BRUTON'S TYROSINE KINASE AND AUTOREACTIVE B LYMPHOCYTES: ROLES IN DEVELOPMENT, SURVIVAL, AND DISEASE

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To my mother, Susan Katherine Thisell,

Who assured me, despite my doubts, that I would love biology.

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LIST OF ABBREVIATIONS

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BCR	B cell receptor
BLNK	B cell linker
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BTK	Bruton's tyrosine kinase
Btk^{flox}	LoxP-flanked *Btk*
$CreER^{T2}$	Tamoxifen-inducible Cre
DAG	Diacylglycerol
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-related kinase
FcγR	Fc gamma receptor
FcεR	Fc epsilon receptor
FO	Follicular
FolRβ	Folate receptor beta
GC	Germinal center
GPI	Glucose-6-phosphate isomerase
HEL	Hen egg lysozyme
IgH	Heavy chain of the BCR
IgL	Light chain of the BCR
IP_3	Inositol 1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITK	Interleukin-2-inducible T cell kinase

K/BxN	Arthritis model produced when KRN+/+ B6 are crossed with NOD
LN	Lymph node
LPS	Lipopolysaccharide
MAL	MyD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MAV-1	Mouse adenovirus type 1
M-CSF	Macrophage-colony stimulating factor
MD4	Mice expressing transgenic BCR that binds HEL
MFI	Mean fluorescence intensity
ML5	Mice expressing soluble HEL
MS	Multiple sclerosis
MZ	Marginal zone
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Phosphoryl-choline
pDC	Plasmacytoid dendritic cell
PH	Pleckstrin homology
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PIP5K	Phosphatidylinositol 4-phosphate 4-kinase
PIR-B	Paired immunoglobulin-like receptor B

PKCβ	Protein kinase C β
PLCγ2	Phospholipase-C-gamma-2
poLN	Popliteal lymph node
PTEN	Phosphatase and tensin homologue
RA	Rheumatoid arthritis
RAG	Recombination activating gene
SH2	SRC-homology 2
SH3	SRC-homology 3
SHIP-1	SH2 domain-containing inositol-5-phosphatase-1
SHM	Somatic hypermutation
SHP-1	SH2 domain-containing phosphatase-1
SLC	Surrogate light chain
SLE	Systemic lupus erythematosis
STA	Serum transfer arthritis
SYK	Spleen tyrosine kinase
T1	Transitional 1
T1D	Type 1 diabetes
T2	Transitional 2
TCR	T cell receptor
Tfh	T follicular helper
TI-II	T independent-type II
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
XLA	X-linked agammaglobulinemia

CHAPTER I

BACKGROUND AND RESEARCH GOALS

Introduction

B lymphocytes serve an obvious and important function in the human immune system, producing antibodies and helping stave off infectious disease. B lymphocytes also drive the immune reaction by presenting antigen to cognate T cells and producing inflammatory cytokines. However, when a B cell is autoreactive, these functions can be turned against self and result in autoimmune disease. Rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis (MS) are just a few examples of systemic autoimmune diseases in which damaging autoantibody may cause havoc by targeting self-molecules and activating the innate immune system (1). Autoreactive B cells are also uniquely capable of fueling T cell-driven organ specific autoimmunity, by binding autoantigens through specific membrane-bound antibody called the B cell receptor (BCR), processing them, and presenting to autoreactive T cells. In type 1 diabetes (T1D), T lymphocytes are responsible for the actual destruction of insulin-producing beta cells. However, mouse models have shown that B cells are necessary to present antigen to T cells and initiate disease (2-5). These autoimmune diseases result when there is a failure of immune tolerance, either because of leaky central tolerance or a loss of peripheral tolerance mechanisms such as anergy (6).

B cell tolerance, both central and peripheral, is governed by B cell signaling. One protein of particular interest is Bruton’s tyrosine kinase (BTK), as

mouse models have shown that BTK plays a unique role in the development of autoreactive B cells (7-9). Understanding the ways in which BCR signaling mediates tolerance, and how it governs the development and actions of autoreactive B lymphocytes, is crucial to inform the ways in which we treat autoimmune disease. The goal of this work is to further understanding of BTK's role in development, survival, and function of autoreactive B lymphocytes.

Autoreactive B cell development: An overview

Generation of the B cell receptor

In mammals, B cells develop in the fetal liver (10), fetal bone marrow (11), and adult bone marrow (12, 13). B cells are defined by several immunological markers, but most importantly each single B cell expresses a single specific BCR. A simplified model of B cell development in the bone marrow is shown in Figure 1.1. The discrete steps of B cell differentiation correlate with the formation of the BCR, which is generated through somatic rearrangement of the variable (V), diversity (D) and joining (J) gene segments, a process discovered by Susumu Tonegawa and Nobumichi Hozumi (14). The B cell lineage forms from hematopoietic stem cells, which are committed to becoming B cells when common lymphoid progenitors transition into pre-pro-B cells (15). Pre-pro B cells no longer retain the ability of multilineage differentiation, and instead upregulate B cell specific genes and become pro-B cells. During these stages, the heavy chain of the BCR (IgH) is rearranged. Two recombination activating genes

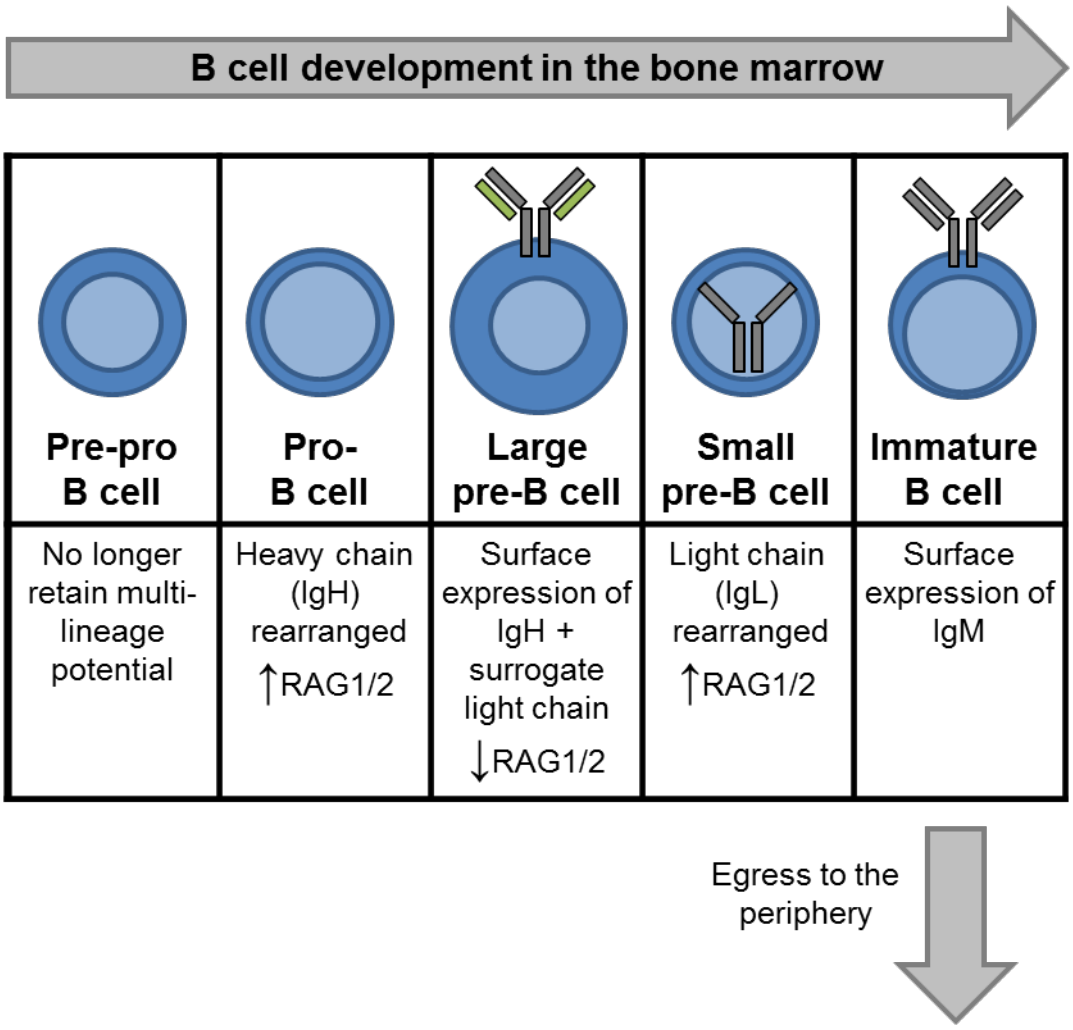


Figure 1.1: The majority of B cells develop in the bone marrow. B lymphocytes are committed to the B cell lineage upon transition to pre-pro B cells. In the pro-B cell stage, RAG1/2 is expressed and the heavy chain of the BCR rearranged. If successful, IgH pairs with the surrogate light chain. This pre-BCR is expressed on the membrane and the B cell transitions into a large pre-B cell, downregulates RAG1/2, and undergoes proliferation. Large pre-B cells transition to the small pre-B cell stage, upregulate RAG1/2 and rearrange the light chain of the BCR. Finally, if the rearrangement of IgL is productive, the B cell expresses IgM and the B cell reaches the immature stage.

(RAGs), RAG1 and RAG2, are largely responsible for Ig recombination, and the loss of either of these proteins results in a block in both B and T cell development (16, 17). If recombination is successful, IgH is paired with the surrogate light chain (SLC), forming the pre-BCR. This serves as a signal to reduce RAG1/2 expression and for chromatin remodeling to reduce accessibility of the IgH alleles (18-20). Pro-B cells then become large pre-B cells, proliferate, and transition into small pre-B cells that re-express RAG1/2. This initiates rearrangement of the BCR light chain (IgL). The IgH and IgL components form IgM and the pre-B cell transitions into an immature B cell (15, 21-23). The formation of a specific receptor is a highly regulated event that allows B cells to function in the adaptive immune system, and V(D)J rearrangement allows the formation of many receptors from a limited set of gene segments. However, the recombination itself is random and therefore, V(D)J rearrangement is a double-edged sword: it generates autoreactive receptors (24).

