNγ by Th1 cells is associated with the pathogenesis of SLE and strongly correlates with disease activity [186]. Analysis of steady state IFNγ expression levels in splenic $CD4^+$ T cells of autoimmune-prone mice demonstrated significantly higher mRNA transcript levels from T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice compared to *Nba2* mice, as well as higher frequencies of IFNγ-producing T_{FH} cells (**Figure 3.1B,C**). Given that IFNγ has been shown to induce BAFF production in myeloid and epithelial cells [17, 165], we hypothesized that increased BAFF levels in *Nba2;Tnfrsf17*$^{-/-}$ mice compared to *Nba2* mice may result from elevated IFNγ production by BCMA deficient T_{FH} cells. In *lyn*$^{-/-}$ autoimmune-prone mice, which have elevated serum BAFF levels, passive transfer of total $CD4^+$ and $CD8^+$ T cells isolated from BR3 deficient mice resulted in decreased IFNγ production, suggesting that BR3 signaling in a subset of T cells mediates IFNγ release [165]. To test whether signaling through BR3 on T_{FH} cells contributes to IFNγ production, splenic T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-}$

$^{/-}$ mice were cultured in the presence or absence of recombinant BAFF. Results demonstrated that T_{FH} cells stimulated with BAFF significantly increased IFNγ production compared to control cells, with higher amounts of IFNγ produced by T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice (**Figure 3.1D**).

Confirmation that signals transduced by BAFF through BR3 on T_{FH} cells induced IFNγ secretion was determined by adding a BR3 neutralizing mAb to the cultures, which reduced IFNγ levels. These data indicate that supra-physiologic levels of BAFF can directly stimulate T_{FH} cells *in vitro* to enhance IFNγ production via BR3, while BCMA expression in autoimmune-prone T_{FH} cells can blunt this response. We have previously reported increased numbers of DCs and serum BAFF levels in *Nba2;Tnfrsf17*$^{-/-}$ and *lpr;Tnfrsf17*$^{-/-}$ mice compared to *Nba2* and *lpr* animals [133]. Depletion of $CD4^+$ T cells in *lpr;Tnfrsf17*$^{-/-}$ mice also revealed decreased serum BAFF titers, suggesting a link between $CD4^+$ T cells and BAFF production [133].

Reciprocal production of IFNγ by T_{FH} cells and BAFF by dendritic cells. To evaluate the relative contribution of BAFF generated by activated DCs to mediate IFNγ production in T_{FH} cells of autoimmune-prone mice, we measured IFNγ levels in supernatants of co-cultures with activated $CD11c^+$ splenic DCs and T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice. To this end, we administered lipopolysaccharide (LPS) to WT mice which led to substantial activation of DCs as measured by an upregulation of CD86 and increased transcription of BAFF (**Figure 3.2A,B**).

Figure 3.1

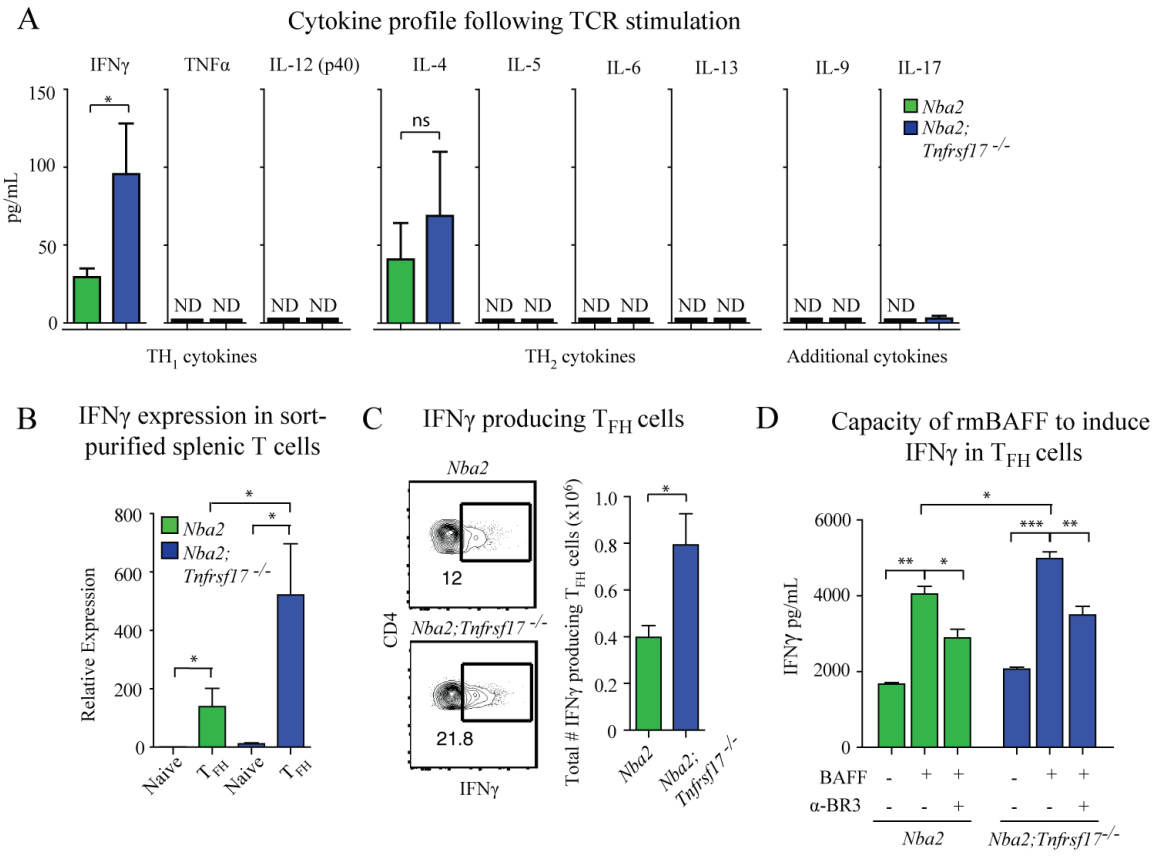
A
Cytokine profile following TCR stimulation
IFNγ
TNFα
IL-12 (p40)
IL-4
IL-5
IL-6
IL-13
IL-9
IL-17
pg/mL
150
100
50
0
*
ns
ND ND
Nba2
Nba2; Tnfrsf17 -/-
TH1 cytokines
TH2 cytokines
Additional cytokines
B
IFNγ expression in sort-purified splenic T cells
Relative Expression
800
600
400
200
0
Naive
TFH
C
IFNγ producing TFH cells
Nba2
Nba2;Tnfrsf17 -/-
CD4
12
21.8
IFNγ
Total # IFNγ producing TFH cells (x10^6)
1.0
0.8
0.6
0.4
0.2
0
D
Capacity of rmBAFF to induce IFNγ in TFH cells
IFNγ pg/mL
6000
4000
2000
0
**
***
BAFF
α-BR3
Nba2
Nba2;Tnfrsf17-/-

Figure 3.1.IFNγ production is increased in T_{FH} cells of BCMA deficient autoimmune-prone mice. (A) Sorted $CD4^+$ T cells from 25-week-old *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice were cultured with anti-CD3/CD28 for three days. Th1 and Th2 cytokines in culture supernatants were measured by Luminex® analysis. Bars indicate mean ± SEM from three individual mice per strain. ND – not detected (**B**) Gene expression values of *IFNγ* in sorted naïve $CD4^+$ T cells and T_{FH} cells relative to *Gapdh* of *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice. Expression of *IFNγ* in naïve T cells from *Nba2* mice was set to one. Data represent mean ± SEM from three mice per strain. (**C**) Representative flow cytometry plots and bar graphs showing percentages and total numbers of T_{FH} cells staining positive for intracellular IFNγ. Splenocytes were stimulated with PMA/ionomycin in the presence of Golgi Stop™ for 5 hours before staining. Data represent mean ± SEM from three mice per strain. (**D**) Purified $CD4^+$ T cells from 5-6 month old mice were incubated in the presence or absence of rmBAFF with or without an anti-BR3 blocking antibody. IFNγ production was measured in the culture supernatant by ELISA following three days of culture. Combined data from 5 mice/genotype. Statistics determined with a one-way ANOVA (**B,D**) or Student's t-test (**C**) and denoted as follows: *p <0.05, **p <0.01, ***p<0.001.

Sorted splenic dendritic cells were cultured at varying ratios with T_{FH} cells in the presence or absence of a BR3 neutralizing mAb. The results demonstrated that T_{FH} cells from *Nba2;Tnfrsf17*$^{-/-}$ mice produced higher basal levels of IFNγ compared to *Nba2* mice (**Figure 3.2C**). Addition of DCs to the cultures significantly increased IFNγ production by T_{FH} cells from both *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice at a high DC:T_{FH} cell ratio (1:12), with the IFNγ levels induced by DCs on T_{FH} cells from *Nba2;Tnfrsf17*$^{-/-}$ mice substantially higher. Cultures of lower DC:T_{FH} cell ratios (1:18; 1:24) failed to induce IFNγ from T_{FH} cells of *Nba2* mice in contrast to T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice, which produced IFNγ Blocking BR3 reduced the IFNγ production by T_{FH} cells but not to basal levels, suggesting that in addition to BAFF signaling, activated DCs provide additional signals to promote IFNγ production in T cells in this system. Taken together, these data demonstrate that physiologic levels of BAFF from DCs are sufficient to drive IFNγ production in T_{FH} cells via BR3, and suggest heightened sensitivity of T_{FH} cells in autoimmune-prone mice to BAFF stimulation in the absence of BCMA.

To investigate further the role of BR3 and BCMA in T_{FH} cells of autoimmune-prone mice to control IFNγ production and reciprocally support BAFF expression in DCs, we established an *in vitro* culture system to measure how IFNγ signaling affects BAFF expression in DCs. We first examined the capacity of recombinant IFNγ to induce the expression of BAFF in WT and IFNγR1$^{-/-}$ dendritic cells. Twenty-four hours after IFNγ stimulation, BAFF mRNA transcript levels were increased in WT dendritic cells compared to IFNγR1$^{-/-}$ dendritic cells and unstimulated control cells (**Figure 3.2D**). To formally test whether increased IFNγ production by T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice compared to *Nba2* mice affects BAFF expression levels in dendritic cells, culture

supernatants from T_{FH} cells stimulated with recombinant BAFF to promote IFNγ production were first prepared (**Figure 3.1D and data not shown**). Supernatants were then added to WT and IFNγR1$^{-/-}$ dendritic cells, and cultured in the presence or absence of an IFNγ blocking mAb. Dendritic cells do not express BCMA or BR3 and therefore cannot respond to residual recombinant BAFF within the T_{FH} cell supernatants (**Figure 3.2E**). After 24 hours, BAFF mRNA transcript levels in dendritic cells were measured. Only supernatants from BAFF-stimulated T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice was sufficient to induce BAFF expression in WT dendritic cells compared to supernatants from T_{FH} cells of *Nba2* mice and unstimulated T cells (**Figure 3.2F**). Moreover, neutralizing IFNγ activity in supernatants from T_{FH} cells of *Nba2;Tnfrsf17*$^{-/-}$ mice reduced BAFF expression in WT DCs to steady state levels. IFNγR1$^{-/-}$ dendritic cells failed to upregulate BAFF mRNA transcript levels when cultured with supernatants of T_{FH} cells regardless of BAFF stimulation.

Figure 3.2

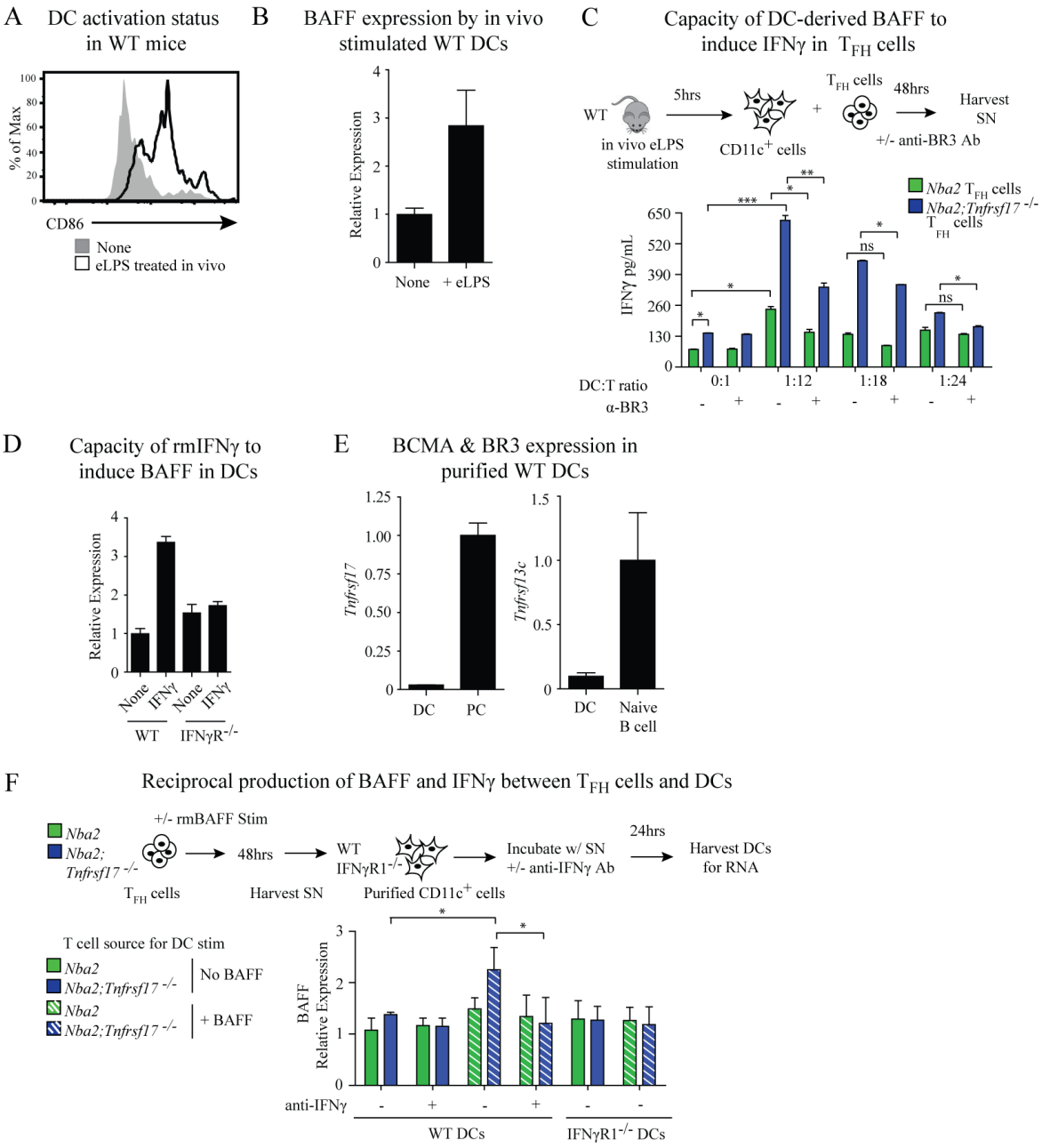
A
DC activation status in WT mice
% of Max
CD86
None
eLPS treated in vivo
B
BAFF expression by in vivo stimulated WT DCs
Relative Expression
None
+ eLPS
C
Capacity of DC-derived BAFF to induce IFNγ in T_FH cells
WT
in vivo eLPS stimulation
5hrs
CD11c+ cells
T_FH cells
48hrs
Harvest SN
+/- anti-BR3 Ab
Nba2 T_FH cells
Nba2;Tnfrsf17 -/- T_FH cells
IFNγ pg/mL
DC:T ratio
0:1
1:12
1:18
1:24
α-BR3
D
Capacity of rmIFNγ to induce BAFF in DCs
Relative Expression
None
IFNγ
WT
IFNγR-/-
E
BCMA & BR3 expression in purified WT DCs
Tnfrsf17
Tnfrsf13c
DC
PC
Naive B cell
F
Reciprocal production of BAFF and IFNγ between T_FH cells and DCs
Nba2
Nba2; Tnfrsf17 -/-
T_FH cells
+/- rmBAFF Stim
48hrs
Harvest SN
WT
IFNγR1-/-
Purified CD11c+ cells
Incubate w/ SN
+/- anti-IFNγ Ab
24hrs
Harvest DCs for RNA
T cell source for DC stim
Nba2
Nba2;Tnfrsf17 -/-
No BAFF
+ BAFF
BAFF Relative Expression
anti-IFNγ
WT DCs
IFNγR1-/- DCs

Figure 3.2. BAFF and IFNγ amplify the production of each other in dendritic cells and BCMA deficient T_{FH} cells. (**A,B**) Representative histograms showing CD86 surface expression and BAFF mRNA transcript levels are induced in sorted $CD11c^+$ DCs of WT mice following a 5 hour *in vivo* treatment with 10mg LPS. Data are representative of three independent experiments. (**C**) Titrating numbers of sorted DCs from spleens of WT mice treated with LPS were co-cultured with T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice ± BR3 blocking antibody. IFNγ concentrations in culture supernatant were measured by ELISA. (**D**) DCs from WT and IFNγR1$^{-/-}$mice were stimulated with IFNγ for 24 hours. Gene expression values of *Tnfsf13c* (BAFF) relative to *Gapdh* in DCs of the indicated mice were determined by qPCR. The expression of *Tnfsf13c* in unstimulated WT DCs was set to one. (**E**) Gene expression values of *Tnfrsf17* (BCMA) and *Tnfrsf13c* (BR3) relative to *Gapdh* in sorted WT DCs. Expression levels of BCMA in plasma cells (PC) and BR3 in naïve B-cells served as positive controls and were set to one. Data shown represent three independent experiments (**F**) T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice were stimulated with recombinant BAFF for 48 hours and culture supernatants collected. Sorted DCs from WT and IFNγR1$^{-/-}$ mice were stimulated with T_{FH} cell-derived culture supernatant ± IFNγ blocking antibody for 24 hours. Gene expression values of *Tnfsf13c* in DCs were determined as described in panel **D**. (**C,D,F**) Data representative of three independent experiments. Data and error bars represent mean ± SEM. Statistics determined using a one-way ANOVA (**C,F**), and denoted as follows: *p <0.05, **p < 0.01, ***p <0.001, ns – not significant.

Neutralization of IFNγ ameliorates splenic cell frequency and autoimmune features in BCMA-deficient auto-immune prone mice. Finally, we tested whether blocking IFNγ in *Nba2;Tnfrsf17*$^{-/-}$ mice would decrease serum BAFF levels and reverse the increased frequency of T_{FH} cell-mediated B-cell responses in these mice. Anti-IFNγ mAb given three times weekly for four weeks resulted in markedly reduced numbers of splenic dendritic cells and serum BAFF levels (**Figure 3.3A**). T_{FH} cells and plasma cells were also reduced in these mice, leading to significantly lower serum autoantibody titers (**Figure 3.3A**). In addition, heightened levels of serum IFNγ was measured in SLE patients compared to healthy controls, although the difference did not achieve statistical significance. However, we noted a strong correlation between serum IFNγ levels and the percentage of $CD4^+BR3^+$ cells in SLE patients and a trend towards significance between serum IFNγ levels and serum BAFF levels in patients (**Figure 3.3B**). Collectively, these data indicate that signals transduced through BR3 and BCMA in T_{FH} cells of autoimmune-prone mice play opposing roles in promoting IFNγ production, with higher levels of IFNγ produced in T_{FH} cells lacking BCMA. This in turn leads to greater BAFF expression in dendritic cells that amplifies IFNγ production and abnormal $CD4^+$ T cell responses, including T_{FH} cell accumulation.