Development and escape to the periphery

It is estimated that 70-80% of developing B cells in the bone marrow are autoreactive (25). Before these autoreactive B cells reach maturity, most are successfully removed through negative selection. Negative selection occurs when a B cell binds autoantigen during development, and is removed from the B cell repertoire by the processes of receptor editing or clonal deletion. However, some signal through the BCR is necessary, in a process termed “positive selection.” This is antigen-independent, low-level signaling that indicates that the

B cell has successfully rearranged its BCR. Therefore, the level of signaling is integral to B cell development, and a B cell must both successfully rearrange its IgH and IgL chains and survive developmental checkpoints that ensure that it is not autoreactive. There is some evidence that negative selection begins as early as at the pre-BCR stage. Keenan *et al* found that mice deficient in the surrogate light chain (SLC) exhibited more B cells with autoreactive IgH, leading to increased anti-nuclear antibodies in serum. This was attributed to the escape of pre-B cells from negative selection (26). However, there is also evidence that the pre-BCR itself is autoreactive, and this autoreactivity signals the successful rearrangement of IgH that allows a B cell to continue to mature (27-29). While the evidence of negative selection at the pre-BCR stage is conflicting, it is very clear that most negative selection occurs at the immature B cell stage. At this point, immature B cells are exposed to autoantigen in the microenvironment. They still maintain expression of RAG1/2, and if their receptor is autoreactive undergo a process called receptor editing. First, editing occurs at the Igκ locus, forming a new IgL, which pairs with IgH and forms a BCR with a new specificity. If recombination is unsuccessful or the B cell remains autoreactive, the Igκ locus is inverted or deleted and the Igλ locus is recombined (30-34). Receptor editing is an effective mechanism of central tolerance induction. However, even immature B cells that undergo receptor editing may remain autoreactive. If an autoreactive immature B cell fails to effectively rearrange its receptor and remains autoreactive, clonal deletion initiates by the mechanism of programmed cell death (35-37). Signaling through the BCR governs the process of central

tolerance. If the BCR is not successfully recombined, there is no signal and the B cell dies. However, if the cell is strongly autoreactive, it will be negatively selected. These processes of negative selection are highly effective, but autoreactive B cells still escape to the periphery, and there is evidence that this process is leakier in genetic backgrounds that favor autoimmune disease (38-41).

Mature autoreactive B cells and peripheral tolerance

After successful generation of the BCR, immature B cells egress from the bone marrow and travel to the periphery. Splenic B cell subsets, and their markers, are depicted in Figure 1.2. In the spleen, when B cells first arrive they are termed “transitional 1” (T1). They then become transitional 2 (T2). Both T1 and T2 B cells express high levels of IgM, but T2 B cells upregulate IgD, complement receptor 2 (designated CD21) and the Fc epsilon RII (FCεRII, designated CD23), while T1 B cells remain negative for these markers (42-45). These two transitional subsets are the precursors to marginal zone (MZ) and follicular (FO) B cells, the fully mature B cell subsets. MZ B cells are named for their residence in the outer white pulp of the spleen, between the red pulp and the marginal sinus. They do not circulate, swiftly respond to blood borne pathogens, and express high levels of CD21, the complement receptor 2 (46). FO B cells form the bulk of mature naïve B cells. They reside in the spleen and lymph nodes in the B cell follicle, which is adjacent to the T cell zone. This allows activated T cells and FO B cells to interact during the immune reaction. In

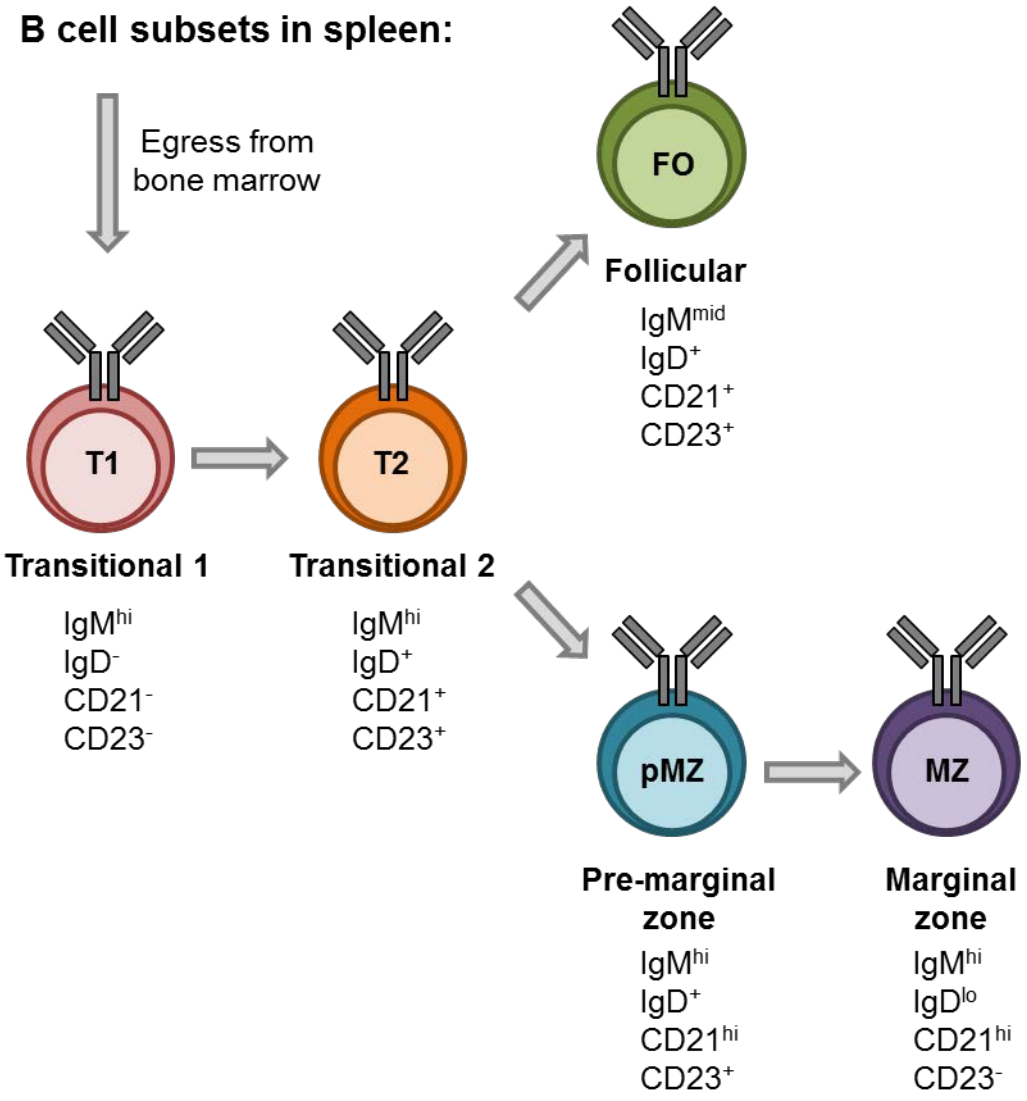


Figure 1.2: B cell maturation in the spleen. After development and undergoing central tolerance mechanisms in the bone marrow, B cells egress to the spleen. There, transitional 1 and 2 B cells are the precursors to the mature B cell subsets. T2 B cells transition to follicular B cells, or to pre-marginal zone B cells that in turn become marginal zone B cells. Follicular B cells reside in the follicles in the spleen and lymph nodes, and may recirculate. Marginal zone B cells reside in the outer white pulp of the spleen, where they can quickly encounter blood-borne antigen. Markers listed are for determination of B cell subsets by flow cytometry.

addition, FO B cells circulate through peripheral blood and the bone marrow (47). The specificity of the B cell, and thus the strength of signal through the BCR, is thought to determine the subset of the cell (48).

When autoreactive B cells escape central tolerance in the bone marrow, to avoid autoimmunity they must be controlled by peripheral tolerance. The primary mechanism of peripheral tolerance is anergy, in which a B cell is exposed to antigen but is functionally unresponsive. In this state, the B lymphocyte fails to flux calcium, proliferate, or produce antibody in response to antigen. This was first studied in mice treated *in utero* or as neonates with fluorescein conjugated to human γ globulin. High doses resulted in a reduction in the number of fluorescein-binding B cells, but lower doses showed no reduction in cell number but the B cells that remained exhibited tolerance to the antigen. The autho rs referred to this induced tolerance as “anergy” (49). Anergy has also been studied extensively in the context of transgenic mice, in which already recombined IgH and/or IgL sequences are provided, resulting in a fixed BCR-specificity (50-53). One such study used mice expressing a transgenic BCR that recognized hen egg lysozyme (HEL). These “MD4” animals were bred to “ML5” animals, which express soluble HEL. In the MD4xML5 offspring, B cells exhibited normal development until they reached the spleen, where they are low in number. The surviving splenic anti-HEL B cells arrest and acquire an anergic phenotype. Anti-HEL B cells from MD4xML5 mice failed to proliferate or produce antibody in response to HEL. B cells from these mice also exhibited poor responses to both T cell help and the innate stimulus of LPS. Finally, they found the anergy

phenotype was B cell-intrinsic, as there was no evidence of suppression from other splenic cells (50, 54). In contrast, if MD4 are bred to animals expressing membrane-bound HEL, B cell depletion is much more severe, resulting from a developmental block between immature and transitional B cells (55). The MD4xML5 model is only one method of studying anergic B cells. In the anti-insulin transgenic model, which allows study of a physiologic autoantigen, B cells are anergic but reach the FO stage and also exhibit large marginal zones (51, 53). This difference in B cell development may be due to the differing affinities of the autoreactive BCR for its antigen. ML5 transgenic B cells have an affinity for HEL of approximately 2×10^{-9} M, whereas anti-insulin B cells bind autologous rodent insulin at 1×10^{-7} M (50, 53, 56). This difference may allow anti-insulin B cells to reach mature subsets, while anti-HEL B cells do not. Additionally, avidity must play a role, as is shown by the fact that membrane-bound antigen induces clonal deletion (central tolerance) in the anti-HEL anergy model while soluble antigen induces anergy (peripheral tolerance). Low affinity or avidity for autoantigen is more likely to induce receptor editing or anergy, while high affinity or avidity favors deletion (57, 58). Throughout B cell development, the level of signaling through the BCR regulates autoreactive B cell survival and function.

Failure of peripheral tolerance leads to autoimmune disease

If an autoreactive B cell escapes central tolerance, it will hopefully be controlled by peripheral tolerance. When peripheral tolerance fails, autoimmunity can become autoimmune disease. In some cases, autoreactive B cells can drive

disease even if they retain an anergic phenotype. This is accomplished through antigen presentation to cognate autoreactive T cells. In the non-obese diabetic (NOD) mouse model of T1D, B cells drive disease through precisely this mechanism (3-5). Anti-insulin transgenic B cells have been used to study antigen presentation in this model. Even though insulin-specific B cells remain anergic, and do not proliferate or produce antibody to insulin, they remain able to efficiently present antigen to and activate cognate T cells (51). Anergy is not a requirement for autoreactive B cells to cause damage through antigen presentation. Antigen presentation by autoreactive B cells is also required to drive certain murine models of experimental autoimmune encephalomyelitis (EAE), a model for human MS, though it is not known if these autoreactive B cells are also anergic (59-61). Additionally, B cells that could not secrete antibody could still present antigen to T cells and drive disease in a transgenic model of lupus (62).

While in some cases autoreactive B cells maintain anergy and yet still present autoantigen to autoreactive T cells, in other cases tolerance is broken and autoreactive B cells begin to proliferate and produce antibody. In some cases, this loss of tolerance may be due to signaling through various TLRs (63, 64) or perhaps through cognate interactions with T cells (65). After loss of tolerance, B cells may form germinal centers and there undergo affinity maturation and isotype switching. In non-autoreactive B cells, these functions are incredibly important to form highly-specific, effective immune responses. Affinity maturation occurs through the process of somatic hypermutation (SHM). In the

dark zone of the germinal center, B cells proliferate and insert mutations into the antibody variable region loci, leading to changes in affinity. Upon transit to the light zone, only the high affinity receptors are positively selected. B cells continue to cycle between the dark and light zones and undergo SHM, resulting in high affinity antibodies that are most effective against pathogens (66). Of course, when tolerance is broken and this process occurs in autoimmune disease, it can result in higher affinity antibodies to self. SHM results in higher affinity B cell clones to dsDNA in the MRL/lpr murine lupus model (67) and higher affinity anti-peripherin B cells in the NOD model of T1D (68). There is also evidence of affinity maturation in human studies of patients with RA (69), MS (70-72), Sjogren's syndrome (73, 74) and T1D (75).

Isotype switching is a second crucial process of B cell response to antigen. Naïve B cells express IgM and IgD concurrently. During the course of an immune response, B cells can switch the constant regions of their heavy chains, resulting in IgG^+, IgA^+, or IgE^+ B cells. Which isotypes are formed depends on the nature and the route of the stimulating antigen (76). Isotype switching in autoimmunity drives disease by enabling activation of innate immune functions through FcγR signaling or the recruitment of complement. In RA, a diverse antibody repertoire is associated with increased disease severity. Undifferentiated arthritis patients with autoantibody responses consisting of IgM, IgG1, IgG3 and IgA anti-cyclic citrullinated peptide (anti-CCP) antibody are more likely to progress to RA than patients who do not (77). Diverse antibodies also predict radiographic damage risk and resistance to treatment with biologics (78-

80). The importance of class-switched antibody to autoimmune disease is also clear in SLE, in which autoantibodies class switch to IgG and undergo SHM in order to mediate disease (81). Though central tolerance does effectively decrease autoreactive B cell specificities, and peripheral tolerance controls certain B cells that escape, when these mechanisms fail autoimmunity results.

Endogenous autoreactive B cell subsets

Autoreactive B cells occur in normal mouse and human B cell repertoires. Examples of murine autoreactive endogenous B cells are listed in Figure 1.3. In mice, a possible population of endogenous anergic B cells was first described by Allman *et al*. Much like T2 B cells, the identified population was positive for the immaturity marker CD93 and for CD23, but exhibited low levels of surface IgM (43). First termed T3 B cells because of this phenotype, the field has shifted towards the designation of “anergic population 1” (An1), due to the fact that the cells exhibited classical hallmarks of anergy, such as functional silencing and low expression of IgM. An1 cells are enriched for autoreactive specificities, fail to mount immune responses, and have a short half-life compared to non-autoreactive B cells (82, 83). A similar subset is evident in humans, and is increased in autoimmune disease (41, 84, 85).

A second autoreactive-prone, endogenous B cell subset further complicates the picture of autoreactive B cells. B cells that have thus far been discussed are, specifically, termed “B2” B cells. Another subset of B cells, B1 cells, were first discovered in 1983 as a small subset of $CD5^+$ B cells in the

Endogenous autoreactive-prone B cell subsets

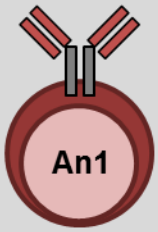
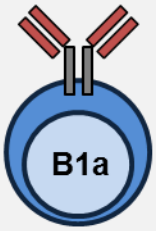
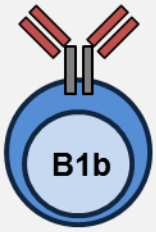
	Anergic population 1	B1 cells	
			
Cell surface phenotype	IgM^{low}, CD23^{+}, CD93^{+}	IgM^{hi}, CD19^{+}, B220^{lo}, CD5^{+} CD11b^{+} in peritoneal cavity, CD43^{+} in spleen and bone marrow	IgM^{hi}, CD19^{+}, B220^{lo}, CD5^{+}, CD11b^{+}
Major site of development	Bone marrow	Fetal liver, self-renewing	Fetal liver, self-renewing

Figure 1.3: Endogenous autoreactive B cell subsets in the mouse. Autoreactive-prone B cell subsets include the anergic population 1 (An1) that have low IgM expression and do not proliferate or produce antibody in response to stimulus. B1a and B1b cells are innate-like, autoreactive-prone B cells. Listed are the cell surface phenotype and sites of development for these autoreactive B cell populations.

spleen, and they were thought to be associated with autoimmunity (86). It is now known that two subsets of B1s exist, those that are $CD5^+$ and those that are $CD5^-$. $CD5^+$ B1 cells are designated B1a, while $CD5^-$ B cells are designated B1b. B1 and B2 cells can develop from either fetal or adult tissues, but B1 cells develop primarily from the fetal tissues and bone marrow has preferential potential for development of B2 cells (87). B1s express a limited BCR repertoire that is highly enriched for polyreactive receptors, meaning they may have a low level affinity for multiple antigens (88-90). They exhibit slow turnover and are self-renewing (91). Most importantly, their polyreactive specificities allow them to recognize both pathogens and autoantigen (92). However, B1 cell may also have more specific binding, and interestingly, autoantibody-producing B1 cells may be positively selected during development. Wild-type mice are known to develop B1 cells that bind to the autoantigen Thy-1. However, in mice lacking the Thy-1 antigen (CD90), neither anti-Thy1 specific B1 cells nor antibody developed (93). Despite, or even because of, their autoreactivity, B1s serve important functions in the immune system. It is estimated that 80% of serum IgM is B1 derived (94). This IgM, called natural antibody, is a polyreactive IgM pool secreted by B1s in the spleen and the bone marrow (95, 96). The autoreactivity of natural IgM actually performs helpful functions, such as binding self-antigen produced by cell death and assisting in its clearance (97, 98). It also may enforce tolerance, and is known to be protective in mouse models of atherosclerosis (99-103). The polyreactive nature of natural IgM also serves as a barrier against pathogen replication before the adaptive immune response (92, 104-108) and enhances B2

cell-dependent IgG responses (104, 109). B1 cells can also rapidly respond to tissue injury and form a vital part of the immune response to polysaccharide antigens (105, 110-113). Finally, B1s may also contribute to immunity through production of IL-10, a cytokine that is generally accepted as anti-inflammatory (114, 115). However,IL-10 may not always be a tolerogenic cytokine, as blocking IL-10 was protective in a mouse model of SLE and has been shown to drive autoantibody production in B cells from human SLE patients (116, 117). A $CD5^+$ B cell subset has also been reported in humans, which also produces polyreactive IgM (118-121). However, it is clear that CD5 alone is not sufficient to identify human B cells, as it can be upregulated during activation (122-125). In one report, CD20, CD27, and CD43 expression has identified another polyreactive IgM-producing B cells, containing both $CD5^+$ and $CD5^-$ subsets, in umbilical cord and adult peripheral blood (126-128). This poly-reactive, innate-like B cell subset is conserved in mice and humans, and serves important immune functions. It shows that some autoreactivity may actually be conserved in B cells for a reason, so that B1 cells can develop and perform their necessary functions. B1 cell development is governed by BCR signal strength, much like other autoreactive B cell functions (129, 130). Yet again, we find that B cell signaling contributes to development and function. Of particular interest, for several reasons, is the signaling protein Bruton's tyrosine kinase (BTK).