Figure 3.3

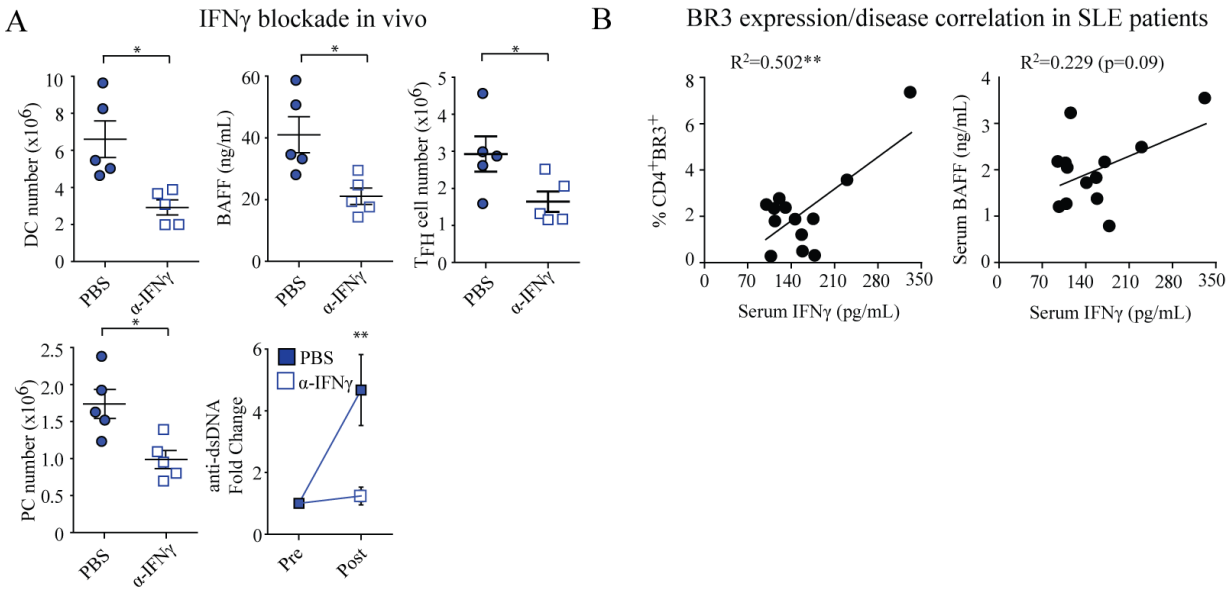

Figure 3.3 IFNγ corresponds with disease severity in mice and humans. (**A**) *Nba2;Tnfrsf17*$^{-/-}$ mice were treated for 4 weeks with an anti-IFNγ blocking antibody. The total numbers of DCs, T_{FH} cells, and PCs, as well as serum IFNγ, BAFF, and dsDNA IgG concentrations were measured following treatment. Each symbol represents an individual animal. (**B**) Pearson's correlation between the percentages of serum IFNγ and $CD4^+BR3^+$ T cells (left) or serum BAFF levels (right) in SLE patients. Each symbol represents an individual patient. Data and error bars represent mean ± SEM. Statistics determined using a Student's t test (**A**), two-way ANOVA (**A,** dsDNAIgG), or linear regression of Pearson's correlation (**B**) and denoted as follows: *p <0.05, **p < 0.01, ***p <0.001.

Discussion

Despite evidence suggesting a role between IFNγ-producing $CD4^+$ T cells and BAFF-producing myeloid cells, the specific $CD4^+$ sub-population, source of BAFF, and receptor mediating this interaction in the context of autoimmunity has not been clearly established. Our data further lend support and extend an emerging model wherein abnormalities in IFNγ production facilitate excess BAFF production in myeloid cells such as dendritic cells [165]. In the absence of BCMA, increased numbers of IFNγ-producing T_{FH} cells are present in autoimmune-prone mice, and these produce more IFNγ when signals through BR3 were transduced by BAFF. This in turn leads to heightened BAFF expression in dendritic cells, which promotes the abnormal survival of autoreactive B-cells and uncontrolled germinal center responses. Interestingly, we observed higher serum IFNγ levels in SLE patients compared to healthy subjects, with the levels of IFNγ correlating with the frequencies of $BR3^+CD4^+$ T cells and increasing proportionally with serum BAFF levels. Although IFNγ production is linked to the accumulation of T_{FH} cells in autoimmunity, the precise role for IFNγ produced by T_{FH} cells in the germinal center reaction leading to autoantibody production is unknown. Our finding that IFNγ-producing T_{FH} cells accumulate in germinal centers of spleens from autoimmune-prone mice is consistent with the notion that both T_{FH} cell-mediated help and cytokines regulate the germinal center B-cell response. This, together with the demonstration that the BAFF-BCMA axis controls T_{FH} cell homeostasis in murine autoimmunity underscores the importance of increased levels of both BAFF and IFNγ in driving aberrant germinal center responses and autoimmunity.

Summary Model

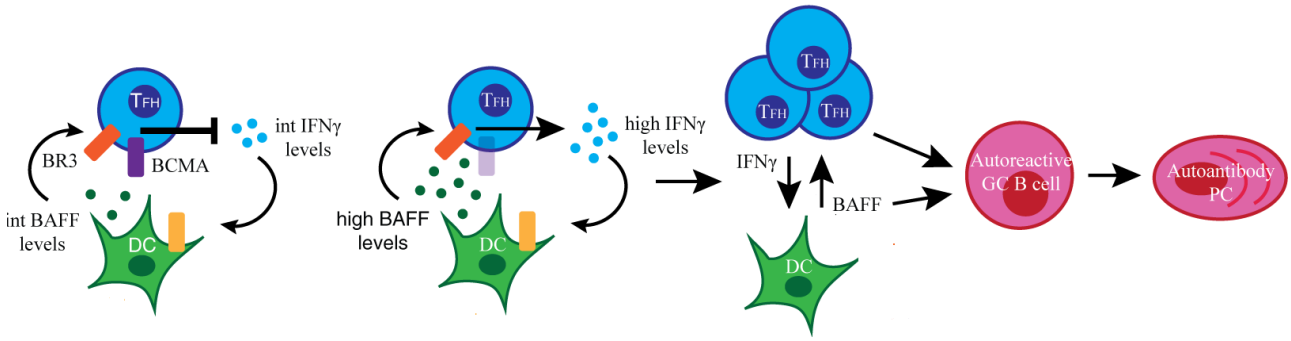

Figure 3.4. Dendritic cells and T follicular helper cells participate in a feedback loop driven by BAFF and IFNγ production. In autoimmune prone mice, upregulation of BCMA by T_{FH} cells prevents aberrant signaling by BAFF through BR3, limiting IFNγ production and thus dampening over-activation of dendritic cells. Loss of BCMA in T_{FH} cells results in strong signaling through BR3, which leads to hyper-production of IFNγ, which can act on dendritic cells to promote BAFF production. The cyclic process mediated by BAFF and IFNγ, produced by dendritic cells and T_{FH} cells, respectively, promotes the generation of auto-reactive GC B cells and auto-reactive PCs. Blocking either of these cytokines in BCMA-deficient autoimmune-prone hosts can ameliorate disease.

CHAPTER 4

Neutrophils Contribute to Excess Serum BAFF Levels and Promote $CD4^+$ and B Cell Responses in Autoimmunity

Excess circulating BAFF levels in both lupus-prone mice and SLE patients are associated with a loss of B cell tolerance and autoantibody production [19, 91, 174]. In lupus-prone mice, neutralizing BAFF activity reduces both the frequency of peripheral B cells and activation of T cells, which is sufficient to prevent and treat the disease [139, 192]. Yet, the mechanisms that control excess BAFF production in autoimmunity and which BAFF-producing cells contribute to disease pathogenesis are largely unknown.

The innate and adaptive arms of the immune system are thought to play essential roles in the development of SLE [193]. Neutrophils are a critical component of the innate immune system and the first line of defense against invading pathogens through uptake and destruction of microorganisms. The contribution of neutrophils to SLE pathology has been largely attributed to their ability to produce type I IFNs [193]. In addition, neutrophils undergo cell death by releasing neutrophil extracellular traps (NETs) that provide a source of autoantigens [194-197]. Neutrophils produce BAFF that is stored intracellular as preformed molecules, which are released when cells are stimulated with IFNγ [198]. Neutrophils also express a membrane-anchored form of BAFF that is cleaved to a biologically active soluble form after stimulation [199]. Recently, a subset of human neutrophils has been shown to provide help to splenic B cells through the production of BAFF that enhances antibody production [200]. Thus, neutrophils may be a key cellular source of BAFF in SLE that contribute to abnormal B cell responses. Given our findings that dendritic cells are a critical source of BAFF and contribute to dysregulated T_{FH} cell homeostasis and function, we asked whether neutrophils may contribute to disease by also interacting directly with T_{FH} cells.

Materials

Mice.Tnfrsf17$^{-/-}$, B6.Fas^{lpr}/J, B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$, *Nba2*, and *Nba2Tnfrsf17*$^{-/-}$ mice, fully backcrossed onto the C57BL/6 (B6) strain, were previously described [139, 177]. Age-matched WT B6 mice were purchased from NCI. Mice were screened by PCR to determine inheritance of the entire *Nba2* locus, the loss of *Tnfrsf17*, and *lpr* mutation as previously described [139]. All experiments were performed on individual mice using four-six month-old female mice unless indicated. Mice were randomized to experimental conditions and no mice were excluded from data analysis. Mice were housed in a specific pathogen-free animal facility at the University of Virginia. All animal procedures were conducted in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

Neutrophil Isolation Protocols and Staining Procedure. Spleens were removed from mice and were homogenized into a single-cell suspension using Dulbecco's Phosphate Buffered Saline (Gibco, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (HyClone, Rockford, IL), designated isolation buffer. Red blood cells were lysed by re-suspending spleen cells in ammonium chloride-Tris buffer and incubating on ice for 10 minutes. Splenocytes were washed and re-suspended in isolation buffer at a concentration of $1x10^6$ cells/mL. Four different isolation techniques were used to develop a protocol optimal for purifying spleen resident neutrophils and maintaining neutrophil viability to enable subsequent functional analyses.

EasySep® isolation technique: First, negative selection by magnetic beads using the EasySep® protocol (Stemcell Technologies, BC, Canada), designed for isolating

murine neutrophils from peripheral blood and bone marrow, was performed as per the manufacturer's instructions. The commercial EasySep® antibody cocktail contains mAbs against mouse CD4, CD5, CD11c, B220, CD49b, CD117, Ter119, and F4/80 of undisclosed concentrations.

OAC isolation technique: This technique used the EasySep® protocol, but was supplemented with additional antibodies specific for contaminating cell populations present in inflamed murine spleen. This modified magnetic bead isolation technique is referred to as the optimized antibody cocktail (OAC). For the OAC protocol, the commercial antibody cocktail provided by EasySep® (50µL per $1x10^6$ total cells/mL) was supplemented with the following biotin-conjugated mAbs: anti-CD3 (clone 145-2C11; used at 3.25µg/mL), anti-Ter119 (clone TER119; used at 0.25µg/mL), and anti-B220 (clone RA3-6B2; used at 2.25µg/mL), all purchased from BD Biosciences; anti-F4/80 (clone BM8; used at 4.35µg/mL) and anti-CD11c (clone N418; used at 4.35µg/mL) from BioLegend; anti-CD19 (clone 1D3; used at 4.35µg/mL) and anti-NK1.1 (clone PK136; used at 4.35µg/mL) from eBioscience. Spleen cells were incubated with antibodies at 4˚C for 15 min and were subsequently washed with isolation buffer. Cells were then resuspended in isolation buffer (100×10^6 cells/mL), streptavidin microbeads were added (270µL/mL; MiltenyiBiotec, Auburn, CA), and the cell mixture was incubated for 15 min at 4˚C. Cells were washed and resuspended in isolation buffer at $2.5x10^6$ cells/mL. Neutrophils were isolated using the EasySep® magnet (Stemcell Technologies) by pouring off the non-adherent cell fraction.

Density gradient centrifugation isolation technique: This method used a density gradient to isolate neutrophils by centrifugation of splenic single-cell suspensions on

Ficoll (GE Healthcare) as previously described for peripheral blood neutrophils [201, 202].

Electronic cell sorting: Finally, splenic neutrophils were purified using a cell sorter. For cell sorting, red-blood cell lysed-splenocytes were stained at a concentration of 1 x 10^7 cells/mL with anti-CD11b FITC or anti-CD11b PE (clone M1/70; eBioscience; used at 5μg/mL for FITC, 1μg/mL for PE) and anti-Ly6G PE-Cy7 (cloneIA8; BioLegend; used at 0.25μg/mL) for 30 minutes on ice. Immediately before sorting, cells were re-suspended in Live/Dead Fixable Aqua (Invitrogen, Grand Island, NY) as per the manufacturer's instructions. All steps were performed on ice and cells were spun at 175 x g for 8 minutes at 4˚C.

In vivo pristane treatment and neutrophil depletion. Three-month-old female mice were given a single i.p. injection of 0.5 ml PBS (Gibco) or TMPD (2,6,10,14-tetramethylpentadecane), commonly known as pristane (Sigma-Aldrich), as previously described [178]. After one month, animals were euthanized and spleen and serum were prepared for flow cytometric analysis of neutrophils, T- and B-cell populations, and ELISA to measure serum BAFF, IFNγ, and autoantibody titers. To deplete neutrophils, B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice were given i.p. injections of 400μg rat IgG (Sigma-Aldrich) or 1A8 (BioXCell) every other day for 4 weeks. Following treatment, mice were euthanized and spleen and serum were harvested for analysis.

In vitro assays. CD4$^+$ T cells from spleens of WT mice were isolated to greater than 90% purity (Miltenyi MACS® kit). Neutrophils from spleens of lupus-prone mice were isolated to greater than 90% purity, as previously described [203]. All cells were cultured in complete RPMI (Gibco) supplemented with 10% FBS (Gibco) unless

otherwise indicated. Cell viability was determined using live/dead AQUA (Invitrogen) according to manufacturer's instructions. To ascertain any alterations in neutrophil function as a result of the isolation method, an *S. aureus* uptake assay was performed. Purified neutrophils were added to 96 well U-bottom plates at 5 x 10^4 cells/well in 100µL of complete media. AlexaFluor488-labeled *S. aureus* particles (Molecular Probes, Invitrogen; used at 5 µL per well) were added either with or without 1 hour pre-treatment with an opsonizing reagent, derived from *S. aureus* specific rabbit polyclonal IgG antibodies (Molecular Probes). Cells were incubated at 37˚C for 2 hours. Cells were washed twice to remove excess, non-adherent bacteria and were stained with Live/Dead Fixable Aqua (Invitrogen) and then fixed in 1% formaldehyde/PBS. Prior to data acquisition, the Alexafluor488 on the surface of the cells was quenched with trypan blue to eliminate detection of surface-bound bacteria that had not been internalized, as previously described [204]. For T cell and neutrophil co-cultures, $CD4^+$ T cells were labeled with CellTrace Violet™ (Life Technologies) and cultured (5 x 10^4) alone or with equivalent numbers of splenic neutrophils in the presence of 1µg/ml anti-CD3 (145-2C11, CedarLane Labs) for 3 days. In some cases, 10µg/ml of anti-BR3 blocking antibody was added (R&D Systems). T cells were analyzed by flow cytometry for proliferation by CellTrace Violet™ dilution and culture supernatants were analyzed for IFNγ, IL-4, and IL-17 production by ELISA. To measure the frequency of IFNγ-producing $CD4^+$ T cells, spleen cells were cultured in the presence of 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop™ (BD Biosciences). After 5 hours of stimulation, $CD4^+$ T cells that produced IFNγ were measured by intracellular flow staining.

Flow cytometry. Cells were spun at 800rpm (130G) for 8 minutes and maintained on ice for all manipulations. Cells were stained at a concentration of 1-2 $x10^6$ cells/well in a 100µl volume. Following staining, cells were fixed in 1% formaldehyde (Fisher) or Fix/Perm Buffer (eBioscience). Mouse antibodies included the following: Ly6G-PE or Ly6G-PECy7 (IA8), CD16/CD32-Alexa647 (93), CD64-PE (X54-5/7.1), CD62L-PE or CD62L-APC (MEL-14), CD11a-FITC (M17/4),GR-1-FITC (RB6-8C5), all from BioLegend;CD11b-FITC or CD11b-PE (M1/70), CD18-biotin (M18/2), CD4-EF450 (GK1.5), all from eBioscience; IFNγ-PE (XMG1.2), B220-PE (RA3-6B2), CD138-APC (281-2), GL-7-FITC, all from BD Biosciences. Streptavidin was used on the following fluorophores: FITC, PE, APC, EF450, APC-Cy7. Viability was determined using LIVE/DEAD® Fixable AQUA (Life Technologies). Samples were acquired on a CyAn™ ADP (Beckman Coulter) and analyzed using FlowJo software version 9.3.3 (TreeStar Inc.). Gates were first set on live cells, singlets, and FMO (fluorescence minus one) controls.

ImageStream. To confirm the morphology of neutrophils, cells were stained with anti-CD11b-FITC and anti-Ly6G-PE-Cy7 as described above. Neutrophils were fixed and permeabilized using the BD Cytofix/Cytoperm Kit according to manufacturer's instructions (Becton Dickinson). DAPI (Life Technologies) was used for nuclear staining. Samples were acquired on an ImageStreamX (Amnis) at a 60X magnification and analyzed using IDEAS software (Amnis).

Histology and immunofluorescence. Frozen 5µm tissue sections were stained with the following reagents: PNA-FITC (Sigma-Aldrich); IgD-EF450 (11-26) and CD11b-PE (M1/70) from eBioscience; Ly6G-APC (IA8), CD11c-biotin (N418) from BioLegend;

BAFF-PE (121808) from BD Bioscience;C3-FITC (CL7503F) from CedarLane Labs;IgA-biotin or IgG-biotin from Southern Biotech. Streptavidin-EF450 was purchased from eBioscience. Sections were analyzed on an Axio Imager 2 with Apotome (Zeiss). Magnification is provided in the figure legend. Quantification of BAFF intensity in sections was performed using the open source software CellProfiler (Broad Institute) and is given in arbitrary units (A.U.). H&E staining was performed as previously described [139].

ELISAs. Murine BAFF (Apotech) and IFNγ (R&D) levels in culture supernatant or sera were measured by ELISA. IL-4 and IL-17 levels in culture supernatant were measured using the following reagents from eBioscience: anti-mouse IL-4 (11B11), anti-mouse IL-17 (MM17F3), anti-mouse IL-4 biotin (BVD6-24G2), anti-mouse IL-17 biotin (17B7). Recombinant murine IL-4 and IL-17 were used to generate a standard curve. Serum autoantibody titers were measured as previously described [139].