Bruton's tyrosine kinase: A multi-functional protein of the immune system

BTK in BCR signaling

BTK is a member of the TEC family of non-receptor tyrosine kinases. It consists of five domains, made up of 659 amino acids (131). BTK functions in antigen-specific BCR signaling, as well as in signaling by CD40, various Toll-like receptors (TLRs), the Fcε receptor (FcεR) and the Fcγ receptor (FcγR) (132-139). The most profound effects of BTK loss are exhibited in the B cell compartment, and the most studied role of BTK is its role in BCR signaling (140). The mature BCR consists of membrane-bound antibody, formed by V(D)J rearrangement, and the Igα/Igβ heterodimers that form the cytosolic signaling component. Each B cell expresses 2 X 10^5 identical BCRs. Antigen engagement by these BCRs instigates a signaling cascade resulting in B cell activation, which is summarized in Figure 1.4. When a naïve B cell encounters its antigen, immunoreceptor tyrosine-based activation motifs (ITAMs) on Igα and Igβ are phosphorylated by the SRC-family kinase LYN (141). Another SRC-family kinase, spleen tyrosine kinase (SYK), docks to the dually-phosphorylated ITAMs and activates through autophosphorylation (142). Concurrently, LYN phosphorylates ITAMs on CD19, a BCR coreceptor, enabling the binding and activation of phosphoinositide-3-kinase (PI3K) (143). PI3K in turn generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_3 is a signaling mediator that recruits BTK to the membrane by the pleckstrin homology (PH) domain. At BTK's N-terminus, the

PH domain binds to PIP_3, localizing BTK from the cytoplasm to the cell membrane. This domain is critically important to BTK's function. The *xid* mouse model, once known as the CBA/N, lacks the PH domain and exhibits a phenotype that is identical to the homozygous *Btk* knockout, which lacks the entire BTK protein (132, 140, 144-147). Recruitment of BTK to the cell membrane allows docking through its SRC-homology 2 (SH2) domain to phosphorylated tyrosines on the adaptor protein B cell linker (BLNK), which in turn allows phosphorylation of Y551 on BTK by LYN and SYK (148-150). The SH2 domain of BTK allows its docking to BLNK; additionally, other proteins can dock to BTK through the SRC-homology 3 (SH3) domain. The SH3 domain enables BTK to function as an adaptor in addition to its function as a kinase (148, 151-154). One function this adaptor allows is recruitment of phosphatidylinositol 4-phosphate 4-kinase (PIP5K), which functions as a feedforward mechanism to facilitate BTK's own activation (154). Finally, at the C-terminal domain of BTK is the kinase domain, which is responsible for the phosphorylation and activation of phospholipase-C-gamma-2 (PLCγ2) (155-157). PLCγ2 cleaves PIP_2 into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (131, 158). IP_3 initiates calcium flux when it binds to its receptor on the endoplasmic reticulum (ER), initiating a cascade that results in downstream activation of the transcription factor nuclear factor of activated T cells (NFAT). The production of DAG activates protein kinase C β (PKCβ) and results in the eventual activation of nuclear factor kappa B (NFκB) and various mitogen-activated protein kinase (MAPK) pathways (134, 135, 159, 160).

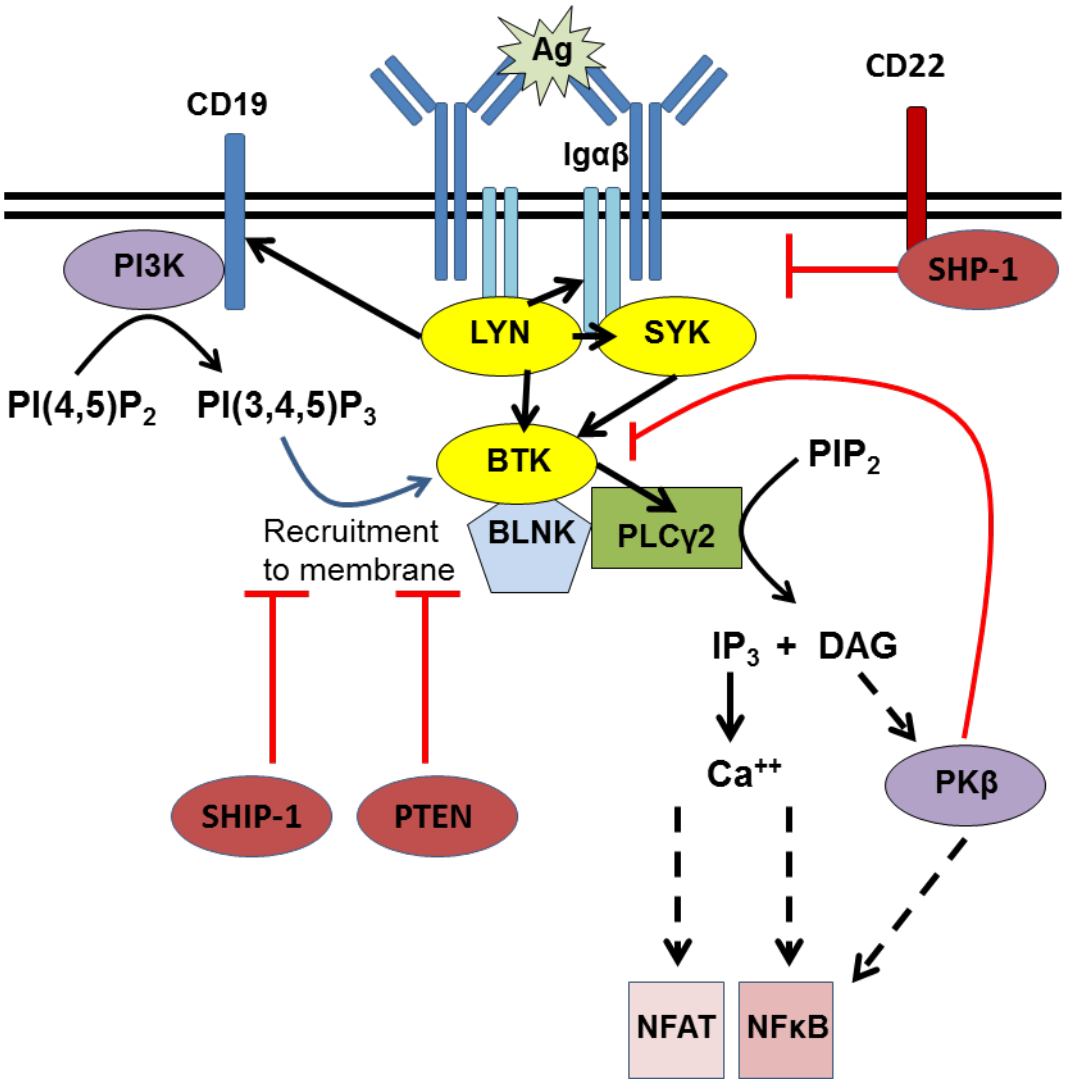


Figure 1.4: B cell receptor signaling and negative regulation. Antigen engagement of the BCR results in a phosphorylation cascade that activates the B lymphocyte. The receptor tyrosine kinase LYN phosphorylates Igαβ, CD19, and SYK. The phosphorylation of CD19 results in generation of PIP_3, which in turn recruits BTK to the membrane. BTK can be phosphorylated by LYN or SYK, and its activation allows it to in turn phosphorylate and activate PLCγ2. PLCγ2 generates the second messengers IP_3 and DAG, resulting in calcium flux, activation of PKβ, and the eventual activation of transcription factors. This signaling cascade is negatively regulated by the phosphatases shown by red circles. SHP-1 is a phosphatase recruited to CD22, which is able to dephosphorylate tyrosines on Igα and BTK. SHIP-1 and PTEN both inhibit the generation of PIP_3, therefore inhibiting the recruitment of BTK to the plasma membrane.

Negative regulation of BTK

BTK activation in B cells is regulated by various phosphatases. The localization of BTK at the plasma membrane can be inhibited by the action of two phosphatases, including phosphatase and tensin homologue (PTEN) and SH2 domain-containing inositol-5-phosphatase-1 (SHIP-1). Both of these phosphatases catalyze the dephosphorylation of PIP_3 into PIP_2 (161, 162). However, it is important to note that PTEN generates $PI(4,5)P_2$, which can feed back into the PI3K pathway, while SHIP-1 generates $PI(3,4)P_2$, which cannot. This difference may affect the level and the durability of negative regulation by these enzymes. Another regulator of BTK is SH2 domain-containing phosphatase-1 (SHP-1), which is recruited through paired immunoglobulin-like receptor B (PIR-B) and sialic acid-binding Ig-like lectins such as CD22 and Siglec-G. SHP-1 is able to dephosphorylate tyrosines on BTK and other signaling mediators like Igα (163). Additionally, the activation of PKCβ by DAG is a feedback inhibitor of BTK, phosphorylating the S180 residue in BTK's linker domain that negatively regulates BTK function (164). PTEN, SHIP-1, and SHP-1 are all possible actors in the regulation of B cell anergy, which is discussed in more detail later in this chapter.

BTK and B cell function: Clues from mouse models

In mice, the loss of early signaling mediators, such as SYK or Igμ, results in total B cell deficiency (165-167). BCR signaling is completely dependent upon these initiating signals. Without them the B cell cannot confirm that a B cell

receptor has been formed and the B cell is blocked in the early stages of B cell development. BTK seems to function differently, as an amplifier of signal rather than an on/off switch. There are some apparent differences between BTK-sufficient and BTK-deficient B cells in murine bone marrow, though the total number and turnover kinetics of pre-B cells are unchanged (140, 146, 168). BTK-deficient mice have significantly reduced Igλ light chain usage and a slight (three hour) delay in the emergence of IgL-positive B cells (153). In addition, BTK/TEC and BTK/BLNK double knockouts do show a block in the pro-B cell stage. This implies that BTK may have a role in pre-BCR signaling, but some redundancy is in place to allow for B cell development in the single BTK knockout (169, 170). BTK-deficient B cells show increased proliferative response to IL-7 *in vitro*, so it may be that any defects caused by BTK loss are offset by increased proliferation (152).

Despite the relatively normal B cell development in the bone marrow of *Btk*-deficient animals, there is a ~50% decrease in the total number of mature B cells (140). This is due to a developmental block at the T2 B cell stage (43, 45, 140, 171). Concurrently, numbers of follicular B cells are reduced in the spleen. BTK is generally not considered to be of particular importance in MZ B cell development, though it may have a function in the selection of low-affinity BCRs to that compartment (172-175). It is important to note that BTK-negative B cells do have a selective disadvantage when in direct competition to BTK-positive B cells. *Btk* is an x-linked gene. In female mice that expressed an inserted *LacZ* reporter in place of BTK, BTK-deficient *LacZ* expressing B cells were almost

absent from the mature B cell compartments (146). These data point to a selective disadvantage of BTK-deficient B cells, and indeed, *Btk*+/- female mice are indistinguishable from WT in B cell function and in autoimmune disease (137).

BTK-deficiency in humans: X-linked agammaglobulinemia

Mutations in BTK also cause immunodeficiency in humans, in whom BTK loss causes a much more severe B cell depletion than seen in the murine phenotype. X-linked agammaglobulinemia (XLA) was first reported by Colonel Ogden C. Bruton in 1952 in a report describing a boy lacking humoral immune responses, which resulted in a high susceptibility to infection by encapsulated bacteria (176). XLA, which may be caused by over 600 different mutations in the *BTK* gene, results in a severe block at the pre-B cell stage of B cell development (177, 178). Patients have very low serum immunoglobulin levels; they have less than 1% of normal B cell numbers, and no plasma cells. The B cells that remain exhibit high levels of IgM (179), and are enriched for polyreactive, autoreactive-prone BCRs (180). However, patients with XLA are not generally thought to develop autoimmune disease. There has been one report of T1D and a few reports of juvenile arthritis in XLA patients (181-183). One study has found a majority of XLA patients report inflammatory symptoms (184). However, these were not clearly diagnosed autoimmune disease, and these reports may be due to the abnormalities in the myeloid compartment that result from lack of BTK.