RT-PCR. Cells were suspended in RLT/2-ME buffer and total RNA was isolated using the RNAqueous® Micro Kit (Ambion). cDNA was generated using the iScript™ cDNA synthesis kit (Bio-Rad), according to manufacturer's instructions. PCR reactions were performed using iQ™SYBR® Green Supermix (Bio-Rad) and the following primer sequences (IDT Technologies): *HPRT* Fwd-5'- AGC TAC TGT AAT GAT CAG TCA ACG-3', *HPRT* Rev-5'- AGA GGT CCT TTT CAC CAG CA-3', *Tnfrsf17* Fwd-5'-GGC GCA ACA GTG TTT CCA CA-3', *Tnfrsf`17* Rev-5'-CTC GGT GTC GGC CTT GTC CA-3'. Samples were run on a BioRadMyiQ™ System.

Statistical analysis. Statistics were determined using Prism software v5.0 (GraphPad Software). All error bars represent the mean ± standard error of the mean. All

p-values were derived from the two-tailed Student's t test, one-way ANOVA, or two-way ANOVA, as appropriate, and stated in the figure legend (*$p<0.05$, **$p< 0.01$, ***$p<0.001$).

Results

Isolation of a purified, non-activated viable population of murine splenic neutrophils. Neutrophils are an important cellular component of the innate immune system that provides immediate protection to the host from infection. Neutrophil infiltration into inflamed peripheral tissues during infection is beneficial for immunity through phagocytosis of microbes, the release of antimicrobial factors, and secretion of pro-inflammatory cytokines. Recent reports further suggest that spleen-infiltrating neutrophils play a role in the adaptive immune response by providing survival signals to B cells. However, neutrophils may have detrimental effects on immunity in inflammatory diseases where their recruitment to lymphoid tissues and activation occur abnormally. To determine the contribution of neutrophils that reside in secondary lymphoid tissues to adaptive immunity, direct evaluation of the functional properties of tissue resident neutrophils is required. We developed a modified magnetic bead isolation approach for purifying neutrophils from inflamed spleens of autoimmune-prone mice by negative selection. Using this approach, we yielded neutrophils with greater than 90% purity without compromising cell viability (**Figure 4.1A,B**). Equally important, the isolation procedure had little effect on the activation of neutrophils and did not impair phagocytic function (**Figure 4.1 C,D**) [203]. This novel approach allows us to interrogate the functional role of murine neutrophils in normal and abnormal immune responses.

Figure 4.1

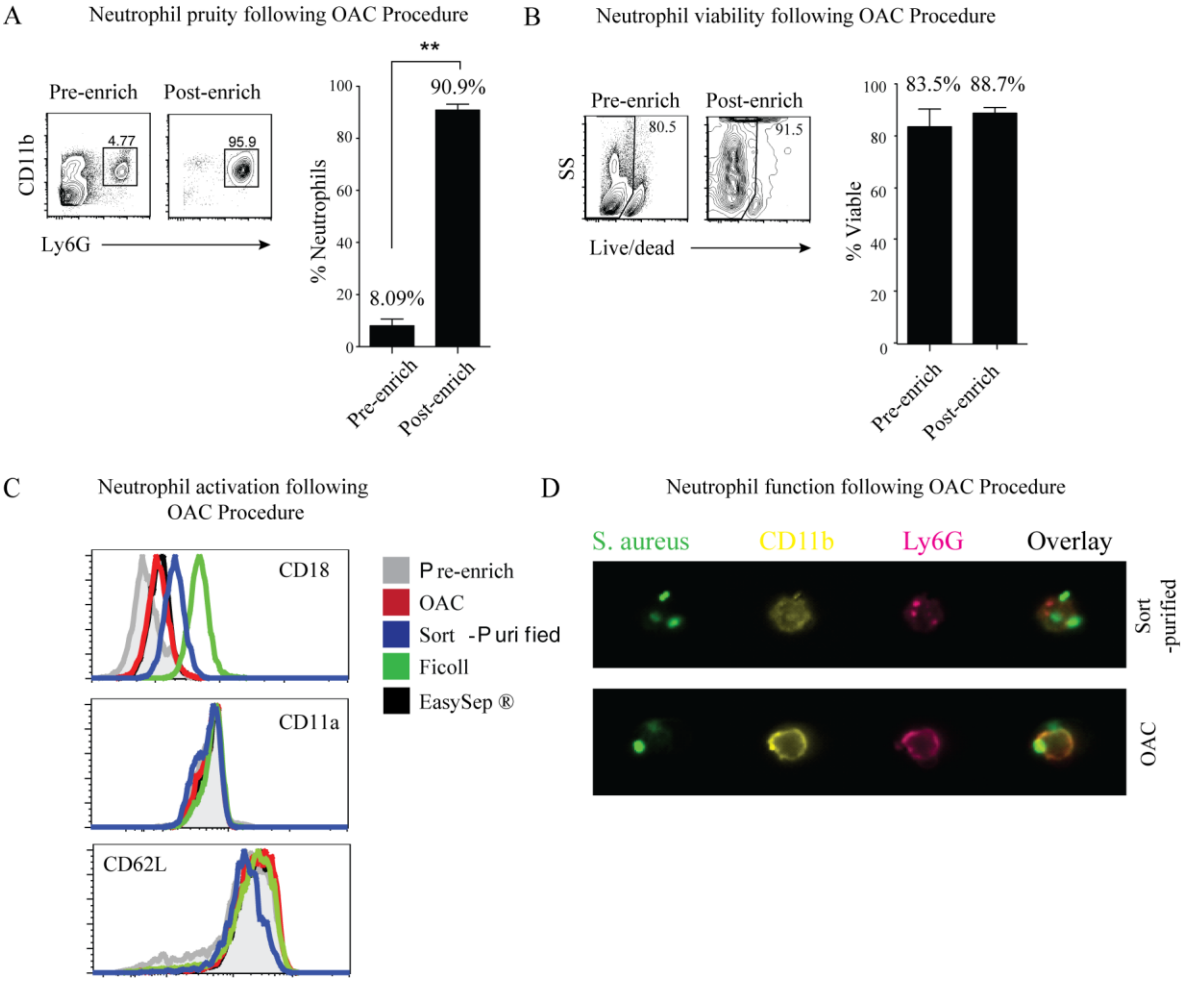
A
Neutrophil pruity following OAC Procedure
Pre-enrich
Post-enrich
CD11b
Ly6G
4.77
95.9
**
90.9%
8.09%
% Neutrophils
Pre-enrich
Post-enrich
B
Neutrophil viability following OAC Procedure
Pre-enrich
Post-enrich
SS
Live/dead
80.5
91.5
83.5%
88.7%
% Viable
Pre-enrich
Post-enrich
C
Neutrophil activation following OAC Procedure
CD18
CD11a
CD62L
P re-enrich
OAC
Sort -P uri fied
Ficoll
EasySep ®
D
Neutrophil function following OAC Procedure
S. aureus
CD11b
Ly6G
Overlay
Sort -purified
OAC

Figure 4.1. Isolation of splenic neutrophils using the OAC protocol generates a pure neutrophil population with limited cell death and activation without impairing function. Spleens were isolated from 5-month old B6.Fas^{lpr}/J and B6 mice. Single cell suspensions were analyzed before (pre-enrich) and after (post-enrich) isolation of neutrophils using the OAC method to determine (**A**) purity and (**B**) viability. (**A,B**) Combined data from four separate animals. (**C**) Expression of the activation markers CD18, CD11a, and CD62L on isolated neutrophils 4 hours after purification. One representative experiment from two independent experiments is shown. (**D**) Isolated neutrophils were incubated in the presence of fluorescently labeled *S. aureus* particles with prior opsonization for 2 hours. Engulfment of opsonized *S. aureus* by neutrophils was confirmed by high-resolution microscopy and flow cytometry using ImageStreamX technology. One representative image from more than 250 images taken from two separate animals for each purification method is shown. Figure adapted from [203].

BAFF levels are influenced by BCMA in murine lupus. We previously demonstrated that lupus-prone mice with a deficiency in BCMA have increased serum BAFF levels [139]. To evaluate further the role of BCMA on the expression of BAFF in autoimmunity, we studied the BAFF-producing innate immune cells in spleens of female naïve C57BL/6 (WT) mice and congenic lupus-proneB6.Fas^{lpr}/J mice, and compared mice sufficient or deficient in the gene *Tnfrsf17* that encodes BCMA. Twenty-five-week old mice were analyzed due to robust differences in the amount of circulating BAFF among the genotypes.B6.Fas^{lpr}/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice showed increased serum BAFF levels, with significantly higher BAFF levels in the absence of BCMA compared to WT and *Tnfrsf17*$^{-/-}$controls, indicating that heightened BAFF levels were dependent on *lpr* predisposition (**Figure 4.2A**).

Our lab has demonstrated an increased frequencies of splenic CD11c^{+} DCs in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to B6.Fas^{lpr}/J mice [139], suggesting that the heightened level of circulating BAFF in these mice may be derived from elevated numbers of BAFF-producing DCs. Interestingly, hematoxylin and eosin staining of spleen sections revealed significant accumulation of polymorphonuclear (PMN) leukocytes with a neutrophil phenotype in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to the other strains (**Figure 4.2B**). PMNs in the spleens of B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice were found in abundance surrounding enlarged follicles with a poorly defined mantle zone, whereas fewer PMNs surrounding follicles were observed in spleens ofB6.Fas^{lpr}/J mice. Analysis of spleens from WT and *Tnfrsf17*$^{-/-}$ mice indicated that PMNs were found predominantly within the mantle zone. These data suggest that neutrophils, together with dendritic cells,

may contribute to excess BAFF production in autoimmunity and are modulated in cell frequency or BAFF production by BCMA.

To determine whether BCMA deficiency affects the accumulation and BAFF expression of DCs and neutrophils, we examined these cells by immunofluorescence staining for Ly6G and CD11c and quantified BAFF co-localization in spleens of mice. Confocal imaging confirmed increased frequencies of $CD11c^+$ DCs in spleens of B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to the other strains (**Figure 4.2C, left panel**). Despite increased splenic $CD11c^+$ DCs in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice, we observed no differences in $CD11c^+$ cells that co-localized with BAFF expression compared to the other strains. To verify this observation, we quantified the intensity of fluorescently stained BAFF on each $CD11c^+$ cell as a measurement of the amount of BAFF protein using the image analysis software, CellProfiler [205]. No significant differences in BAFF intensities co-localizing to $CD11c^+$ DCs were measured among the mouse strains (**Figure 4.2C right panel**). We also did not observe any differences in BAFF intensities co-localizing to $F4/80^+$ cells, suggesting that BCMA deficiency does not affect BAFF protein expression in splenic macrophages (**data not shown**).

Figure 4.2

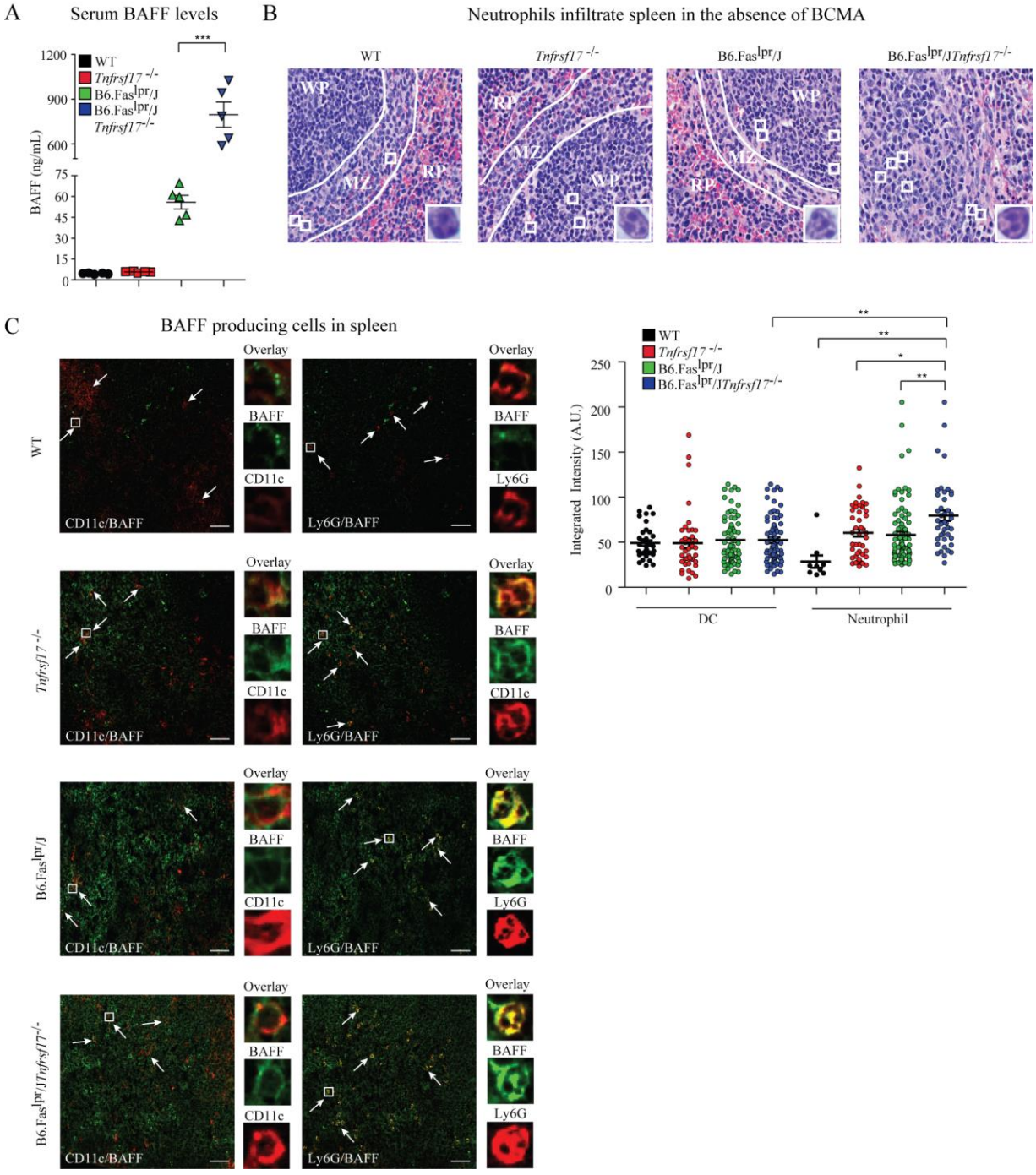
A
Serum BAFF levels
BAFF (ng/mL)
WT
Tnfrsf17 -/-
B6.Fas lpr/J
B6.Fas lpr/J Tnfrsf17 -/-
***
B
Neutrophils infiltrate spleen in the absence of BCMA
WT
Tnfrsf17 -/-
B6.Fas lpr/J
B6.Fas lpr/J Tnfrsf17 -/-
WP
MZ
RP
C
BAFF producing cells in spleen
Overlay
BAFF
CD11c
Ly6G
CD11c/BAFF
Ly6G/BAFF
Integrated Intensity (A.U.)
DC
Neutrophil

Figure 4.2. Neutrophils and dendritic cells contribute to elevated BAFF levels in the absence of BCMA. (**A**) BAFF levels were measured in sera from mice of the indicated genotype. Each symbol represents a single animal. (**B**) Spleen sections from 6 month-old mice were stained with H&E. Each arrow represents a neutrophil identified by nuclear structure, with the boxed neutrophil magnified in the inset image. Magnification at 40X. Shown are representative images from 5 mice per genotype. RP – red pulp, FO – follicle, MZ – mantle zone. (**C)** Left panels: spleen sections from 6-month-old mice were analyzed for BAFF-producing cells by immunofluorescence. BAFF – green, CD11c or Ly6G – red. Scale bar represents 50μm. Arrows represent cells with co-localization of BAFF and either CD11c or Ly6G, with the boxed cell magnified in the inset images. One representative image from 4 mice of each genotype is shown. Right panel: quantification of the intensity of BAFF staining from spleen-resident $CD11c^+$ DCs and $Ly6G^+$ neutrophils was measured by CellProfiler. Each symbol represents an individual cell within a single histological sample from a minimum of 3 distinct samples/genotype. Error bars indicate mean $\pm$ SEM. Statistics determined using a one-way ANOVA with a Tukey post test (**A,C**),and denoted as follows: *p <0.05, **p <0.01, ***p <0.001.

Neutrophils are phenotypically defined as $Ly6G^+$ $CD11b^+$ and have been shown to be potent BAFF producers [200, 206, 207]. To investigate further whether BCMA deficiency controls BAFF production via neutrophils, we examined Ly6G and BAFF expression in spleen. WT and *Tnfrsf17*$^{-/-}$mice had few $Ly6G^+$ cells present in spleen (**Figure 4.2C**). In contrast, B6.Fas^{lpr}/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice consistently had higher frequencies of splenic $Ly6G^+$ cells. Quantification of BAFF intensity per $Ly6G^+$ cell demonstrated increased BAFF intensities of $Ly6G^+$ cells in spleens of *Tnfrsf17*$^{-/-}$ and B6.Fas^{lpr}/J mice, with higher BAFF intensities in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to WT controls (**Figure 4.2C**).Taken together, these data indicate that BCMA deficiency in lupus-prone mice controls the abnormal accumulation of splenic $CD11c^+$ DCs and neutrophils, and suggests that neutrophils contribute to excess circulating BAFF levels in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice.

BCMA deficiency in lupus-prone mice promotes accumulation of spleen-resident neutrophils. We next evaluated the steady state frequencies of splenic neutrophils in 6-week-old and 25-week-old mice. No differences in the percentages and numbers of $Ly6G^+$ $CD11b^+$neutrophils were observed among 6-week-old mice (**Figure 4.3A and data not shown**). In contrast, B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice showed significantly higher numbers of splenic neutrophils at 25 weeks of age compared to the other strains. ImageStream analysis confirmed that the $Ly6G^+CD11b^+$cells isolated from spleens of B6.Faslpr/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice were neutrophils, as defined by the classic poly-morphonuclear granulocyte morphology (**DAPI staining; Figure 4.3B**). We did not observe any differences in the frequencies of $Ly6G^+CD11b^+$ neutrophils in the bone

marrow of lupus-prone mice compared to control animals, suggesting that the increased frequency of splenic neutrophils in lupus-prone mice resulted from accumulation and not increased neutrophil development (**data not shown**). Increased frequencies of splenic neutrophils were also found in BCMA deficient congenic *Nba2* lupus-prone mice [139, 177], which provided independent confirmation that BCMA expression is critical for restraining accumulation of neutrophils in autoimmunity (**Figure 4.3C**). Finally, to determine whether these findings were limited to murine autoimmune susceptibility genes, we examined splenic neutrophils in WT and *Tnfrsf17*$^{-/-}$ mice under environmentally induced lupus conditions after treatment with the inflammatory agent pristine [178]. *Tnfrsf17*$^{-/-}$mice had significantly higher numbers of splenic neutrophils compared to WT mice following pristane treatment and mock-treated controls (**Figure 4.3D**). These data indicate that BCMA is a key regulator of splenic neutrophil accumulation in autoimmunity.

Figure 4.3

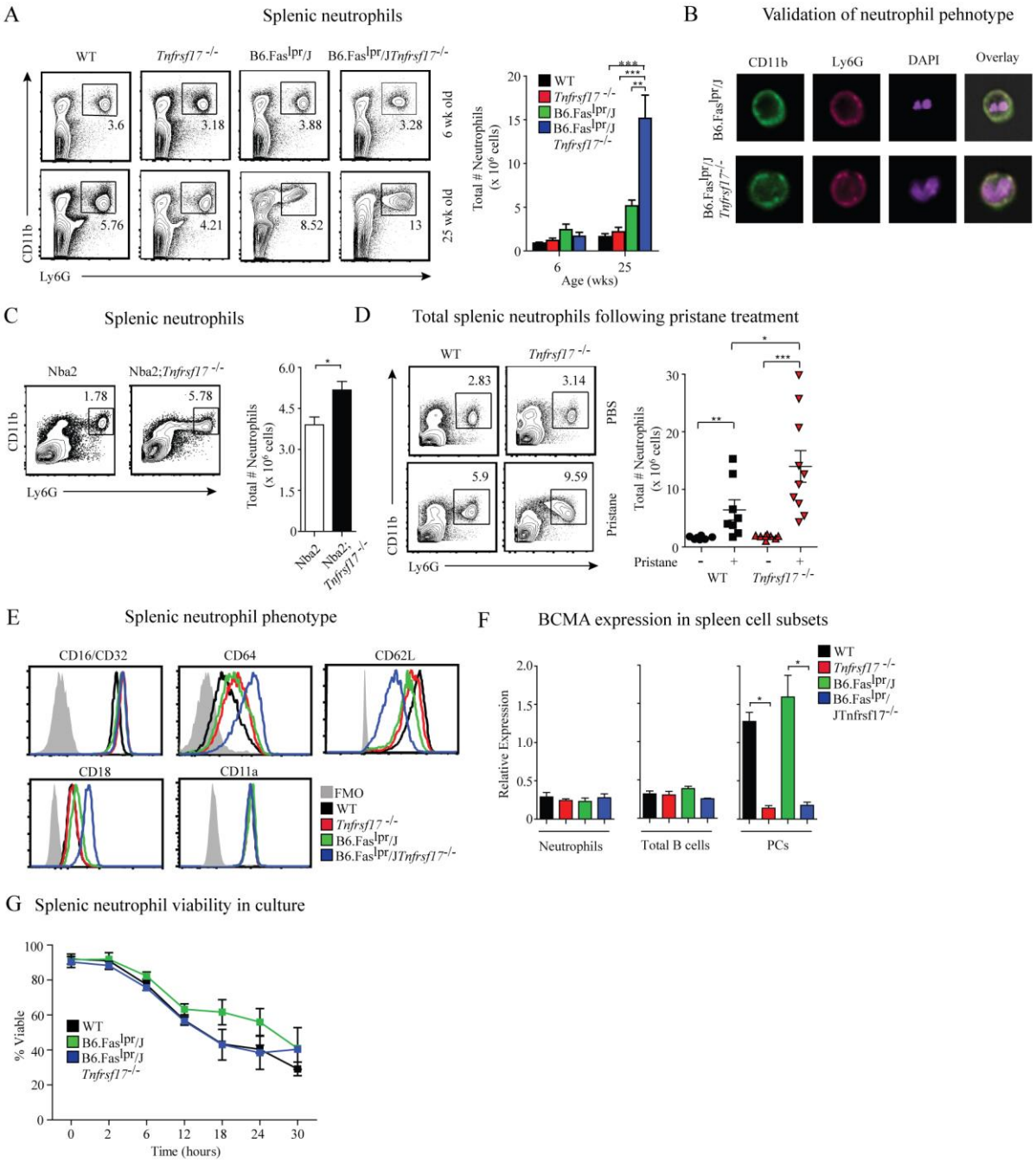
A
Splenic neutrophils
WT
Tnfrsf17 -/-
B6.Fas lpr/J
B6.Fas lpr/JTnfrsf17-/-
6 wk old
25 wk old
3.6
3.18
3.88
3.28
5.76
4.21
8.52
13
CD11b
Ly6G
Total # Neutrophils (x 10^6 cells)
Age (wks)
B
Validation of neutrophil pehnotype
CD11b
Ly6G
DAPI
Overlay
C
Splenic neutrophils
Nba2
Nba2;Tnfrsf17 -/-
1.78
5.78
D
Total splenic neutrophils following pristane treatment
2.83
3.14
5.9
9.59
PBS
Pristane
Pristane
E
Splenic neutrophil phenotype
CD16/CD32
CD64
CD62L
CD18
CD11a
FMO
F
BCMA expression in spleen cell subsets
Relative Expression
Neutrophils
Total B cells
PCs
G
Splenic neutrophil viability in culture
% Viable
Time (hours)

Figure 4.3.Neutrophils accumulate and have an activated phenotype in spleens of lupus-prone in the absence of BCMA.(**A**) The frequency and total number of splenic neutrophils ($CD11b^{+}Ly6G^{+}$) was determined in mice of the indicated age and genotype. Left panel: representative flow cytometry plots. Right panel: combined data from 6-9 mice/genotype. (**B)** Neutrophil morphology was validated using ImageStream technology by measuring co-localization of CD11b, Ly6G and the presence of a multi-lobed nucleus using DAPI. One representative image/genotype from at least 100 images/genotype is shown. Images were taken at 60X. (**C**) The frequency of neutrophils in spleens of *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice were quantified from 9 mice per strain. (**D)** Three-month-old WT and*Tnfrsf17*$^{-/-}$ mice were injected with a single dose of either PBS or pristane. After 4 weeks, the frequency of neutrophils in spleens of mice was determined. Left panel: representative flow cytometry plots. Right panel: total numbers of neutrophils for each group; each symbol represents an individual animal. Combined data from two independent experiments. (**E)** Representative histograms showing the activation state of neutrophils freshly isolated from spleens of mice. One histogram from 5 individual mice/genotype analyzed. (**F)** Gene expression values of *Tnfrsf17*relative to *HPRT* in sorted cells from spleens of mice were determined by qPCR. The expression of *Tnfrsf17* in BCMA deficient animals for each cell population was set to one. Combined data from three independent mice/genotype. (**G**) The percent of viable neutrophils was measured at the indicated time-points from purified splenic neutrophils using LIVE/DEAD® Fixable AQUA. Combined data from 3 mice/genotype. Error bars indicate mean $\pm$ SEM. Statistics determined with a one-way ANOVA using a Tukey post-test (**A,D,F**) or Student's t-test (**C**), and denoted as follows:*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A previous report has demonstrated that increased BAFF production by neutrophils is linked with an elevated activation state [198]. Following activation, neutrophils upregulate the low affinity IgG Fc receptors (CD16/CD32), the high affinity receptor for IgG (CD64), and lymphocyte function associated antigen (LFA-1) (comprised of CD11a and CD18), as well as downregulate CD62L expression [208-210]. Therefore, we tested whether the increased frequency of splenic BAFF-producing neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice was associated with cell activation using these markers. We observed minimal differences in membrane expression levels of CD11a and CD16/CD32 on neutrophils among the strains (**Figure 4.3E**). In contrast, neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice had markedly higher expression levels of CD64 and CD18, and lower CD62L compared to neutrophils from control mice. Splenic neutrophils from*Nba2*;*Tnfrsf17*$^{-/-}$mice also expressed increased levels of CD64 and CD18, and decreased CD62L expression (**data not shown**). These data indicate that the absence of BCMA in lupus-prone mice contributes to an activated phenotype of spleen-resident neutrophils.

To determine if this neutrophil effect was due to intrinsic expression, and therefore directly involved in controlling the accumulation of splenic neutrophils in lupus-prone mice, we analyzed BCMA expression by measuring mRNA transcript levels. We found that BCMA was not expressed in neutrophils from B6.Fas^{lpr}/J mice compared to WT, *Tnfrsf17*$^{-/-}$, and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice (**Figure 4.3F**). Plasma cells from WT and B6.Fas^{lpr}/J mice served as a positive control, whereas naïve B cells that do not express BCMA served as negative controls. Additionally, no differences in the viability of purified neutrophils among the mouse strains were observed (**Figure 4.3G**). These

data indicate that the accumulation and activation of neutrophils inB6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ spleens is not due to BCMA deficiency in neutrophils, but results from the loss of BCMA in other cell types.

Neutrophils co-localize with CD4$^+$ T cells in spleens of lupus-prone mice and contribute to T cell response through a BAFF-dependent mechanism. Low numbers of BAFF-producing neutrophils have been detected in the perifollicular regions of spleens from healthy humans [200]. To determine further where neutrophils accumulate in spleens of lupus-prone mice under steady state conditions, we stained tissue sections for Ly6G and CD11b, as well as for PNA and IgD to identify B cell follicles (PNA$^-$IgD$^+$) and germinal centers (PNA$^+$IgD$^-$). We detected Ly6G$^+$CD11b$^+$neutrophils in the perifollicular area of spleens from WT mice (**Figure 4.4A**). Neutrophils were also detected outside B cell follicles and germinal centers in spleens of*Tnfrsf17*$^{-/-}$and B6.Fas^{lpr}/J mice, with even more perifollicular neutrophils that extended outward, beyond the follicular mantle in spleens of B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice. Interestingly, neutrophils were positioned in the T cell zones of *Tnfrsf17*$^{-/-}$, B6.Fas^{lpr}/J andB6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to WT mice, as measured by co-localization of Ly6$^+$ and CD4$^+$cells (**Figure 4.4B**). These data suggest that splenic neutrophils accumulating in the T cell zones ofB6.Fas^{lpr}/J mice, and even more inB6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice, may interact with CD4$^+$ T cells.

Figure 4.4

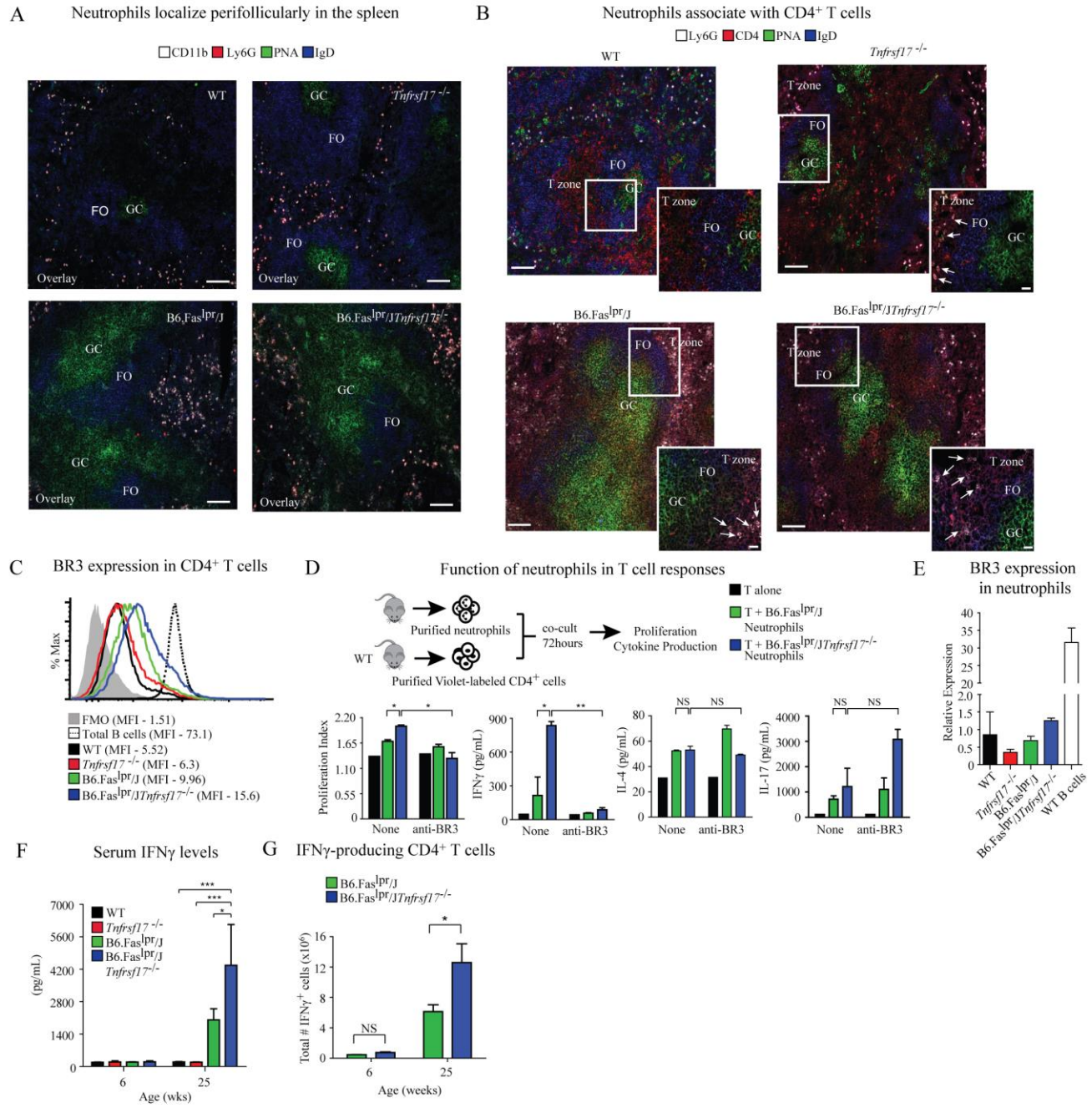
A
Neutrophils localize perifollicularly in the spleen
CD11b Ly6G PNA IgD
WT
Tnfrsf17 -/-
B6.Fas lpr/J
B6.Fas lpr/JTnfrsf17 -/-
FO
GC
Overlay
B
Neutrophils associate with CD4+ T cells
Ly6G CD4 PNA IgD
T zone
C
BR3 expression in CD4+ T cells
% Max
FMO (MFI - 1.51)
Total B cells (MFI - 73.1)
WT (MFI - 5.52)
Tnfrsf17 -/- (MFI - 6.3)
B6.Fas lpr/J (MFI - 9.96)
B6.Fas lpr/JTnfrsf17 -/- (MFI - 15.6)
D
Function of neutrophils in T cell responses
Purified neutrophils
WT
Purified Violet-labeled CD4+ cells
co-cult 72hours
Proliferation Cytokine Production
T alone
T + B6.Fas lpr/J Neutrophils
T + B6.Fas lpr/JTnfrsf17 -/- Neutrophils
Proliferation Index
IFNγ (pg/mL)
IL-4 (pg/mL)
IL-17 (pg/mL)
None
anti-BR3
NS
E
BR3 expression in neutrophils
Relative Expression
WT
Tnfrsf17 -/-
B6.Fas lpr/J
B6.Fas lpr/JTnfrsf17 -/-
WT B cells
F
Serum IFNγ levels
(pg/mL)
Age (wks)
G
IFNγ-producing CD4+ T cells
Total # IFNγ+ cells (x10⁶)
Age (weeks)

Figure 4.4.Neutrophils co-localize in T cell zones and influence CD4$^+$ T cell responses in a BAFF-dependent manner. Confocal microscopy of spleen sections demonstrating (A) *in situ* localization of neutrophils and (**B)** co-localization of neutrophils and CD4$^+$ T cells. Scale bar indicates 100μm. Scale bar of inset indicates 20μm.Images are representative of at least 3 images from four mice/genotype. (**C**) Representative histograms showing BR3 expression on CD4$^+$ T cells from spleens of 3mice/genotype. Total B cells (B220$^+$) and a FMO stain served as controls. (**D)** Purified neutrophils from spleens of lupus-prone mice were co-cultured with CD4$^+$ T cells from WT mice at a 1:1 ratio ± BR3 blocking antibody. After 3 days of culture, CD4$^+$ T cell proliferation was measured by CellTrace™ Violet dilution and cytokine concentrations in culture supernatant were measured by ELISA. Combined data from 3 mice/genotype. 1 of 3 independent experiments with similar results is shown. (**E)** Gene expression levels of *Tnfrsf13C* (BR3) in purified neutrophils from mice of the indicated genotype. Samples were normalized to *HPRT* and the expression in WT animals was set to one. Total B cells are shown as a positive control. Combined data from 3 mice/genotype. (**F)** Serum IFNγ levels from 6- and 25-week-old mice of the indicated genotype were measured by ELISA. Combined data from 5-9 mice/genotype. (**G)** Total splenocytes from mice of the indicated genotype were cultured in the presence or absence of PMA/ionomycin with GolgiStop™. The frequency of IFNγ-producing CD4$^+$ T cells was assessed following 5 hour *in vitro* stimulation. Combined data from 4 mice/genotype at 6 and 25weeks of age. Error bars indicate mean $\pm$ SEM. Statistics determined with a two-way ANOVA using a Bonferroni post test (**D**), or a one-way ANOVA with a Tukey post test (**F,G**), and denoted as follows:*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns – not significant.

We hypothesized that the accumulation of splenic neutrophils in T cell zones of lupus-prone mice may influence $CD4^+$ T cell responses through a BAFF-dependent mechanism. We and other groups have reported that murine splenic mature $CD4^+$ T cells express low levels of the BAFF receptor BR3 [162, 163, 211] (**Figure 2.8E**). Consistent with these studies, we found that BR3 is expressed at low levels on splenic $CD4^+$ T cells of all mouse strains compared to the high expression levels of BR3 on B cells (**Figure 4.4C**). BR3 expression levels on $CD4^+$ T cells from lupus-prone mice were slightly higher, as measured by mean fluorescence intensity (MFI) values but were not significant. To directly test whether splenic neutrophils from lupus-prone mice affected $CD4^+$ T cell function, we developed a co-culture system to measure neutrophil-mediated $CD4^+$ T cell responses *in vitro*. Purified neutrophils from B6.Fas^{lpr}/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice were co-cultured with mature resting $CD4^+$ T cells from WT mice and T cell proliferation and cytokine production in the culture supernatants were measured. Using this assay, we measured greater $CD4^+$ T cell proliferation from co-cultures with neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared to B6.Fas^{lpr}/J and T cells cultured alone (**Figure 4.4D**). Confirmation that neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice induced T cell proliferation by producing BAFF that signals through BR3 on $CD4^+$ T cells was determined by adding a BR3 neutralizing mAb to the cultures, which reduced T cell proliferation(**Figure 4.4D**). Neutrophils do not express BR3, BCMA or TACI and therefore cannot respond to BAFF (**Figure 4.4E, data not shown**). These data are consistent with our earlier findings that neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice have a more robust activated phenotype compared to neutrophils from B6.Fas^{lpr}/J mice (**Figure 4.3E**).

Previous work using an OT-II system suggests that neutrophils may induce both Th1 and Th17 differentiation [212]. To determine whether autoimmune-prone neutrophils are driving different effector cell subsets, we co-cultured resting WT $CD4^+$ T cells with neutrophils from B6.Fas^{lpr}/Jor B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice and measured IFNγ, IL-4 and IL-17 in culture supernatant to determine the relative differentiation of Th1, Th2, and Th17 responses, respectively. The results demonstrated that T cells cultured alone produced low amounts of IFNγ, IL-4 and IL-17 (**Figure 4.4D**). Addition of neutrophils from both B6.Fas^{lpr}/JandB6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice to the cultures increased IFNγ production by T cells, with the IFNγ levels induced by neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice substantially higher. Neutrophils from both B6.Fas^{lpr}/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice promoted minimal IL-4 responses and induced equivalent levels of IL-17 (**Figure 4.4D**). To evaluate the relative contribution of BAFF generated by neutrophils from lupus-prone mice to mediate cytokine production in $CD4^+$ T cells of WT mice, we utilized a BR3 blocking mAb. Blocking BR3 reduced IFNγ production by T cells to basal levels, indicating that BAFF produced by splenic neutrophils is responsible for IFNγ production in $CD4^+$ T cells in this system. However, blocking BR3 had no impact on IL-4 production. Interestingly, blocking BR3 increased the IL-17 production by T cells when cultured with neutrophils from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice but not B6.Fas^{lpr}/J mice; however this increase was not statistically significant (**Figure 4.4D**).