The differences between the mouse and human phenotypes of *BTK*-deficiency lead to the question- is it appropriate to study BTK function in the mouse model? An important point is that patients treated with BTK inhibitors have a full repertoire of mature B cells. Indeed, treatment with BTK inhibitors does not recapitulate the XLA-phenotype (185). This could be due to the fact that BTK inhibitors only target the kinase domain and do not affect the function of BTK as an adaptor, or it could be that mature B cells respond differently to losing BTK than developing B cells do. Mouse models allow *in vivo* evaluation of mature BTK-deficient B cells and detailed study of the role of BTK in autoreactive versus non-autoreactive B cells.

BTK-mediated signaling and autoimmunity

The importance of BTK in autoreactive B cells has been shown in several ways. The transgenic overexpression of BTK in a mouse model leads to a SLE-like disease, that is associated with spontaneous germinal center formation and autoantibody production (9). Another model in which BTK is constitutively activated results in spontaneous production of autoreactive IgM plasma cells (186). In contrast, lowering of BTK levels to 25% of normal decreases autoantibody production and the autoimmune syndrome that is produced in *Lyn*-deficient mice (8). My lab has shown that the loss of BTK is protective in the NOD model of T1D, and in the anti-insulin transgenic model the loss of BTK results in a loss of 95% of anti-insulin B cells (137, 187). This block in anti-insulin

B cell development is much more severe than the block seen in non-autoreactive B cell development in either the B6 or NOD mouse models (137, 140), revealing that transgenic autoreactive B cells rely more on BTK for their development than non-autoreactive B cells. This also proves to be true in endogenous autoreactive subsets. Autoreactive-prone B1 cells and anergic An1 cells are both absent in *Btk*^{null} models (43, 140, 187). The differential dependence of autoreactive B cells on BTK may be due to aberrant signaling.

BCR signaling is dysregulated in autoreactive B cell populations.

Anergic B cells, including the MD4xML5 model and endogenous An1 cells, exhibit elevated intracellular free calcium and activation of ERK, a terminal kinase of the BCR activation pathway (188). In the Ars/A1 model of anergy, in which B cells express a transgenic low-affinity receptor for self-antigen, ITAMs on Igα/β are monophosphorylated. This conformation allows LYN to bind but not SYK, which results in activation of the inhibitory protein SHIP-1 and its adaptor Dok-1 (189). Indeed, the loss of SHIP-1 breaches tolerance in the same model (190), showing that SHIP-1 effectively mediates anergy in this specific model. This mechanism may be responsible for maintaining anergy in endogenous An1s, which show an increase in phosphorylation of Dok-1 (82). The anti-HEL MD5xML5 model of anergy exhibits increased levels of PTEN, though this is not seen in other models and may be due to the relatively high affinity of the transgenic BCR to its autoantigen (191). It is clear that negative signaling enforces anergy.

In many ways, the B1 cell subset is similar to anergic B cells in terms of BCR signaling. B1s have high levels of cytoplasmic free calcium, but are unable to flux more calcium in response to a BCR-stimulus (192). B1s do not proliferate in response to BCR-crosslinking, and exhibit high constitutive ERK phosphorylation (193-195). However, they do not seem to be negatively regulated by SHIP-1; rather, the sialic-acid binding protein Siglec-G has been identified as a strong negative regulator of BCR-induced calcium flux in B1as. This effect is most likely mediated through recruitment of SHP-1 (196). Interestingly, deletion of SHP-1 in the Ars/A1 model resulted in those B cells assuming a B1-like phenotype (190). It appears that though anergic autoreactive B cells and the B1 subset show signs of activation such as high resting calcium and ERK phosphorylation, these cells are kept in check by consistent negative regulation.

BTK and antigen presentation

There is some evidence that BTK has a role in antigen presentation, which may influence disease protection seen in some autoimmune models. One study found that B cells from *xid* mice were less able to internalize anti-IgM, and subsequently reduced in the ability to present antigen to T cells. This deficiency was linked to BTK regulation of actin dynamics, though the mechanism was not completely clear (197). However, in a human study, dendritic cells from XLA patients were equally able to present antigen and activate T cells (198). This difference could be due to murine versus human differences or differences

between B cell and dendritic cell antigen presentation. In contrast, *Btk*-deficient B cells from the anti-insulin transgenic mouse model can efficiently internalize insulin, so the loss of BTK may not completely abrogate antigen presentation by autoreactive B cells (187). Alternatively, the internalization of non-crosslinking autoantigens may be regulated differently than internalization of a crosslinking antigen like anti-IgM. Possible regulation of antigen internalization and presentation is only one way in which BTK may regulate the immune response to autoantigen or pathogens.

BTK-mediated signaling and the immune response

The loss of BTK results in signaling defects through the BCR, and also complete loss of B cell subsets such as B1s. It is predictable; therefore, that BTK plays a role in the immune response to antigen. The role of BTK in the immune response was first studied in the *xid* model. In those early studies it was established that the loss of BTK had differing effects depending on the type of immunization. *Btk*-deficiency results in an inability to respond to T cell-independent type II antigens (140, 147, 199). We now know that this defect is most likely due, at least in part, to the lack of B1 cells in *Btk*-deficient mice, which are important for the response to these antigens (112, 113, 200). T-dependent antibody responses are also reduced after initial immunization (201); however, these responses can be somewhat recovered in the secondary response to boosting (140, 147, 199, 202). Pathogen responses, by definition more complicated than immunization with model antigen, can also be affected by the

loss of BTK. In particular, pathogens that require responses by B1 cells result in more severe disease in *Btk*-deficient models. *Xid* mice are more susceptible to various strains of *Streptococcus pneumoniae* (203). It is now clear that *S. pneumoniae* resolution requires B1 cell action (105). *Btk*-deficient mice cannot clear mouse adenovirus type 1 (MAV-1), a pathogen that requires T-cell independent immune function (204). In contrast, *Btk*-deficient mice can generate sufficient immune responses to traditionally T cell-dependent immunizations to protect them from subsequent challenge (205), and seem to respond effectively to certain infections such as *Candida albicans* (206). However, in spite of the fact that BTK-deficient mice seem to partially overcome defects in T cell-dependent immunity with sufficient stimulus, the loss of BTK is still protective against the development of SLE. Even though antigen, B cells, and T cells are all available, *Btk*-deficiency in SLE models has been shown to reduce anti-DNA autoantibodies and protect from disease (207-212). It may be that BTK loss impacts the available autoreactive B cell specificities, as is seen in the severe block in the development of anti-insulin B cells as compared to non-autoreactive B cells (187). It is also possible that BTK-deficiency more strongly inhibits the entry of autoreactive B cells into the germinal center, as compared to non-autoreactive B cells. A third possibility is that BTK-deficiency affects the innate immune cells that are responding to autoantibody.

BTK in innate cells and innate signaling

Macrophages, neutrophils, mast cells, and dendritic cells all express BTK (138, 213, 214). However, the role of BTK in these cells is less clear than its role in B lymphocytes. Arthritis models that have studied the role of BTK in innate immune cells have most often focused on FcγR mediated phagocytosis and cytokine production by macrophages, and mostly relied on BTK inhibition (138, 215-219). However, many of these inhibitors are known to have off-target effects (138, 220, 221). Genetic deletion of BTK has pointed to some interesting effects in innate cells. In one report, *Btk*-deficient bone marrow derived dendritic cells (BMDCs) exhibited increased T cell stimulatory activity. This phenotype was linked to their reduced ability to secrete IL-10, resulting in increased levels of MHC class II and CD86 in response to lipopolysaccharide (LPS) stimulus (214). BTK loss may also affect neutrophils, though the literature conflicts. *Btk*-deficient neutrophils in mouse models have been reported to have decreased E-selectin mediated recruitment and decreased granules per cell (222, 223); however, human *BTK*-deficient neutrophils are shown to have either no loss of effector function (224) or even produce more reactive oxygen species (225).

Effects of BTK loss on innate immune cells are most likely due to its roles in innate signaling. Mast cells are reported to depend upon BTK for FCεR signaling (139). BTK is involved in TLR signaling, and binds to TLRs 4, 6, 8 and 9. It can also associate with MyD88 and MyD88 adaptor-like protein (MAL), key proteins involved in certain TLR signal transduction (226). Peripheral blood mononuclear cells (PBMCs) from humans with XLA were unable to produce

tumor necrosis factor alpha (TNFα) in response to the TLR4 agonist LPS, though IL-6 production was unaffected (227, 228). However, when monocytes from the XLA pateints were treated with macrophage-colony stimulating factor (M-CSF), TNF production was rescued. This was linked to an increase in the protein kinase TEC, which may be redundantly expressed in mature macrophages (227). There is evidence that BTK is important in TLR-signaling through phosphorylation of MAL (136, 229, 230). That there is a role for BTK in TLR signaling is clear, but the specifics and in which cells BTK is most important is an area that needs further study.

It is important to remember that BCR and innate signaling are not discrete pathways. BCR engagement and TLR engagement together can interact and bring together the innate and adaptive immune systems. This interaction can affect antigen presentation and the quality and duration of the B cell response (231). Antigen engagement by the BCR influences subcellular localization of TLRs (232), and the colocalization of TLR9 and the BCR with in the autophagosome is dependent on BTK (233). This interaction of innate receptor and BCR signaling perhaps explains the evidence that innate signaling can influence the loss of tolerance in autoreactive B cells (63, 64, 234).

Research Goals

The purpose of this project is to define the contribution of BTK to the autoreactive B cell during its development, survival, and function. In Figure 1.5, I

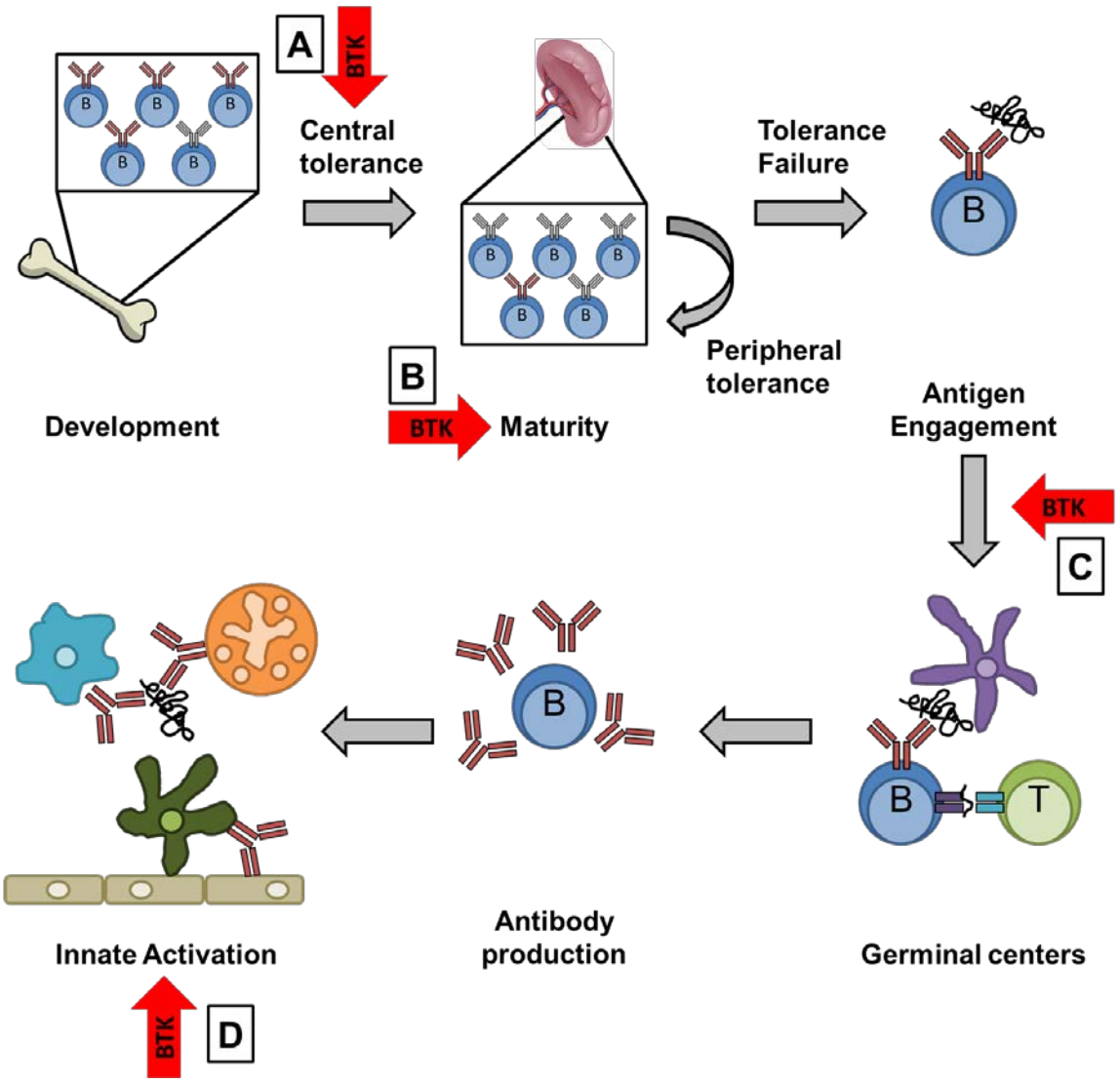


Figure 1.5: BTK has multiple roles throughout the autoimmune process. Autoreactive B cells develop in the bone marrow and are culled by central tolerance. Some escape to the periphery, where they may either maintain or lose peripheral tolerance. The loss of tolerance results in a response to autoantigen, possible antigen presentation to autoreactive T cells, and the generation of a germinal center. Autoreactive B cells in germinal centers produce high affinity autoantibody, which can bind to autoantigens by the variable region and to innate cells by FcRs. Activated innate cells can then instigate inflammation and disease pathology. BTK is known to contribute to autoreactive B cell development (A), to BCR signaling (C) and to innate signaling (D). In addition, it is hypothesized that BTK may contribute to the survival of autoreactive B cells in peripheral organs (B) and in the transition to the germinal center (C).

summarize the stages of the autoreactive B cell “life cycle,” which has been extensively discussed in this chapter. To review, autoreactive B cells are estimated to form 70-80% of the developing B cells in the bone marrow (25). Most of these are culled by central tolerance mechanisms, such as receptor editing and clonal deletion, but some escape and reach maturity in the spleen (43, 82). These B cells must be controlled by peripheral tolerance mechanisms such as anergy. However, anergy can fail by allowing antigen presentation function, such as in the NOD model of T1D (51), or tolerance may be lost resulting in B cell proliferation and autoantibody production, as in autoimmune arthritis or lupus (6, 69). If an autoreactive B cell responds to autoantigen, it can be recruited into germinal centers with autoreactive T cells and undergo SHM and isotype switching. High-affinity, switched autoantibodies mediate disease by activating innate immune cells.

BTK is known or hypothesized to contribute to this cycle of autoimmunity in several places, detailed in Figure 1.5 A-D. It is clear that BTK regulates the development of autoreactive B cells and their transition to the periphery (A) as An1, B1, and transgenic anti-insulin B cells are all impacted more by the loss of BTK during development than non-autoreactive B cells (7-9, 140, 187). A commonality of autoreactive B cells is their dysregulated signaling, so it follows that BTK may be more necessary to these cells than others. Because of this dysregulated signaling, autoreactive B cells may require BTK for survival as well as development (B), and my research uses the first models available to test this hypothesis. BTK is also known to affect receptor signaling, as can be expected

from its role as an adaptor and in activating PLCγ2, and may play a role in either the transition into the germinal center or the quality of the germinal center response (C). Finally, BTK is also expressed in innate cells (D), and its loss can impact signaling through TLRs and FcRs. This loss of signaling may result in reduced innate responses to autoantibody.

In chapter II of this thesis, I will detail my work studying BTK in autoimmune and immune-complex mediated arthritis. Autoimmune arthritis, which depends upon autoreactive B and T cells forming germinal centers, B cells producing antibody, and innate immune destruction of targeted tissue, will be compared to immune-complex mediated arthritis, a method which assesses innate immune function. This chapter furthers understanding of the role of BTK in autoreactive B cell development and in innate signaling as it contributes to arthritis.

Previous BTK-deficient models rely on total genetic knockout, meaning that BTK is absent from all stages of development, in all tissues. This has prevented study of the role of BTK in the survival and function of mature autoreactive B cell subsets. In chapter III, I will highlight work in which I used a novel inducible knockdown model of BTK, enabling me to assess the role of BTK in B cells that have reached maturity. These data, for the first time, detail the role of BTK in mature An1s, B1s and anti-insulin B cells. Chapter III will also use the inducible knockdown model in a study of a T-independent immunization, uncoupling the role of BTK in B cell development versus in response to immunization. Collectively, the work in this thesis provides critical knowledge on

cellular dependence upon BTK. These data demonstrate how the role of BTK differs in each stage of the autoimmune process.

CHAPTER II

THE ROLE OF BRUTON'S TYROSINE KINASE IN AUTOIMMUNE AND IMMUNE COMPLEX-MEDIATED ARTHRITIS

Abstract

Autoreactive B cells drive autoimmune arthritis by producing harmful autoantibodies, which bind and activate innate receptors. B cell receptor (BCR) signaling regulates these autoreactive B cells, and is in part mediated by the signaling protein Bruton's Tyrosine Kinase (BTK). BTK inhibition has been shown to be effective in prevention of both spontaneous autoimmune arthritis, which depends upon both the innate and adaptive immune systems, and in immune complex-mediated arthritis, which depends only on innate cells. However, these inhibitors are known to have off-target effects. In these studies, I have used genetic deletion of BTK to determine its role in the adaptive and innate immune responses that drive inflammatory arthritis. The loss of BTK was protective against the development of arthritis in K/BxN mice, which depend upon both adaptive and innate immunity. *Btk*-deficiency resulted in severely reduced B lymphocytes at every splenic developmental stage. Germinal center B cells were significantly reduced, with a subsequent loss of T follicular helper cells, despite the fact that BTK is not expressed in T cells. Autoantibody was severely decreased, while total IgG was only mildly affected, indicating a specific reduction in autoreactive B cells. However, *Btk*-deficiency was not protective in serum-transfer arthritis, which relies only on innate immunity. These data show that the contribution of BTK in disease protection is mainly due to its role in B cell signaling, rather than in innate immune cells.

Introduction

Bruton's tyrosine kinase (BTK) is a signaling protein expressed in B cells, macrophages, neutrophils, mast cells, and dendritic cells (138, 213, 214). In B lymphocytes, BTK propagates signaling through the B cell receptor (BCR), toll-like receptor 4 (TLR4) and CD40 (133-135, 137, 140, 160). In myeloid cells, BTK mediates signaling by various TLRs, Fcε receptor (FcεR), and Fcγ receptors (FcγR) (136, 138, 215). Importantly, there is increasing evidence that BTK plays a critical role in autoreactive B cell development and regulation. Autoreactive-prone B cell subsets are more severely depleted in BTK-deficient mouse models than non-autoreactive B cell subsets. This includes innate-like, autoreactive prone B1 cells and anergic autoreactive An1 B cells, which are both absent in BTK-deficient mice (43, 140, 187). BTK-deficiency depleted mature anti-insulin B cells by 95% in a transgenic mouse model (187), compared to a 20% loss of normal B cells in the non-transgenic mouse (137). Transgenic BTK overexpression results in spontaneous germinal center and plasma cell generation. These reactions are autoreactive, and produce anti-nuclear antibody that causes autoimmune pathology (9). Similarly, the constitutive expression of BTK results in autoreactive IgM-producing plasma cells (186). Conversely, decreased levels of BTK result in increased B cell tolerance. Lowering BTK levels is enough to protect against the autoimmune disease produced in LYN-deficient mice (8), and the loss of BTK is protective against the development of Type 1 diabetes (T1D) in the non-obese diabetic (NOD) mouse model (137). In

this chapter, I study the role of BTK in another autoimmune disease, antibody-mediated arthritis.