As previously mentioned, excess production of IFNγ by $CD4^+$ T cells is associated with the pathogenesis of SLE [187]. Analysis of serum IFNγ titers in lupus-prone mice demonstrated significantly higher levels from B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice with age compared to B6.Fas^{lpr}/J mice and control animals, which had little IFNγ (**Figure**

4.4F). Moreover, increased numbers of IFNγ-producing $CD4^+$ T cells in spleens of B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice were observed compared to B6.Fas^{lpr}/J mice (**Figure 4.4G**). These data suggest that the elevated amount of BAFF generated by neutrophils in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice compared toB6.Fas^{lpr}/J mice signals through BR3 on $CD4^+$ T cells to preferentially drive the differentiation of Th1 cells.

Depletion of neutrophils reduces excess BAFF and IFNγ levels and autoimmunity in B6.Fas^{lpr}/JTnfrsf17$^{-/-}$mice. The above *in vitro* findings support the possibility that, like DCs present in spleens of B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice and *Nba2;Tnfrsf17*$^{-/-}$ mice [139, 213], neutrophils may also serve as a key source of BAFF for promoting IFNγ production and humoral autoimmunity *in vivo*. To address this possibility, we examined the impact of antibody mediated (anti-Ly6G [214]) *in vivo* depletion of neutrophils in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice on BAFF and IFNγ production, germinal center B cell responses, and autoantibody titers after 4 weeks of treatment. Administration of the Ly6G depleting antibody reduced splenic neutrophil frequencies by 80% (**Figure 4.5A**). Neutrophil depletion resulted in a substantial decrease in both serum BAFF and IFNγ levels (**Figure 4.5B, C**). We found reduced numbers of $CD4^+$ T cells, including IFNγ-producing T cells, in spleens ofB6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice compared to isotype antibody-treated mice (**Figure 4.5D**). Since reduced levels of BAFF and $CD4^+$ T cell help would be expected to affect peripheral B cell responses, we evaluated spleen cellular responses. Mice that were neutrophil depleted had significantly decreased numbers of B cells, germinal center B cells, and plasma cells (**Figure 4.5E**). Moreover, we observed reduced serum anti-dsDNA IgG titers and immune complex deposition in kidneys of mice after

neutrophil depletion (**Figure 4.5F, G**). These data indicate that neutrophils drive autoimmunity by promoting abnormal BAFF and IFNγ production.

Discussion

Neutrophils play a key role in the innate host defense to infection [215, 216]. They eliminate pathogens through phagocytosis, the release of antimicrobial products, and the release of NETs that trap and destroy invading pathogens. In addition, neutrophils have emerged as important regulators of adaptive immune responses to infection by modulating antibody production, dendritic cell activation, and antimicrobial T cell responses. The abnormal accumulation and function of neutrophils are linked with autoimmune diseases such as SLE, yet their role in disease pathogenesis is unclear. Neutrophils derived from SLE patients have impaired phagocytic capacity, increased activation state and, in some patients, a propensity to release NETs [193, 195, 217]. Thus, neutrophils in autoimmunity may contribute to the presentation of auto-antigens, leading to autoantibody production. Neutrophils are also a source of cytokines, including BAFF that are important for B cell development, differentiation, and survival [198-200, 206]. In lupus-prone mice and SLE patients, increased circulating BAFF levels correlate with increased numbers of peripheral B cells, B cell hyperactivity, and autoantibody production [173, 218]. Thus, neutrophils may also play immunoregulatory roles in autoimmunity in secondary lymphoid organs by producing excess BAFF that promotes survival of autoreactive B cells and the production of autoantibodies. Here we found that the accumulation, activation, and BAFF expression of neutrophils in lupus-prone mice are increased further in the absence of BCMA.

Figure 4.5

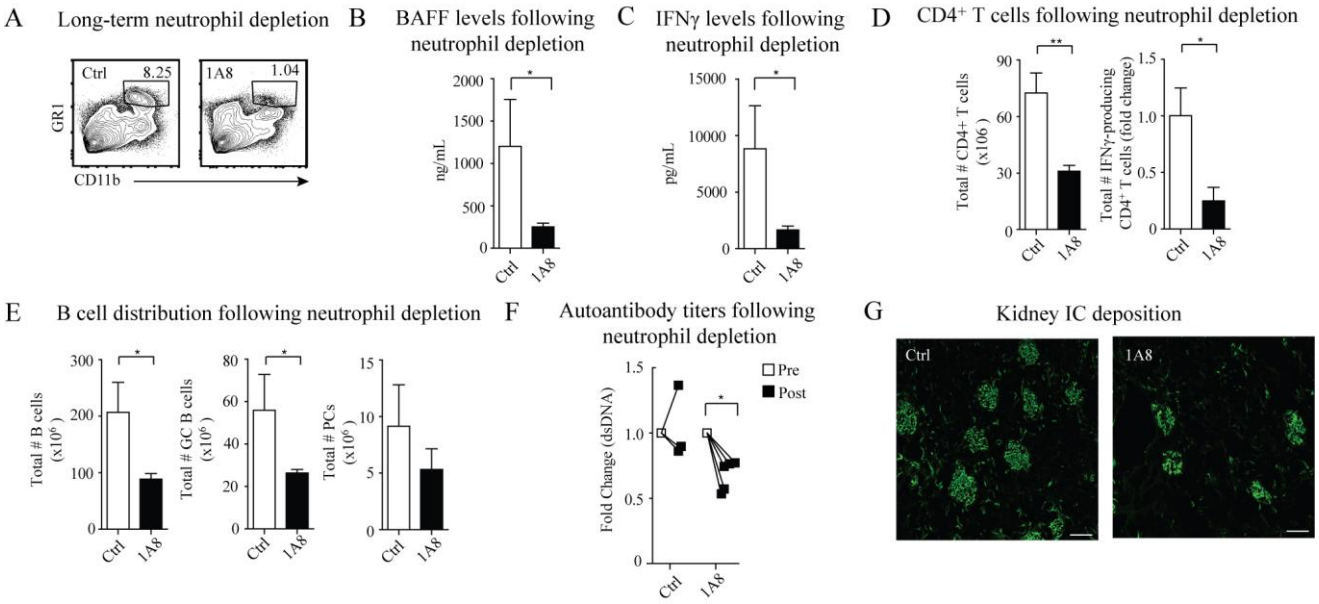
A
Long-term neutrophil depletion
Ctrl
8.25
1A8
1.04
GR1
CD11b
B
BAFF levels following neutrophil depletion
ng/mL
2000
1500
1000
500
0
Ctrl
1A8
C
IFNγ levels following neutrophil depletion
pg/mL
15000
10000
5000
0
Ctrl
1A8
D
CD4+ T cells following neutrophil depletion
Total # CD4+ T cells (x106)
90
60
30
0
Ctrl
1A8
Total # IFNγ-producing CD4+ T cells (fold change)
1.5
1.0
0.5
0
Ctrl
1A8
E
B cell distribution following neutrophil depletion
Total # B cells (x10⁶)
300
200
100
0
Ctrl
1A8
Total # GC B cells (x10⁶)
80
60
40
20
0
Ctrl
1A8
Total # PCs (x10⁶)
15
10
5
0
Ctrl
1A8
F
Autoantibody titers following neutrophil depletion
Pre
Post
Fold Change (dsDNA)
1.5
1.0
0.5
0
Ctrl
1A8
G
Kidney IC deposition
Ctrl
1A8

Figure 4.5. Depletion of neutrophils ameliorates autoimmune disease. B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$mice were treated with the anti-Ly6C (1A8) neutrophil depleting antibody or a control antibody. After 4 weeks, sera, kidney and spleen were analyzed for markers of autoimmune disease. (**A)** Representative flow cytometry plots showing reduced frequency of neutrophils (CD11b^{+}GR1^{+}) in spleens after treatment. One plot from 5 mice/group is shown. **(B,C)** Serum BAFF and IFNγ levels were measured after treatment by ELISA. Combined data from 5 mice/genotype. (**D)** Total numbers of CD4^{+} T cells and IFNγ-producing CD4^{+} T cells were quantified from spleens of mice after treatment. Combined data from 5 mice/group. (**E)** Total numbers of B cells, GC B cells (CD19^{+}GL7^{+}CD138^{-}), and PCs (CD19$^{+/low}$CD138^{+}) in spleens of mice after treatment were determined by flow cytometry. (**F**) Serum dsDNA IgG titers of mice before and after treatment were determined by ELISA. Each symbol represents the fold change of autoantibody titers from an individual mouse after treatment. (**G)** IgG and C3 deposition in kidneys of mice was measured after treatment. One representative image from 5 mice/group is shown. **(A-G),** One of two independent experiments with similar results is shown. Error bars indicate mean $\pm$ SEM. Statistics determined with a Student's t-test (**B-E**), or a two-way ANOVA using Bonferroni post test (**F**), and denoted as follows: *p <0.05, **p <0.01.

We also show that neutrophils from BCMA deficient lupus-prone mice induce $CD4^+$ T cell proliferation and IFNγ production in a BAFF-dependent manner more potently compared to neutrophils from BCMA sufficient lupus-prone mice. These findings correlated with higher BAFF and IFNγ serum levels, as well as increased frequencies of IFNγ-producing $CD4^+$ T cells, in BCMA deficient lupus-prone mice compared to control animals. Reduced BAFF and IFNγ serum levels, decreased frequencies of IFNγ-producing T cells, GC B cells, and autoantibody production after neutrophil depletion indicated the involvement of neutrophils in these autoimmune traits.

The relevant BAFF receptor(s) through which increased BAFF levels promote lupus is unknown. Given that signals transduced through BR3 on mature B cells and through BCMA on plasma cells support cell survival, it has been thought that the expression of these receptors on B lineage cells is largely responsible for BAFF-mediated B cell hyperactivity and autoantibody production in SLE. However, both *lpr* and *Nba2* mice deficient in BCMA unexpectedly develop an accelerated lupus-like disease that is associated with aberrant BAFF levels, germinal center formation, and $CD4^+$ T cell-dependent autoantibody production [139], suggesting an important role for BCMA in the context of murine lupus. Our findings indicate that BCMA critically influences BAFF production by controlling the accumulation and activity of spleen-resident neutrophils. BAFF signaling through BR3 expressed on T cells has been shown to induce proliferation and cytokine production. [162, 164, 165, 211]. Thus, apart from excess BAFF directly affecting the activation and survival of autoreactive B cells, BAFF-dependent regulation of $CD4^+$ T cell responses may also contribute to B cell hyperactivity and loss of tolerance in autoimmunity.

Although B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ neutrophils induce CD4^{+} T cell proliferation and IFNγ secretion through BAFF, IL-4 and IL-17 production were driven via an alternative mechanism. These findings are consistent with previous reports demonstrating that neutrophils induce IL-17 via an MHC-dependent mechanism [212, 219]. Importantly, in autoimmune-prone mice, neutrophils localize within the T cell zone *in situ*, suggesting our *in vitro* findings are biologically relevant. Although the role of type I IFNs in SLE has been well established, the significance and impact of the type II IFNs, has only more recently been appreciated [220-222]. Furthermore, multiple groups have shown the benefits of disrupting the IFNγ signaling pathway in autoimmune disease. Both MRL/*lpr* and NZBWF1 mice deficient in IFNγ or the IFNγ receptor showed significant improvement in disease [186, 223, 224]. Our findings suggest that neutrophils may contribute to increased IFNγ production. Consistent with this notion, depletion of neutrophils *in vivo* led to a substantial reduction in serum IFNγ and IFNγ-producing CD4^{+} T cells, as well as an overall improvement in disease severity.

BCMA expression was not detected in neutrophils. This finding indicates that BCMA deficiency in neutrophils themselves is not involved in the differences of neutrophil accumulation and activation betweenB6.Fas^{lpr}/J and B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ animals. We propose that the overall inflammatory environment in B6.Fas^{lpr}/J*Tnfrsf17*$^{-/-}$ mice affects neutrophil accumulation compared to B6.Fas^{lpr}/J mice [139]. How neutrophils are recruited to secondary lymphoid organs is unknown; however, IFNγ has been shown to mediate neutrophil recruitment to the lung following tissue injury or infection [225, 226]. Thus, further work will be necessary to determine whether a feedback loop exists between BAFF-producing neutrophils and IFNγ-producing CD4^{+} T

cells in the recruitment of neutrophils in autoimmune disease. Taken together, our findings demonstrate a role for neutrophils in the pathogenesis of murine lupus via a T cell-mediated mechanism.

Summary Model

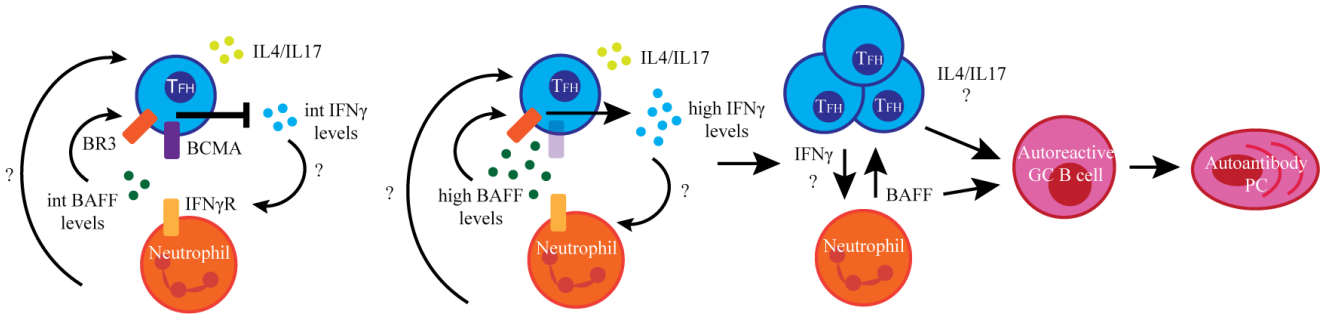

Figure 4.6. Neutrophils contribute to excess serum BAFF levels and promote CD4$^+$ and B cell responses in autoimmunity. In autoimmune prone mice, neutrophils re-localize to the T cell zone and interact with CD4$^+$ T cells through both BAFF-dependent and –independent mechanisms to promote cytokine production. Upregulation of BCMA by T_{FH} cells prevents aberrant signaling by BAFF through BR3, limiting IFNγ production. Although the IFNγ receptor is expressed by neutrophils, and other reports have shown that IFNγ is an inducer of BAFF expression by neutrophils, the role of T_{FH}-derived IFNγ in perpetuating BAFF in neutrophils is unknown in our model. Loss of BCMA in T_{FH} cells results in strong signaling through BR3, which leads to hyper-production of IFNγ. Presumably, the cyclic process mediated by BAFF and IFNγ, produced by neutrophils and T_{FH} cells, respectively, promotes the generation of auto-reactive GC B cells and auto-reactive PCs. The role of IL4 and IL17 in the disease process is unknown. Depletion of neutrophils in BCMA-deficient autoimmune-prone hosts can ameliorate disease, underscoring the importance of these cells.

CHAPTER 5

Regulation of T Follicular Helper Cells and the GC Response by T Follicular Regulatory Cells

(Preliminary Data)

Optimal T_{FH} cell generation and GC formation are paramount to generating a robust and long lasting immune response [5, 55]. Data from numerous groups support a model where access to a limited number of T_{FH} cells promotes the development of only the highest affinity GC-B cells that will ultimately become high affinity memory B cells and PCs [5, 9]. In agreement with this, outgrowth of T_{FH} cells, as occurs in the absence of $CD4^+$ regulatory cells, results in a reduced frequency of antigen specific PCs following protein immunization [66]. Furthermore, following pathogen clearance, resolution of the GC is required to prevent unwanted GC responses that could lead to the development of autoimmunity. The subset of regulatory T cells critical for maintaining T_{FH} homeostasis during the GC response is known as T follicular regulatory (T_{FR}) cells [59, 66, 67].

Despite our appreciation of the existence of T_{FR} cells, little is known about how these cells control the GC response. *In vitro* studies have demonstrated that T_{FR} cells induce suppression of $CD4^+$ T cell proliferation, but the mechanism of this suppression has not been addressed [59, 66, 67]. Studying the role of T_{FR} cells *in vivo* has been complicated by the inability to specifically deplete T_{FR} cells [66, 67]. Therefore, whether T_{FR} cells act by directly suppressing T_{FH} cells, GC-B cells, inducing tolergenic DCs, or by an alternative mechanism is unknown. Furthermore, how T_{FR} cells function in the context of aberrant outgrowth of T_{FH} cells, such as in the setting of autoimmune disease, is completely open to investigation. Given that the absence of BCMA leads to a significant increase in the frequency and effector function of T_{FH} cells in autoimmune-prone mice, we sought to characterize the T_{FR} cell population in the presence or absence of BCMA.

Methods

Mice.Tnfrsf17$^{-/-}$ and *Nba2*, *Nba2;Tnfrsf17*$^{-/-}$, fully backcrossed onto the C57BL/6 (B6) strain, were previously described [139, 177]. Age-matched wild-type (WT) B6 mice were purchased from NCI. WT, *Tnfrsf17*$^{-/-}$ and *Nba2*, *Nba2;Tnfrsf17*$^{-/-}$ mice were crossed with the B6.FoxP3$^{gfp/gfp}$ reporter mouse [227]. For each experiment, a minimum of 5 mice was used for each strain unless indicated to account for variability in disease phenotype and assess statistical significance. No mice were excluded from the data analysis and no randomization was performed. Mice were housed in a specific pathogen-free animal facility at the University of Virginia.

Study subjects. Females that met the American College of Rheumatology Revised Criteria for SLE and age-matched healthy females were enrolled in this study during routine clinical care through the Division of Rheumatology at the University of Virginia School of Medicine (Charlottesville, VA). Exclusion criteria included patients who were pregnant or receiving the following treatments: i.v.steroids, cyclophosphamide, >20 mg oral prednisone daily, or B-cell and BAFF-depleting therapies. The SLE Disease Activity Index (SLEDAI) measured disease activity. Additional SLE patient blood was purchased from AllCells (Alameda, CA). No blood samples were excluded from the data analysis.