Autoimmune arthritis can result when autoreactive B cells and T cells interact, resulting in the production of damaging autoantibody. B cells also may contribute to pathogenesis by presenting antigen and producing inflammatory cytokines. The autoantibody produced can then bind to FcRs on innate effector cells such as macrophages, mast cells, neutrophils, and natural killer cells, resulting in activation and further inflammation. RA is characterized by inflammation of synovial tissue, cartilage erosion, and the eventual destruction of bone (235). Because of its roles in both B lymphocytes and innate immunity, BTK is a possible target for small molecule inhibition and treatment of this disease. However, only one early mechanistic study has been carried out using genetic models of BTK-deficiency (236). Other work has relied exclusively on BTK inhibitors (215-219, 237) and focused mostly on the role of BTK in innate cells such as macrophages. BTK inhibitors are known to have off-target effects, such as binding and inhibition of interleukin-2-inducible T cell kinase (ITK), affecting T cell function (220). Genetic deletion provides important insight into the mechanism of protection mediated specifically by the loss of BTK. To further study the role of BTK in autoreactive B lymphocytes, and to separate that role from its function in innate cells, I used the K/BxN model of autoimmune arthritis.

The K/BxN model enables study of innate and adaptive immune contributions to arthritis. K/BxN mice are generated from a cross between a KRN mouse, which expresses a transgenic T cell receptor (TCR), and a mouse

expressing MHC class II IA^{g7}, characteristic of the NOD mouse strain. An MHC that is considered autoreactive-prone, IA^{g7} enables recognition of a peptide from glucose-6-phosphate isomerase (GPI) by the KRN TCR (238, 239). This interaction results in the production of anti-GPI autoantibodies and severe arthritis. The disease is more robust in males, and requires both innate and adaptive immunity (240-244). Importantly, K/BxN serum can be transferred into recipients, where it induces immune complex-mediated arthritis that bypasses adaptive immunity and relies only on myeloid immune cells (240, 245). This allows separation of BTK's role in autoreactive B cells versus its function in innate immunity. I generated *Btk*-deficient K/BxN males and studied the progression of arthritis in comparison to *Btk*-sufficient littermate controls. I show that *Btk*-deficiency in the K/BxN model confers significant protection from spontaneous arthritis, results in a severe loss of mature B cells, more dramatic than seen in non-autoreactive models, and a subsequent loss of germinal center B cells and anti-GPI autoantibody. However, total IgG was only slightly reduced, again showing that autoreactive B cells may rely more on BTK than non-autoreactive B cells. In contrast, BTK loss had no effect on the progression of immune complex-mediated arthritis.

Materials and Methods

Mice and Disease Studies

KRN mice were provided by Christophe Benoist and Diane Mathis. *Btk*-deficient NOD mice were derived as previously described (137). Mice were bred and maintained under specific pathogen-free conditions. KRN males were bred to

Btk$^{+/-}$ NOD females, producing *Btk*-sufficient and *Btk*-deficient K/BxN males for spontaneous arthritis studies, assessed weekly for 5 weeks post weaning. For immune complex-mediated arthritis studies, serum from 8-9 week old arthritic *Btk*-sufficient K/BxN males was pooled and injected (200µL, intraperitoneal injection) to produce arthritis in male *Btk*-sufficient and *Btk*-deficient NOD mice. Mice were injected on days 0 and 2 and assessed for arthritis for two weeks. All studies are approved by the Vanderbilt University Animal Care and Use Committee.

Arthritis Scoring

The Chondrex mouse arthritis scoring system was used to assess arthritis progression in the K/BxN and serum-transfer models (https://www.chondrex.com/documents/ Mouse%20CIA.pdf). Briefly, each paw was scored on a scale of 0 to 4, with normal paws scored as 0 and maximally inflamed limbs scored as 4. Scores for all four paws were combined for a total possible arthritis score of 16. For paw thickness measure, hind foot pad paw thickness was measured by a Swiss Precision Instrument (SPI) dial gauge (13-159-9).

Flow Cytometry and Antibodies

Single cell suspensions from spleens, popliteal lymph nodes (LN), and peritoneal cavity were obtained as previously described (137) and stained using fluorochrome or biotin-conjugated antibodies against B220 (RA3-6B2), IgM (µ-

chain specific, Life Technologies), IgD (11-26c.2a), CD21 (76G), CD23 (B3B4), CD4 (RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3, BD Biosciences or N418, eBioscience), CD5 (53-7.3), CD19 (ID3), Fas (Jo2), BCL6 (K11291), GL7, CD44 (IM7, eBioscience), CXCR5 (2G8), PD1 (J43), ICOS (C398.4A, eBioscience), F4/80 (BM8, eBioscience), Ly6G (IA8), and/or CCR7 (4B12). Unless otherwise stated, antibodies are from BD Biosciences. Biotin-conjugated antibodies were secondarily stained with fluorochrome-conjugated streptavidin (BD Bioscience). Dead cells were excluded using 7 Aminoactinomycin D (BD Biosciences), fixable viability dye eFluor® 450 (eBioscience) or Alexa Fluor® 700 Succinimidyl Ester (Life Technologies). Samples were read on a LSRII flow cytometer (BD Biosciences) and data analyzed using FlowJo (Tree Star) software.

Bone Marrow-derived Macrophages

Murine bone marrow cells harvested from femurs were differentiated in RPMI 1640 media (Corning) with 10% FCS (Gibco), 1% antibiotic-antimycotic (Gibco), and 10ng/mL macrophage colony stimulating factor (R&D) for 7 days in non-TC treated polystyrene plates (Fisher). On day seven, attached macrophages were harvested, transferred to 96-well flat-bottom NUNC plates, allowed to adhere, then incubated without stimulus, with 1/20 K/BxN serum, or with 100ng/mL LPS (DIFCO Laboratories) overnight at 37° C. Supernatants were frozen for analysis.

ELISA

Serum IgG (total) and anti-GPI IgG from 8-9 week old *Btk*-sufficient and *Btk*-deficient K/BxN were measured. 96-well flat-bottom NUNC plates were coated with 1µg/mL recombinant mouse GPI (Cloud-Clone Corp) or 2µg/mL goat anti-mouse Ig (Southern Biotech) in PBS overnight at 4ºC. Plates were blocked with 1% BSA in PBS or 10% non-fat dry milk in PBS+0.5% Tween-20 (PBST). Diluted sera (1:3000 or 1:5000) were added to plates. IgG antibodies were detected using goat anti-mouse IgG-alkaline phosphatase (AP) (Southern Biotech). p-Nitrophenyl Phosphate (PNPP) was added to the plate and O.D. read on a Microplate Autoreader (Bio-Tek Instruments) at 405nm. Mouse TNFα Ready-Set-Go!® ELISAs (eBioscience) were performed on BMDM supernatants according to manufacturer's protocol and O.D. read at 450nm.

Histology

Hind paws collected from 8-9 week old *Btk*-sufficient and *Btk*-deficient K/BxN mice were processed as previously described (246). Four blinded observers scored histological samples for inflammation (0, normal; 1, minimal; 2, mild; 3, moderate; 4, severe), cartilage destruction and bone erosion (0, no destruction/erosion; 1, moderate 2, severe).

Whole-body fluorescence imaging

Fluorescence imaging was performed as previously described (247). Cy5-PEG-folate (Nanocs Inc., NY; excitation wavelength - 650 nm, emission - 670 nm) was

injected intravenously (500nmol/kg). Fluorescent imaging was performed after 4 hours by a Pearl Impulse system (LI-COR, Lincoln, NE). Data were collected and analyzed using Pearl Impulse software (LI-COR). Whole-body fluorescence imaging was performed by Wei Han, Ph.D., of Vanderbilt University School of Medicine.

Statistics

Statistics were performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA). P-values for disease curves and FolRβ imaging were calculated using a two-way ANOVA. All other p-values were calculated by unpaired t tests with Welch's correction or the Holm-Sidak method of multiple t tests, as appropriate.

Results

Loss of BTK protects against development of arthritis in K/BxN mice.

To determine BTK contributions to arthritis development, *Btk*-deficiency was introduced to the K/BxN mouse model. *Btk* loss significantly protected against arthritis, as assessed by clinical score and paw thickness (Fig 2.1a,b). By 5 weeks post-weaning, *Btk*-sufficient K/BxN had mean clinical scores of 15.7 (±0.816), compared to *Btk*-deficient K/BxN scores of 9.75 (±2.49), ($p<0.0001$). Paw thickness averaged 3.03mm (±0.156) in *Btk*-sufficient K/BxNs and 2.33mm (±0.271) in *Btk*-deficient K/BxN ($p<0.0001$). H&E stained histologic sections from