Flow cytometry. Mouse spleen single-cell suspensions were prepared and red blood cells were lysed with ammonium chloride–Tris solution. Whole blood was obtained from mice in heparin-treated tubes (Fisher Scientific). Human patient blood was obtained in ACD tubes (BD Bioscience) and was spun down to separate plasma and cellular fractions. Patient sera were prepared with thrombin (Sigma) and peripheral blood mononuclear cells were isolated using a Ficoll gradient (GE Healthcare). For

phenotyping, cells were stained at a concentration of 0.5-2 x10^6 cells/well in a 100µl volume. Mouse antibodies included the following: eBioscience: PD-1-FITC (RMP1-30) or PD-1-biotin (J43), FoxP3-PE (3G3), CD4-EF450 or CD4-PerCP (GK1.5), ICOS-biotin (7E.17G9), Ki-67-PE-Cy7 (SolA15); BD Bioscience: ICOS-PE (7E.17G9), CXCR5-APC or CXCR5-PECy7 (2G8), CD4-PECy7 (RM4-5). Human antibodies included the following: BioLegend: CXCR5-PerCP/Cy5.5 (TG2); eBioscience: PD-1-PE (eBioJ105), PD-1-APC (MIH4), CD4-biotin (OKT4), ICOS-EF450 (ISA-3), FoxP3-PE-Cy7 (PCH101). Streptavidin was used on the following fluorophores: FITC, PE, APC, PERCPCy5.5, EF450, and APC-Cy7. Detection of GFP was determined directly. Cell viability was determined using live/dead AQUA (Invitrogen) according to manufacturer's instructions. Following staining, cells were sorted, fixed in 1% formaldehyde (Fisher Scientific), or suspended in Fix/Perm Wash Buffer (eBioscience). Samples were acquired on a CyAn ADP LX (Beckman Coulter) or sorted with an Influx Cell Sorter (Becton Dickinson) and analyzed using FlowJo software version 9.3.3 (Treestar Inc.).

Histology and immunofluorescence. Frozen 5µm tissue sections were stained with the following reagents: PNA-biotin (Sigma-Aldrich); IgD-PE (11-26c.2a), Streptavidin-EF450 (eBioscience). Sections were analyzed on an Axio Imager 2 with Apotome (Zeiss). Magnification is provided in the figure legend.

Suppression assays. Splenic $PD1^+CXCR5^+FoxP3^-$ T_{FH} cells, $PD1^+CXCR5^+FoxP3^+$ T_{FR} cells, $PD1^-CXCR5^-FoxP3^-$ T_{con} cells and $PD1^-CXCR5^-FoxP3^+$ T_{reg} cells from NP-KLH (Biosearch Technologies)-immunized mice were sorted 7-8 days post immunization to greater than 95% purity. CellTrace Violet (Invitrogen)-labeled effector cells (2 x10^4) (T_{FH} and T_{con}) were cultured with 5 x10^4 irradiated APCs (1500

rad) alone or with titrating numbers of regulatory cells (T_{FR} or T_{reg}) cells in the presence of anti-CD3 (500ng/mL; 145-2C11, CedarLane) for 3 days. Effector cells were analyzed by flow cytometry for proliferation. In some cases, regulatory cells were pre-fixed as previously described [228].

Statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Multiple group comparisons were performed by one-way analysis of variance (ANOVA) and two-way ANOVA was used for analysis of more than two groups when repeated measures were being compared. Two-tailed Student's t-test was used for the analysis of two independent groups. Pearson's correlation coefficient was used to determine the strength of association between two variables. All data were normally distributed with similar variance between the groups. For all tests, a p value less than 0.05 was considered statistically significant and stated in the figure legend (*$p< 0.05$, **$p< 0.01$, ***$p < 0.001$). Prism software v5.0 (GraphPad Software) was used for statistical analyses.

Study approval. All animal procedures were conducted in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Virginia (protocol no. 3506). Female healthy donor and SLE patient blood for this study were obtained with written informed consent from the protocol, Plasma Cells in Systemic Lupus Erythematosus Pathogenesis, which was approved by the University of Virginia Institutional Review Board (protocol no. 16244).

Results

Reduced frequency of T_{FR} cells in autoimmune prone mice and SLE patients. T_{FH} cells are an important component of the normal immune response and critical for the optimal generation of Ag-specific memory B cells and plasma cells through the GC reaction. The outgrowth of T_{FH} cells in the absence of BCMA begs the question; is this strictly an intrinsic consequence of overactive T_{FH} cells, or is there is there a breakdown in the mechanisms that normally control the T_{FH} compartment? To determine whether T_{FR} cells were affected by BCMA deficiency, we assayed the frequency of T_{FR} cells by flow cytometry in WT and autoimmune-prone mice sufficient or deficient for BCMA. Results demonstrated that the frequency of T_{FR} cells was markedly reduced in the absence of BCMA and this was further exacerbated on the autoimmune-prone background (**Figure 5.1A**).

The presence of circulating murine T_{FR} cells was recently demonstrated by the Sharpe group [68]. Our work identified circulating T_{FR} cells in both the murine system as well as the human population as measured by co-expression of PD1, ICOS, and FoxP3 (**Figure 5.1B**). Importantly, we observed a marked reduction in the frequency of T_{FR} cells in SLE patients compared to healthy controls (**Figure 5.1C**). In both the murine system as well as the human patients, the reduced frequency of T_{FR} cells resulted in a substantial skewing of the T_{FH}:T_{FR} ratio (**Figure 5.1C**). The lower the T_{FH}:T_{FR} ratio, the more regulatory cells there are for each effector cell. Considering both the autoimmune-prone mice and the SLE patients had an increase in this ratio compared to controls, this suggests reduced suppression, as each T_{FR} cells would be required to suppress more T_{FH} cells to achieve the same suppressive response as controls.

Figure 5.1

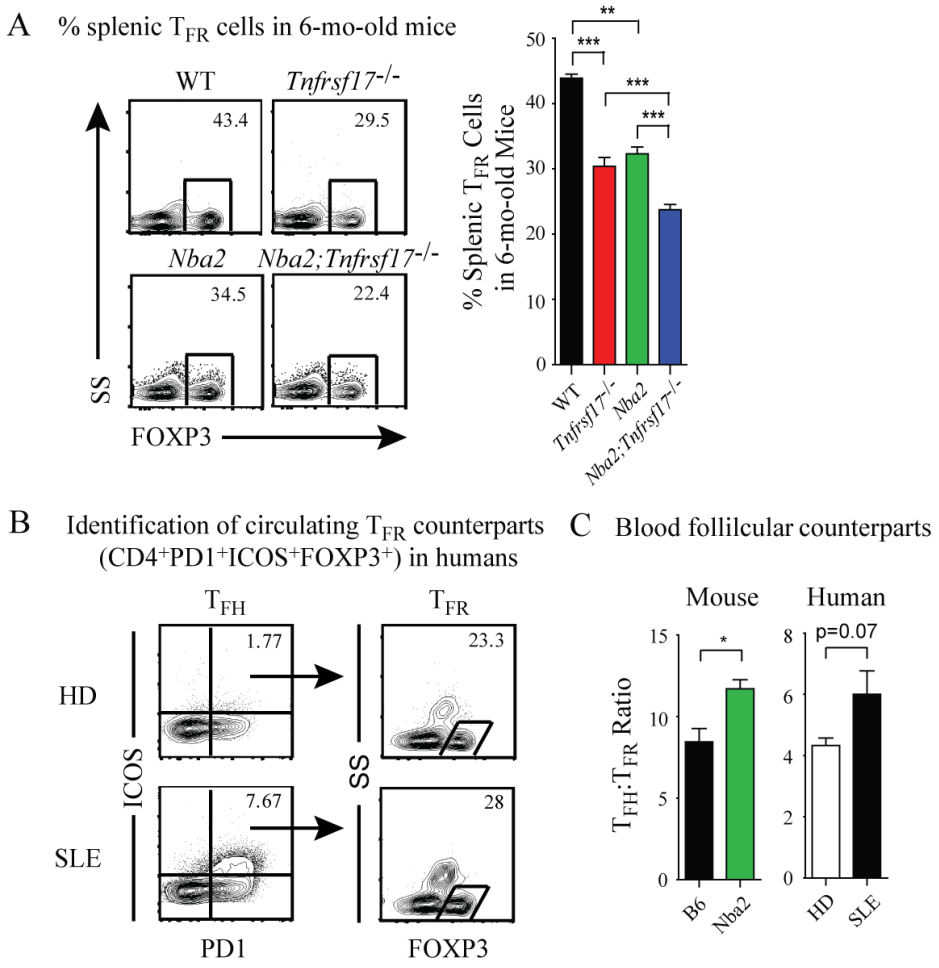
A
% splenic T_FR cells in 6-mo-old mice
WT
Tnfrsf17-/-
43.4
29.5
Nba2
Nba2;Tnfrsf17-/-
34.5
22.4
SS
FOXP3
% Splenic T_FR Cells in 6-mo-old Mice
WT
Tnfrsf17-/-
Nba2
Nba2;Tnfrsf17-/-
B
Identification of circulating T_FR counterparts (CD4+PD1+ICOS+FOXP3+) in humans
T_FH
T_FR
HD
SLE
1.77
23.3
7.67
28
ICOS
SS
PD1
FOXP3
C
Blood follilcular counterparts
Mouse
Human
T_FH:T_FR Ratio
p=0.07
B6
Nba2
HD
SLE

Figure 5.1. T follicular regulatory cells are reduced in frequency in autoimmune-prone mice and SLE patients. (**A**) The percentage of T_{FR} cells was determined in 6 month old female mice of the indicated genotype. Cells were gated on: singlets, live, $CD4^+$, $PD1^+CXCR5^+$. Left panel: One representative contour plot. Right panel: Combined data from 5-9 mice/genotype. (**B**) Identification of circulating T_{FR} counterparts in healthy donors (HD) and SLE patients. 1 representative contour plot from 10 HD and 13 SLE patients analyzed. Cells were gated on: singlet, live, $CD4^+$. (**C**) Ratio of circulating T_{FH} cells and T_{FR} cells in healthy and autoimmune prone mice and humans. Combined data from 5 mice/genotype or 10 HD and 13 SLE patients. Data are presented as mean ± SEM. Statistics determined with the 1-way ANOVA (**A**) or Student's t-test (**C**) and denoted as follows: *P<0.05, ** P<0.01, ***P<0.001.

T_{FR} cells maintain the capacity to proliferate in the absence of BCMA. We next sought to determine whether the decrease in frequency of T_{FR} cells was due to an inherent proliferative defect in the autoimmune-prone mice or in the absence of BCMA. We measured Ki-67 expression in $CD4^+$ T cell subsets in WT mice and BCMA-deficient animals on both the non-autoimmune and autoimmune-prone background. Both T_{FH} and T_{FR} cells strongly proliferated as measured by increased levels of Ki-67 compared to Treg and Tcon cells, irrespective of genotype (**Figure 5.2, left panel**). In contrast to what we predicted based on the reduced frequencies of T_{FR} cells in autoimmune-prone mice and BCMA-deficient animals, the average MFI of Ki-67 in the T_{FR} compartment was higher in autoimmune-prone animals compared to WT controls (**Figure 5.2, right panel**). These findings suggest that in an autoimmune-prone setting and/or the absence of BCMA, failure to properly proliferate is not mediating the defect in frequency of T_{FR} cells.

Figure 5.2

Proliferation of effector and regulatory CD4+ T cell subsets

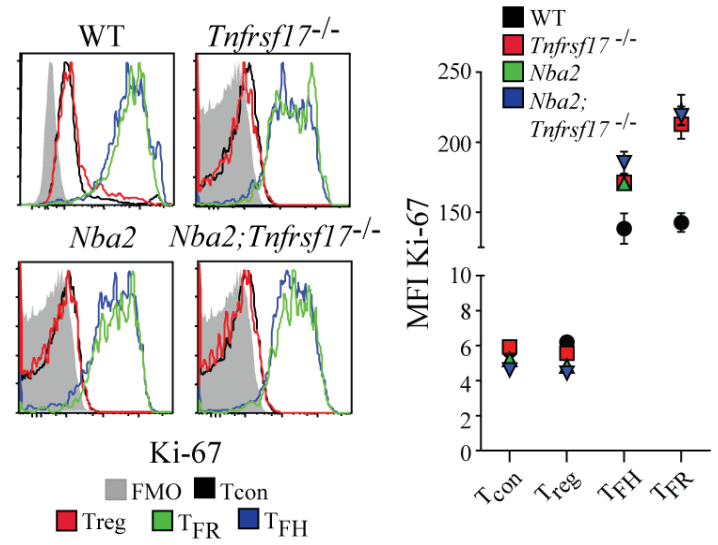

Figure 5.2. T_{FR} cells are highly proliferative. Proliferation was measured in the indicated $CD4^+$ T cell subsets at steady-state in 6 month old female mice by intracellular Ki-67 staining. Left panel: 1 representative histogram from the indicated genotype of 3-5 animals analyzed/genotype. Right panel: Combined data from 3-5 mice/genotype. Data are presented as mean ± SEM.

Generation of FoxP3-gfp expressing autoimmune-prone mice. The modest yet significant decrease in frequency of T_{FR} cells we observe may only partially explain the significant increase in T_{FH} cells. In addition, the maximum number of T_{FH} cells that can be suppressed by any given T_{FR} cell has not been studied. Therefore, we wanted to address whether there were any functional differences between WT and autoimmune-prone T_{FR} cells in the presence or absence of BCMA. To achieve this, we acquired mice that express green fluorescent protein (gfp) driven from the FoxP3 promoter ($FoxP3^{gfp/gfp}$) to be able to sort and interrogate T_{FR} cell function *in vitro* and *in vivo*.

The generation of the $FoxP3^{gfp/gfp}$ mice was described by Fontenot et. al. [227]. Gfp was inserted in frame into the first coding exon. The authors reported no differences between mice expressing the FoxP3-gfp allele and those expressing the normal WT FoxP3 allele. The functionality of the conventional Tregs from both mice was identical *in vitro*. However, the authors never assessed the functionality of T_{FR} cells in this model. $FoxP3^{gfp/gfp}$ mice were crossed with *Tnfrsf17*$^{-/-}$, *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ animals. Identification of the loss of BCMA and the inheritance of the entire *Nba2* locus was performed as described in Chapter 2. Identification of the expression of FoxP3-gfp was performed by flow cytometry (**Figure 5.3A**). To validate the identification of $FoxP3^{+}$ cells *in situ*, WT mice were immunized with NP-KLH to induce a T cell dependent response. Spleens were isolated 2 or 8 days later and stained to visualize the presence of $FoxP3^{+}$ cells (green) in proximity to the B cell follicle (IgD^{+}) and the GC (PNA^{+}). 2 days post immunization, $FoxP3^{+}$ cells were largely excluded from the follicle. However by 8 days post immunization, $FoxP3^{+}$ cells could be found throughout the B cell follicle, with a few $FoxP3^{+}$ cells entering the GC (**Figure 5.3B**).

Figure 5.3

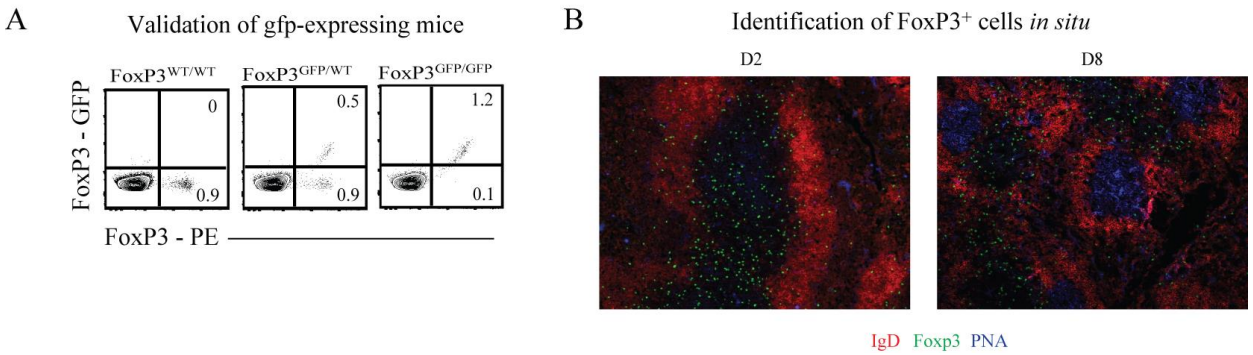

Figure 5.3. Validation of the FoxP3-GFP Model. (**A**) Identification of homozygous mice was assessed by co-staining of FoxP3-GFP and FoxP3-PE in peripheral blood. 1 representative contour plot from at least 5 mice/genotype. (**B**) WT FoxP3$^{gfp/gfp}$ mice were immunized with NP-KLH. 2 or 8 days later, spleens were isolated and stained to visualize the frequency and localization of FoxP3^{+} cells. One representative IF image from 3-4 mice/timepoint. Magnification at 10X.

T_{FR} cells have an enhanced functional capacity in vitro. Although T_{FR} cells have been shown to suppress total (non-Treg) $CD4^+$ T cell proliferation, whether they differentially suppress T_{FR} cells compared to Tcon cells is unknown. Therefore Treg ($CD4^+PD1^-CXCR5^-FoxP3^+$), T_{FR} ($CD4^+PD1^+CXCR5^+FoxP3^+$), T_{FH} ($CD4^+PD1^+CXCR5^+FoxP3^-$) and Tcon ($CD4^+PD1^-CXCR5^-FoxP3\text{-}^+$) cells were sorted based on the gating strategy described in **Figure 5.4A**. Using WT mice, we co-cultured combinations of regulatory cells (Treg and T_{FR}) and CellTrace Violet-stained effector cells (T_{FH} and Tcon) for 72 hours. Our results demonstrated that while conventional Tregs are effective at suppressing Tcons, they have minimal effect on the T_{FH} compartment (**Figure 5.4B**). In contrast, T_{FR} cells are substantially more suppressive than Tregs, irrespective of the effector population. Taken together, these data suggest that T_{FR} cells have an enhanced suppressor capacity compared to Tregs and that T_{FH} cells are largely refractory to normal Treg suppression.

The mechanism of T_{FR} suppression is currently unknown. Therefore, we next tested whether surface molecules or soluble mediators were necessary for T_{FR} function. To prevent secretion of soluble factors, regulatory cells were fixed as previously described [228]. Tcon cells were cultured with fixed Treg cells and T_{FH} cells with fixed T_{FR} cells. Unfixed cells served as positive controls. While both unfixed Treg and T_{FR} cells were able to nearly completely suppress the effector cell proliferation as expected, fixation completely abrogated this response (**Figure 5.4C**). While these experiments do not rule out the possibility of cell-cell contact as being an additional mediator of suppression, it clearly demonstrates that production of soluble factors is required.

Figure 5.4

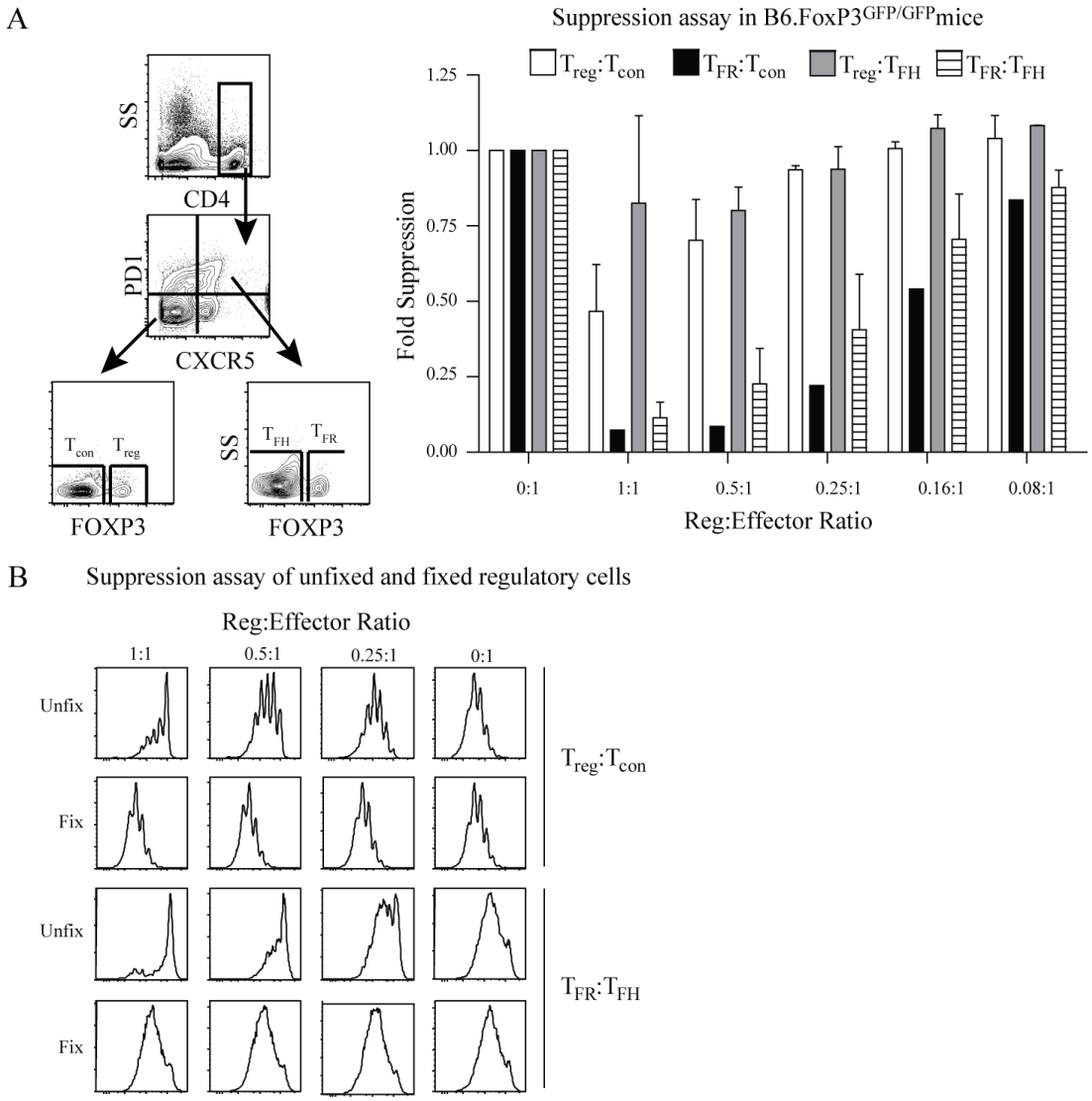
A
SS
CD4
PD1
CXCR5
T con
T reg
FOXP3
SS
T FH
T FR
FOXP3
Suppression assay in B6.FoxP3GFP/GFPmice
Treg:Tcon
TFR:Tcon
Treg:TFH
TFR:TFH
Fold Suppression
1.25
1.00
0.75
0.50
0.25
0.00
0:1
1:1
0.5:1
0.25:1
0.16:1
0.08:1
Reg:Effector Ratio
B
Suppression assay of unfixed and fixed regulatory cells
Reg:Effector Ratio
1:1
0.5:1
0.25:1
0:1
Unfix
Fix
Unfix
Fix
Treg:Tcon
TFR:TFH

Figure 5.4. T Follicular Regulatory cells are highly functional and suppress via soluble factors *in vitro*. (**A**) Left panel; Sorting strategy for the isolation of regulatory and effector subsets using FOXP3$^{gfp/gfp}$ animals. Right panel; WT mice were immunized with NP-KLH. 7 days later, CD4^{+} T cells were isolated from the spleen. Sort-purified effector populations were cultured in the presence of irradiated APCs, α-CD3, and titrating doses of sort-purified regulatory populations for 3 days. Proliferation was measured by CellTrace™ Violet dilution. Fold suppression was determined by setting the proliferation of effector cells cultured in the absence of regulatory cells to 1.0. Combined data from 3-5 experiments/combination of cells. Data are presented as mean ± SEM. (**B**) Cells were isolated as described for (**A**). Immediately following isolation half of the Treg cells and the T_{FR} cells were fixed as described previously [229]. Effector and regulatory cells were then cultured and analyzed as described in (**B**). Individual histograms are shown. 1 of 3 experiments with similar results.

Discussion/Future Directions

The role of T_{FR} cells in immune homeostasis and immune dysregulation are only beginning to be understood. Our preliminary data has identified a defect in the frequency of T_{FR} cells in both autoimmune-prone mice and SLE patients, particularly in the absence of BCMA. Although our work is consistent with a previous report identifying circulating murine T_{FR} cells, we are the first to describe a population of circulating T_{FR}-like cells in humans and to identify a defect within this population in autoimmunity. Future experiments will be necessary to determine whether these human T_{FR}-like cells are functionally equivalent to the described circulating murine population and to the population found in secondary lymphoid organs. In order to achieve this, additional characterization of the human T_{FR}-like cells will be necessary as isolating T_{FR} cells based on their FoxP3 expression, and then interrogating their function, is not possible for humans due to the process of intracellular staining.

Based on our preliminary data, it is currently unclear what mediates the loss of T_{FR} cells in the absence of BCMA. Unlike T_{FH} cells, T_{FR} cells do not express BCMA (**Figure 2.9D and data not shown**). Thus any effect on the T_{FR} population is indirect. This is consistent with our finding that T_{FR} cells from BCMA-deficient animals have no inherent proliferative defect. This data would suggest that although T_{FR} cells are efficiently proliferating in the absence of BCMA and on the autoimmune-prone background, they are not being maintained. An alternate interpretation of these data is that sufficient numbers of T_{FR} cells are not being generated, despite their ability to proliferate once they differentiate. The factors that lead to the differentiation of T_{FR} cells from a subpopulation of Tregs are still being elucidated. Several reports have shown that

the absence of B cells results in a loss of T_{FR} cells [59, 66, 67]. To determine whether specific B cell populations are important for T_{FR} generation, we sort-purified Tregs and cultured them with sort-purified naïve B cells ($B220^{+}IgD^{+}CD95^{-}GL7^{-}CD138^{-}$), GC-B cells ($B220^{+}IgD^{+/-}CD95^{+}GL7^{+}CD138^{-}$), or PCs ($B220^{+/-}IgD^{-}CD95^{-}GL7^{-}CD138^{+}$). Co-culture of Tregs with any of the B cell populations resulted in increased ICOS, Bcl-6, and PD-1 expression, all consistent with a T_{FR} phenotype (**data not shown**). Additional experiments will be required to confirm these findings, determine the mechanism and establish whether defects in the B cell or T_{FH} compartment of BCMA-deficient mice are responsible for the reduced frequency of T_{FR} cells. This work could have profound impacts on SLE treatment, as targeting factors that would support T_{FR} differentiation or function may be a novel mechanism to reduce the T_{FH} frequency and thus, auto-reactive PC response.

Although our data clearly demonstrate a potent suppressive capacity of T_{FR} cells *in vitro*, the mechanism that mediates their enhanced function compared to conventional Tregs is unknown. Ongoing experiments have been designed to elucidate the specific factors being produced by T_{FR} cells that may mediate suppression and differentiate between the two regulatory populations. Specifically, Luminex® assays of supernatant from unstimulated and stimulated T_{FR} cells (and control Tregs) should identify differences in specific cytokines being produced, or the amount of specific cytokines produced. These cytokines could then be targeted using blocking antibodies in *in vitro* co-culture assays to confirm their importance in mediating suppression. In addition, whether any of these properties are altered in autoimmune-prone mice or in the absence of BCMA still need to be addressed as well as T_{FR} functional capacity *in vivo*.

CHAPTER 6

Understanding the Signaling Pathways and Function of BCMA in Health & Disease

BCMA is a Negative Regulator of T_{FH} Cell Responses in Autoimmunity.

An aberrant accumulation of T_{FH} cells is a characteristic feature of a number of autoimmune diseases, including SLE [230] and is recapitulated in pre-clinical lupus-prone mouse models. Although ablation or constitutive activation of a number of lupus susceptibility genes can result in an increased frequency of T_{FH} cells in murine systems, the factors that contribute to abnormal T_{FH} homeostasis in SLE patients is unknown. Using a multi-gene autoimmune susceptibility model and an inflammation model, which closely mimics the predisposition and development of human SLE, we have identified BCMA as an important receptor for restraining T_{FH} cell expansion and helper function in an intrinsic fashion in systemic autoimmunity (**Figure 6.1**).

In the absence of BCMA in T cells, aberrant accumulation and cytokine production by T_{FH} cells, leads to increased expansion of GC B cells and differentiation of autoreactive PCs, resulting in the production of pathogenic autoantibodies and the development of autoimmune disease. We have identified for the first time that autoimmune-prone T_{FH} cells, within the secondary lymphoid organs, express the receptors BR3 and BCMA. In the absence of BCMA, uncontrolled signaling by excess BAFF levels predominantly leads to increased IFNγ production by T_{FH} cells.

Figure 6.1

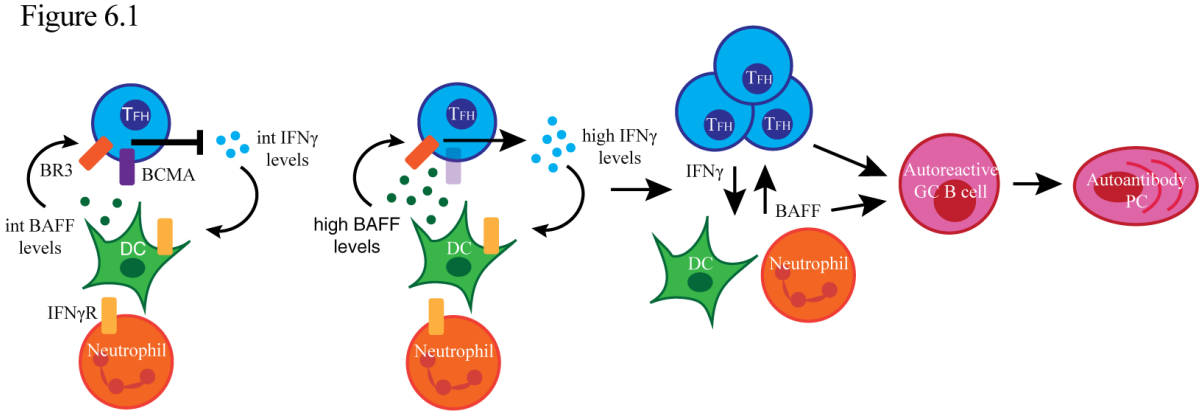

Figure 6.1. BCMA is a negative regulator of T_{FH} cell responses in autoimmunity. T_{FH} cells from autoimmune-prone mice express BR3 and BCMA. When BCMA is present, BAFF signals, coming from DCs and neutrophils, fail to induce appreciable levels of IFNγ. Concurrently, low levels of IFNγ generated by T_{FH} cells, only modestly induces BAFF production by DCs and neutrophils. Loss of BCMA results in strong signals through BR3, and elevated levels of IFNγ, which can then act back on DCs and neutrophils to further enhance their ability to produce BAFF, establishing a pathogenic feedback loop. Interaction between these cell types in the secondary lymphoid organs drives T_{FH} expansion and enhances T_{FH} function, leading to an outgrowth of auto-reactive GC B cells, which ultimately generates auto-reactive PCs.

BCMA & BR3 Signaling in T_{FH} cells.

The signaling pathways transduced by BR3 bound to BAFF that mediate IFNγ production are still being elucidated. Although BR3 signaling is well studied in the context of B cells, whether the same signaling molecules or adaptor proteins are found in T_{FH} cells is unknown. Several molecules that might be prime candidates following BAFF-engagement of BR3 in T cells, are NF-κβ and JNK which in B cells are critical for survival [104, 231]. In T_{FH} cells, activation of either the NF-κβ or JNK pathway may promote T_{FH} cell survival as well as IFNγ production via their interaction with Stat4 or ATF2, respectively, both known inducers of IFNγ [232, 233].

There are several known intracellular signaling molecules and activation stimuli that induce IFNγ production in T cells. TCR stimulation leads to a Mekk4-dependent STAT4-independent activation of IFNγ [234]. In contrast, IL-12 signaling leads to STAT4-dependent IFNγ production [235]. IL-18 is a potent pro-inflammatory cytokine that synergizes with IL-12 to promote IFNγ production using a more intricate pathway that involves stepwise phosphorylation or recruitment of a host of molecules that ultimately results in an ATF-2-dependent activation of IFNγ (**Figure 6.2**) [236-238]. One of the molecules involved in this latter pathway is TRAF6. TRAF6 is a key protein recruited by signals transduced through BR3 in B cells, and is important for BAFF mediated B cell activation and proliferation in mature peripheral B cells as well as malignant B cells [239]. It is interesting to speculate that BAFF signals may usurp this IL-18 mediated pathway and recruit TRAF6 to drive IFNγ production when BAFF levels are particularly high. Alternatively, the TCR mediated pathway of IFNγ activation utilizes JNK, another downstream BR3 target, which may be activated when BAFF levels

are elevated [231]. In combination with TCR stimulation (and consistent with what we observe *in vitro*), both the TRAF6-Stat4 pathway and the Mekk4-ATF2 pathway could act synergistically to promote IFNγ expression. Therefore, at least two shared signaling molecules provide rationale for how BAFF-mediated IFNγ production may occur within the context of T_{FH} cells.

To test whether these pathways are contributing to BAFF mediated IFNγ production in T cells, additional *in vitro* studies would be necessary. Activation of JNK or NF-κβ could be measured by Western blot in purified T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice following culture in the presence or absence of BAFF. To further identify whether JNK or NF-κβ are contributing specifically to IFNγ production following BAFF stimulation, the levels of IFNγ in culture supernatant could be measured following the addition of a JNK or NF-κβ inhibitor to BAFF-stimulated T_{FH} cells from *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice [240, 241]. Even though this work will help address the question of the role of BAFF signaling through BR3 in autoimmune-prone T_{FH} cells, the question of the exact role of BCMA at the molecular level still remains.

The simplest explanation may be that BCMA serves as a BAFF sink, sequestering molecules of BAFF away from BR3, thus preventing unwanted IFNγ production. The same principle applies following engagement of the BAFF ligand as BCMA is reported to share downstream signaling molecules with BR3, such as TRAF6. However, the identification of TRAF6 being an adaptor protein for BCMA signaling was performed in 293 cells transfected with a BCMA plasmid. Whether this recruitment occurs in primary cells, particular T_{FH} cells, is unknown [242].

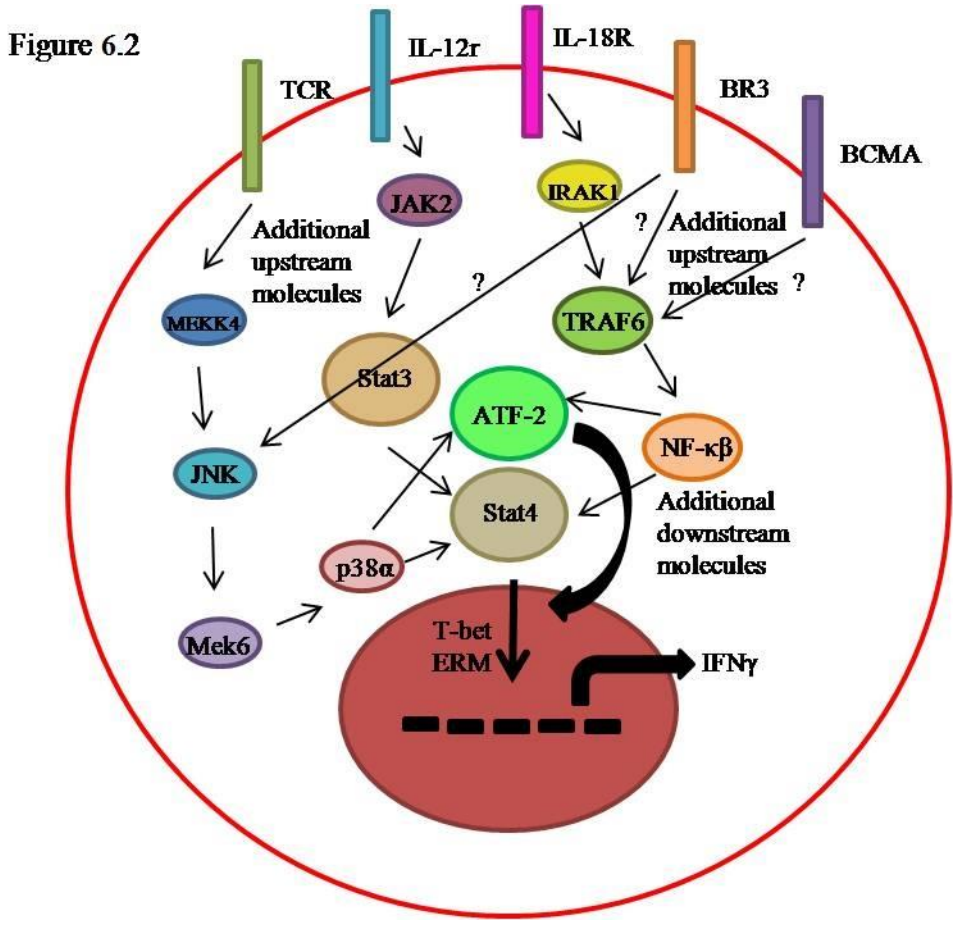

Figure 6.2. Induction of IFNγ gene expression. Multiple signaling pathways converge to induce IFNγ gene expression in $CD4^+$ T cells. Proposed overlap between BAFF signaling and known signaling molecules for the induction of IFNγ in T_{FH} cells. BAFF signaling through BR3 may activate JNK or TRAF6 and lead to Stat4 phosphorylation and IFNγ transcription. BAFF signaling through BCMA may sequester TRAF6 and limit the ability of signals through BR3 to recruit necessary adaptor and signal proteins. However, the JNK pathway would still be intact, allowing for some induction of IFNγ even in the presence of BCMA, consistent with our findings.

A lingering question that remains would be how recruitment of TRAF6 away from the BR3 signaling pathway would not result in BCMA-mediated IFNγ production, if TRAF6 is responsible for how BAFF induces IFNγ. Other co-factors may be recruited following BCMA engagement that bind to TRAF6 and prevent it from activating NF-κβ. Crossing *Nba2* mice onto a $BR3^{-/-}$ background may be useful for addressing this question as BAFF signaling in T_{FH} cells would be restricted to BCMA.

Identifying the Source of Circulating T_{FH} Cells in Murine Samples and Human Patients.

Although we have identified elevated BR3 expression on circulating T_{FH}-like cells in a subset of SLE patients compared to healthy controls, we were unable to identify BCMA expression in any subset of circulating $CD4^+$ T cell. This is in contrast to our finding that spleen-resident T_{FH} cells express both BR3 and BCMA in autoimmune-prone mice. Even though the simplest explanation would be to attribute this discrepancy to differences between mice and man, there is an alternative explanation. Because we do not find BCMA expression in any other $CD4^+$ T cell subset we tested in mice, this suggests that specific cues within secondary lymphoid organs (and specifically areas close to the GC) drive BCMA expression in T_{FH} cells. It is possible that once T_{FH} cells leave this environment, BCMA expression is downregulated, which is why BCMA is not observed in circulating T_{FH} cells.

Alternatively, although circulating T_{FH}-like cells can provide excellent B cell help *in vitro*, the source of these cells is unknown. Circulating T_{FH}-like cells may derive from a pre-T_{FH} population that has not yet upregulated BCMA. In order to fully confirm or refute the expression of BCMA on human T_{FH} cells, access to secondary lymphoid tissue

would be ideal. Since this is more challenging, it is possible that staining of frozen tonsil sections may provide a reasonable substitute. The reagents for measuring BCMA expression in humans are excellent and co-staining for BCMA, CD4, IgD and PNA will provide confirmation of BCMA expression and indicate whether expression is restricted to $CD4^+$ cells outside of the GC or confined to the GC (PNA^+). An alternative approach may be to address some of these questions in mice. Provided enough blood could be obtained, isolation of circulating murine T_{FH}-like cells would allow us to confirm whether BCMA is expressed in the periphery. In addition, isolation of T cells at different phases of activation during the T_{FH} differentiation process (**Figure 1.1**) will provide temporal information as to when BCMA is upregulated and may corroborate the lack of BCMA expression in circulating cells. Moreover, whether BAFF induces IFNγ expression in T_{FH} or circulating T_{FH}-like cells in humans is currently unknown. Preliminary experiments with BAFF stimulated total $CD4^+$ T cells from SLE patients and healthy controls did not yield appreciable levels of IFNγ (**data not shown**). However the low frequency of cells and inability to purify only the T_{FH}-like fraction may have obscured any differences between SLE patients and healthy donors. Another approach, that may be more sensitive and require fewer cells, would be to stimulate bulk $CD4^+$ T cells *in vitro* in the presence of PMA/ionomycin and GolgiStop™ and assess IFNγ production by intracellular flow cytometry.

BAFF Controls T_{FH} Cell Homeostasis in Autoimmunity.

An increase in serum BAFF levels and BAFF-producing cells is an attribute that is observed in the majority of SLE patients. The identity of the source of BAFF in SLE

are not well characterized. In addition, interpretation of the predicted cellular sources in lupus should be carefully considered since circulating cells may not be reflective of what is occurring in the secondary lymphoid organs or at sites of inflammation. Mouse models suggest that myeloid cells within the secondary lymphoid organs are likely a major source of BAFF in autoimmunity. Our studies not only confirm these findings, but further identify $CD11c^+$ dendritic cells as well as neutrophils as prominent sources of BAFF in autoimmunity. Both of these cell populations home to T cell areas of the secondary lymphoid organs and by IHC and IF appear to interact directly with T cells. This interaction may be important for the initial stages of T_{FH} development, but once T_{FH} cells have entered the GC, it is less clear what BAFF source may be driving and maintaining aberrant IFNγ within the T_{FH} compartment, as neither $CD11c^+$ DCs nor neutrophils are found within the GC structure.

Establishing the Role of BAFF in Mediating T_{FH} Responses Within the GC

fDCs appear to be a significant source of BAFF in human tonsil sections[143]. In mice fDCs are also a prominent source of BAFF [243]. However, it is unknown whether fDCs express IFNγR and therefore whether they would be able to participate in a similar feedback loop described above for conventional DCs and neutrophils. Specific ablation of BAFF in the fDC compartment by using the CD21-cre system, and examination of whether there is a reduction in IFNγ-producing T_{FH} cells in our model, would confirm whether following initial interaction between developing T_{FH} cells and either DCs or neutrophils, fDCs are poised to maintain IFNγ production by T_{FH} cells within the GC [244]. Failure to observe any differences in T_{FH} frequency for cytokine production would

suggest that early interactions between pre-T_{FH} cells, DCs and neutrophils imprint a specific phenotype and secretory program in the developing T_{FH} cells, which is maintained in the absence of additional BAFF signals.

We have established a correlation between BAFF levels and T_{FH} homeostasis *in vivo* and have identified a direct effect of BAFF mediating IFNγ production in T_{FH} cells *in vitro*. Yet, how this interaction occurs *in vivo* has proved more challenging. We know that decreasing BAFF levels in autoimmune-prone mice, particularly in the absence of BCMA, reduces the T_{FH} population. However, this method of neutralization of BAFF *in vivo* could not distinguish between direct versus indirect effects mediated through the B cell compartment. To establish the role of BAFF within T_{FH} cells, we would ideally need a system where elevated BAFF levels were maintained, but T_{FH} cells lost the ability to respond to the ligand. Our findings support a model where BAFF is acting through BR3 to promote IFNγ production in T_{FH} cells, which leads to disease exacerbation in the absence of BCMA. Crossing our *Nba2* and *Nba2;Tnfrsf17*$^{-/-}$ mice with the commercially available BR3-floxed animal (B6 (Cg)-Tnfrsf13c^{tm1Mass}/J) and a distal-lck-cre animal, would deplete BR3 specifically in the T cell compartment [245]. A reduced frequency of T_{FH} cells, decreased IFNγ production and an overall amelioration of the autoimmune phenotype would support our *in vitro* findings for the role of BAFF specifically within the T_{FH} compartment. Adoptive transfers and generation of mixed bone marrow chimeras established the role of BCMA within the T_{FH} compartment, but the inability to ensure exact reconstitution, still makes this system less than ideal. To get around these constraints, we have generated BCMA-floxed mice, which allows us to deplete BCMA in specific cell lineages. By crossing these animals with a distal-lck-cre animal, we could

conclusively establish the role of BCMA in the exacerbation of autoimmune disease within T_{FH} cells.

SLE Therapeutic Avenues: Targeting B Cells

Despite our knowledge of the existence of SLE for more than 150 years, the heterogeneous nature of the disease, the challenges in being able to diagnose the disease and the lack of understanding in the etiology and pathogenesis of SLE, has significantly restricted our ability to generate effective therapeutics [246]. Early treatments consisted of regimens that focused on the symptoms of disease and thus consisted of largely steroids, anti-malarials and immunosuppressives [247]. Even though this combination drug treatment substantially reduces morbidity and mortality, it fails to address the upstream mediators of disease and there are significant side effects. As such, targeted immunotherapy has become the mainstay for development of novel therapy.

Numerous reports have identified that auto-reactive PCs play a prominent role in the pathogenesis of SLE [248]. Therefore, biologics aimed at depleting B cells appeared to be a rational approach. Originally developed for the treatment of certain B-cell related cancers, rituximab, a humanized anti-CD20 depleting antibody, has been used in clinical trials to treat SLE symptoms. However, most studies have shown limited improvement with treatment, which may be due to a number of factors including; frequency of administration, concurrent medication, and severity of disease [247]. Furthermore, while CD20 is expressed on all mature B cells, it is downregulated on PCs. Thus pre-existing autoreactive PCs are not removed as effectively and may continue to produce autoreactive Abs. More recently, the proteasome inhibitor, bortezomib, has shown

promise for specifically targeting PCs in mouse models and has been used with some success in isolated incidents for SLE patients refractory to all other treatments [249, 250]. A phase II clinical trial has been established to determine whether this therapy may be beneficial in a larger cohort of SLE patients (www.clinicaltrials.gov).

Early evidence quickly demonstrated a close link between BAFF, APRIL, BCMA, and autoimmunity [91]. In lupus-prone mouse models and SLE patients as well as patients with rheumatoid arthritis, circulating amounts of BAFF are abnormally high and correlate with increased numbers of peripheral autoreactive B cells, heightened B cell activation, and autoantibody production [91, 174, 251]. Pre-clinical studies examining the therapeutic benefit of neutralizing BAFF in lupus-prone mice demonstrated reductions in peripheral B cell numbers as well as reductions in the frequency of plasma cells when both BAFF and APRIL were blocked [192, 252]. Results from these studies provided the basis for developing BAFF antagonists as potent therapeutics for autoimmune disorders where B cell depletion would be predicted to ameliorate disease activity by reducing the numbers of newly activated B cells that secrete pathogenic IgG autoantibodies. There are several agents currently in development that target and neutralize BAFF activity. These agents and their potential for providing clinical benefit to SLE patients have been recently discussed [253-255]. In general, the clinical trials using BAFF-targeting agents have shown less promising clinical response rates than anticipated despite effective depletion of peripheral B cells. However, analysis of the human mAb belimumab (Benlysta) that inhibits soluble BAFF demonstrated clinical benefit in a subset of adult patients with active SLE and was approved in 2011 for treatment of active lupus.

Collectively, the analyses of BAFF-specific inhibitors raise a question regarding the potential of BAFF antagonists for treating autoantibody-mediated autoimmune disorders in which autoreactive B cells have already differentiated into long-lived effector B cells such as memory and/or long-lived plasma cells. This is an important issue since long-lived autoreactive plasma cells have been shown to accumulate not only in the bone marrow but also in secondary lymphoid organs and inflamed tissues of lupus-prone mice [256, 257]. Interestingly, in certain lupus-prone mouse strains neither treatment with antagonists to BAFF nor APRIL reduces autoantibody production and immune complex deposition within kidneys [192], suggesting that terminally differentiated autoreactive plasma cells do not have increased dependency on BAFF/APRIL for survival in contrast to peripheral autoreactive B cells.

Importantly, our findings within the context of autoimmune-prone BCMA-deficient animals, demonstrates that blocking BAFF or a trigger that initiates BAFF production (IFNγ) are effective at reducing the autoimmune phenotype. The amelioration of autoimmunity in our model, in the context of BAFF blockade, stands in contrast to other reports that observed no differences following anti-BAFF therapies [192]. These differences may be due to the phenotype of the model used in other reports. For example, models used by other groups where specific BAFF inhibitors were ineffective had substantial kidney phenotypes, whereas our models had only mild immune complex deposition in the kidney with mild proteinuria. This would suggest that while BAFF inhibition ameliorates certain aspects of disease, it is ineffective at eliminating every phenotype. This notion is further supported by human trials [258].

SLE Therapeutic Avenues: Targeting T_{FH} Cells

Given these findings, preventing the development of PCs and eliminating pre-existing autoreactive PCs is likely to confer substantial benefit to patients. The critical role of T_{FH} cells in promoting the development of PCs through the GC reaction makes them a prime candidate for the next generation of SLE treatment. T_{FH} cells can be controlled on many different levels, including transcriptional regulation, post-transcriptional regulation and post-translational regulation. Targeting any of the key factors in T_{FH} differentiation including Bcl-6, c-Maf, STAT family members, IRF4 and BATF, would substantially reduce T_{FH} development based on mouse studies [55] . However, given the critical function of these factors in other cell types, targeting these molecules may have pleiotropic effects. Therefore, identifying T_{FH}-specific molecules will be paramount to being able to target them therapeutically. Some potential targets include micro RNAs such as the recently identified miR-17~92. Micro RNAs are critical for the development of an optimal T_{FH} response as indicated by mice deficient in the micro RNA biogenesis factor DGCR8 (no generation of any micro RNAs) within the CD4 compartment. These mice fail to form T_{FH} cells following immunization [259, 260]. miR-17~92 contributes to the development of the $CXCR5^{high}Bcl\text{-}6^{high}$ population via its interaction with the tumor suppressor gene PTEN. Although T_{FH} cells are not completed abrogated in mice deficient in miR-17~92, they are substantially reduced [260]. This may be a desirable outcome, as elimination of T_{FH} cells entirely would leave patients susceptible to infection.

An additional therapeutic approach would be to target the products of T_{FH} cells that help drive PC development and T_{FH} cell maintenance. Not surprisingly, the

development of IL-21 blocking reagents and their testing in human patients is currently underway ([261] & www.clinicaltrials.gov). Our *in vitro* as well as *in vivo* findings suggest that IFNγ might represent a promising target as well, as treatment with an anti-IFNγ blocking antibody reduces the major features associated with autoimmunity, including aberrant accumulation of DCs, T_{FH} cells and GC-B cells and serum auto-antibody titers. In addition, our observation that IFNγ serum levels are increased in SLE patients and correlate with serum BAFF levels and BR3 expression in circulating $CD4^+$ T cells support a potential therapeutic benefit in humans. Amgen has developed and is currently testing an anti-IFNγ antibody for the treatment of SLE (www.clinicaltrials.gov). The outcomes of these trials will not only serve to validate our findings and translate our observations in humans, but will inform our decisions for future therapeutic avenues.

SLE Patient Biomarkers.

The heterogeneity of SLE disease pathology, make the identification of a "silver bullet cure" unrealistic. However, the abundance of new biologics and treatment options suggests that identifying specific patient sub-populations, who might benefit from individual or combination therapies, is a more realistic goal. Circulating T_{FH} cells appears to characterize a group of SLE patients with severe disease. Although the correlation between reports is inconsistent, most groups have identified at least one parameter (anti-dsDNA levels, SLEDAI, kidney involvement, etc.) that strongly correlates with the frequency of circulating T_{FH} cells [230]. Our observations in both murine models and human patients are consistent with these findings. Moreover, we have identified several correlations between the percentage of $BR3^+CD4^+$ cells and the titers of anti-DNA

antibodies, serum BAFF levels, and serum IFNγ levels. Using a new cohort of patients to determine whether these parameters can predict disease severity and end organ damage, would be valuable to assess their feasibility as biomarkers.

BAFF Signaling Axis in Immunity.

In combination with signals transduced through the BCR, BAFF signaling through BR3 is critical for regulating B cell survival and the generation of an optimal immune response [262]. BAFF depletion during infection leads to a substantial reduction in Ag-specific IgM responses [263]. Conversely, exogenous BAFF (administered as part of an adenovirus) given at the time of vaccination enhances Ag-specific IgM and IgG titers and maintains the titers for longer periods of time [264]. The effects of these responses have been solely attributed to the direct role of BAFF on the B cell compartment. However, neither of these studies examined the T_{FH} compartment to determine what, if any effect, BAFF exerted within $CD4^+$ T cells.

Although previous reports have demonstrated BR3 expression in the total $CD4^+$ compartment, as well as the activated $CD4^+$ compartment, we find the highest expression on GC-T_{FH} cells. The fact that this observation is made in WT mice, suggests that during the immune response T_{FH} cells are able to respond to BAFF. Consistent with the murine findings, circulating T_{FH} cells express low levels of BR3 in healthy donors. We also observed that mice given the inflammatory reagent, pristane, induced BCMA within the T_{FH} compartment. Whether the inflammatory milieu during an infection or vaccination is sufficient to drive BCMA expression is an area that is completely open to investigation.

Given current published work, the setting that lends itself to the most direct correlation is that of viral infection.

Increases in BAFF levels have been noted following respiratory syncytial virus (RSV), influenza virus and dengue virus [265-267]. In the context of RSV, increased BAFF levels correlate with an improved resolution of virus. Work done by McNamara et al show that this increased BAFF during RSV infection is IFN-dependent (however they only look at the contribution of type I IFNs) [266]. The downstream signaling pathways that mediate this response are still unknown. I would predict that the BAFF signaling axis (irrespective of BCMA expression) is critical for mounting a protective response in this context (**Figure 6.3**). Activation of myeloid cells by toll like receptor signals increases their frequency and production of BAFF. The localization of myeloid cells in the secondary lymphoid organs activates and promotes T_{FH} responses, increasing IFNγ production, which may serve in a feedback loop as it does in autoimmunity, but also helps drive class switching to IgG2a, known to be beneficial for clearing the virus [268-270]. The resolution of the inflammatory initiator intrinsically abrogates the interaction between DCs, neutrophils and T_{FH} cells. Therefore, although substantial work needs to be performed to address the role of the BAFF signaling axis as it relates to T_{FH} cells in immunity, initial reports from other groups suggest our findings may extend to immune protection as well. The relatively low levels of BAFF and the short periods of time where BAFF is overexpressed likely ensures it does not become pathogenic in a normal setting. Therefore, by harnessing the process that becomes pathogenic in autoimmune disease, we may be able to devise stronger vaccines by coupling antigen with factors that will directly stimulate BAFF production or BR3 signaling

Figure 6.3

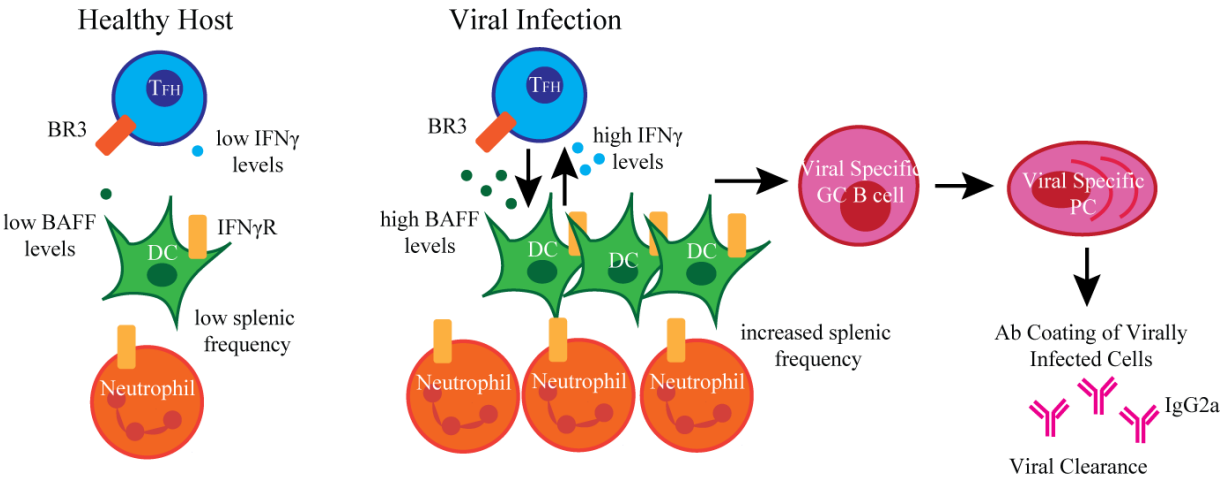

Figure 6.3. Proposed role for BAFF signaling in immunity. T_{FH} cells from healthy hosts express BR3. The low frequency of BAFF-producing cells at steady state fail to induce appreciable levels of IFNγ. Concurrently, low levels of IFNγ generated by T_{FH} cells, only modestly induces BAFF production by local DCs and neutrophils. An influx of activated DCs and neutrophils during a viral infection results in strong signals through BR3, and elevated levels of IFNγ, which can then act back on DCs and neutrophils to further enhance their ability to produce BAFF. A strong T_{FH} response in the context of elevated IFNγ facilitates the generation of viral-specific GC B cells, which undergo CSR to a protective IgG1 isotype, leading to the clearance of virally infected cells.

Concluding Remarks.

The work described in this dissertation has established the BAFF-BCMA axis as a critical intrinsic regulator of T_{FH} homeostasis. We identified both dendritic cells and neutrophils as important sources of BAFF, critical for the development of aberrant T_{FH} cell expansion and function. In the absence of BCMA, BAFF from DCs and neutrophils drive signals through BR3 to induce IFNγ production, which is pathogenic in our model. Furthermore, we provide preliminary evidence that a reduced frequency of T_{FR} cells may contribute to the outgrowth of the T_{FH} compartment in the absence of BCMA. Despite the majority of this work being performed in the context of autoimmunity, the work done in the inflammatory pristane model and the work done by others linking BAFF and IFNγ during infection, suggests our work has implications for multiple disease settings and may provide a framework for the development of novel therapeutics or vaccine strategies.

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