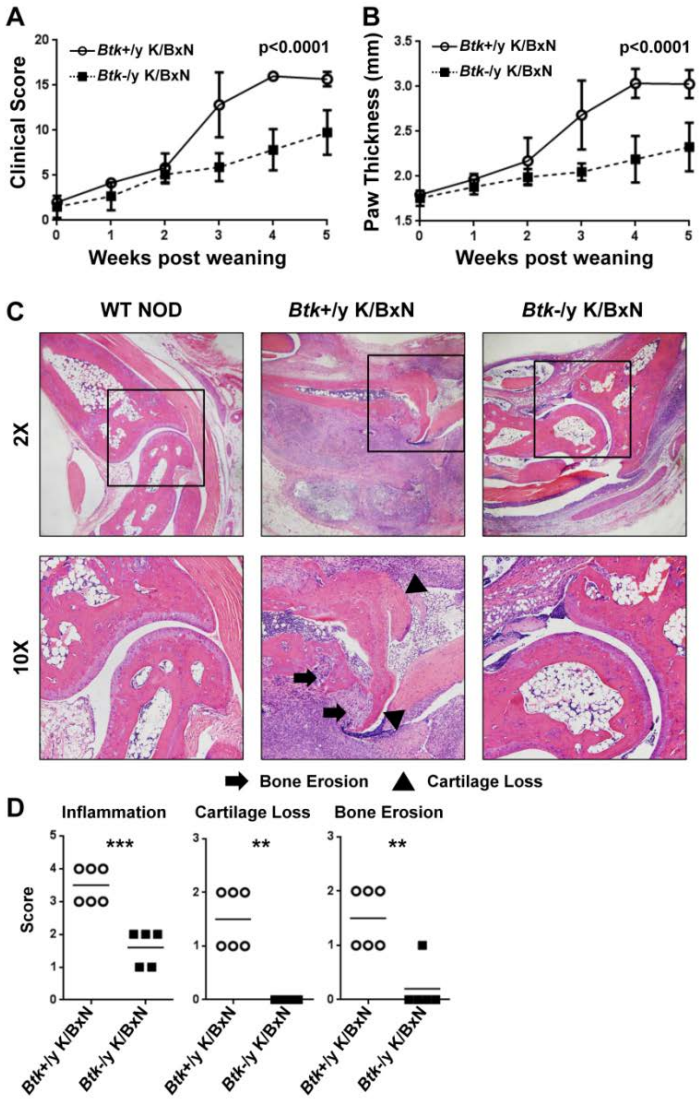


Figure 2.1: *Btk* deficiency is protective against the development of autoimmune arthritis in K/BxN mice. A) and B) *Btk*-sufficient (circles, n=6) and *Btk*-deficient (squares, n=12) K/BxN mice were scored for arthritis for 5 weeks post weaning. Clinical scores (A) were assigned on a scale of 0-4 for each limb and pooled for a total possible score of 16. In addition, paw thickness (mm) was measured by caliper (B). Mean values are shown ± standard deviation. C) Representative H&E staining from right hind paws of WT NOD (left), *Btk*-sufficient K/BxN (middle), and *Btk*-deficient K/BxN (right). 2X magnification (top), 10X magnification (bottom). Arrows indicate areas of bone erosion, triangles indicate cartilage loss. D) Scoring of *Btk*-sufficient (circles, n=6) or –deficient (squares, n=5) for inflammation (top), cartilage loss (middle), and bone erosion (bottom). Inflammation was scored on a scale of 0 to 4, while cartilage loss and bone erosion were scored as 0 to 2. P values were calculated by a 2-way AVOVA with Sidak correction (A, B) **p≤0.01, ***p≤0.001, by unpaired T test with Welch's correction (D).

right hind paws were assessed for inflammation, cartilage destruction, and bone erosion. Fig 2.1c shows a representative, non-arthritic NOD control (left), *Btk*-sufficient K/BxN (middle), and *Btk*-deficient K/BxN (right). Fig 2.1d shows pooled scores of *Btk*-sufficient and –deficient K/BxNs. All three measures show significant differences between genotypes, with *Btk*-deficient K/BxNs exhibiting lower inflammation (*Btk*-sufficient 3.5±0.548, *Btk*-deficient 1.6±0.548, p=0.0003), no cartilage destruction (*Btk*-sufficient 1.5±0.548, *Btk*-deficient 0±0, p=0.0011), and little bone erosion (*Btk*-sufficient 1.5±0.548, *Btk*-deficient 0.2±0.447 p=0.0019). These data demonstrate that lymphocytic infiltration and arthritic damage, and thus arthritis progression, is significantly reduced by BTK loss.

Innate and adaptive immune cells are decreased in Btk-deficient K/BxNs.

To determine the effect of *Btk*-deficiency on immune cell development and survival in K/BxN mice, we used flow cytometry to enumerate T and B lymphocytes, macrophages, neutrophils, and dendritic cells. Fig 2.2a shows representative flow plots of live-gated splenocytes from *Btk*-sufficient (left) and *Btk*-deficient (right) K/BxN, gated to CD4 and CD8 T cells (top) and IgM versus B220 B cells (bottom). In Fig 2.2b, the cell populations are quantified as percent of live lymphocytes (top) or total number of cells (bottom). The percentages of B cells were significantly decreased in *Btk*-deficient K/BxNs (37.8±4.44) compared to *Btk*-sufficient (47.9±6.32) (p=0.0098). Percentages of both $CD4^+$ and $CD8^+$ T cells were significantly increased in *Btk*-deficient K/BxNs (8.46±1.74, 4.65±1.03) compared to *Btk*-sufficient (6.12±1.08, 3.15±0.971) (p=0.0206, p=0.0271), reciprocal to the loss of large numbers of B cells (*Btk*-sufficient=7.18e6±2.37e6,

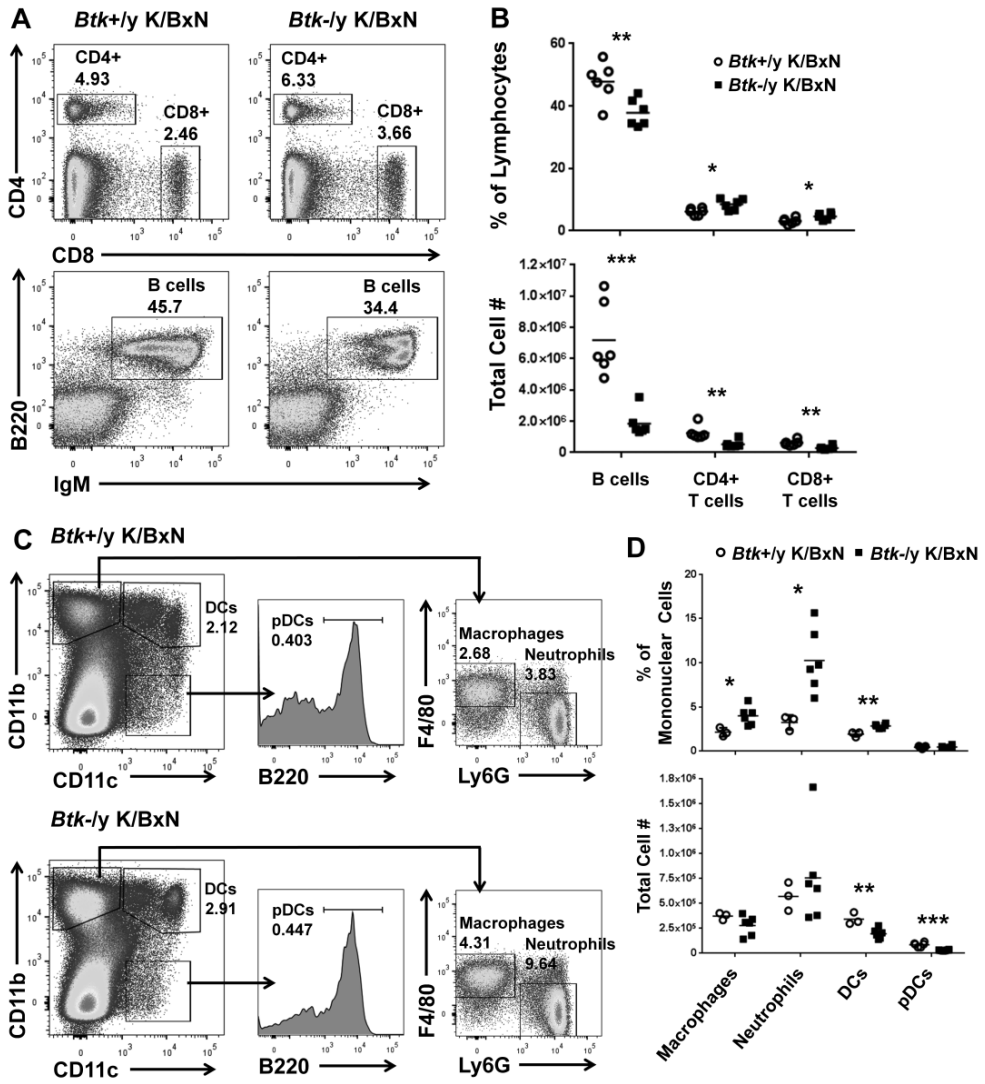


Figure 2.2: Immune cell numbers are decreased in the spleen of *Btk*-deficient K/BxNs compared to *Btk*-sufficient controls. A) Representative flow plots for *Btk*-sufficient (left) and *Btk*-deficient (right) K/BxN, showing total T cells (top) and B cells (bottom). Cells are pre-gated on single live lymphocytes. T cells are gated as $CD4^+$ and $CD8^+$, while B cells are designated as $B220^+/IgM^+$. B) Quantification of T and B cell percentages of lymphocytes (top) and total cell number (bottom) in *Btk*-sufficient (circles, n=6) or –deficient (squares, n=6). C) Innate immune cells are shown by flow cytometry for a representative *Btk*-sufficient (top) or –deficient (bottom) K/BxN. Cells were gated as single live mononuclear cells. $CD11b^+CD11c^-$ cells were gated as $F4/80^+Ly6G^-$ for macrophages or $F4/80^-Ly6G^+$ for neutrophils. $CD11b^+CD11c^+$ cells were designated myeloid dendritic cells (DCs) and plasmacytoid dendritic cells (pDCs) were designated as $CD11b^-CD11c^+B220^+$. D) Quantification of percentages (top) or total numbers (bottom) of innate immune cells in *Btk*-sufficient (n=3-7) or *Btk*-deficient (n=6-8) K/BxN. *p≤0.05, **p≤0.01, ***p≤0.001, as calculated by the Holm-Sidak method of multiple T tests.

Btk-deficient=1.86e6±8.50e5, p=0.0004). However, though T cells do not express BTK, T cells numbers were significantly reduced, ($CD4^+$ *Btk*-sufficient=1.26e6±4.44e5, *Btk*-deficient=5.39e5±2.34e5, p=0.0056; $CD8^+$ *Btk*-sufficient=6.19e5±1.75e5, *Btk*-deficient=2.94e5±1.23e5, p=0.0040), suggesting T cell expansion during arthritic progression in *Btk*-sufficient K/BxNs.

Innate cells were also quantified using CD11b, CD11c, F4/80, Ly6G, and B220 to identify macrophages, neutrophils, myeloid dendritic cells (DCs), and plasmacytoid dendritic cells (pDCs) (Fig 2.2c,d). *Btk*-deficient K/BxN mice had significantly increased percentages of neutrophils (*Btk*-sufficient=3.26±0.843, *Btk*-deficient=10.3±3.57) and macrophages (*Btk*-sufficient=2.15±0.491, *Btk*-deficient=4.00±1.05) (p=0.0140, p=0.0258); however, absolute numbers were not significantly altered. Thus, the higher percentage again reflects the substantial loss in numbers of B cells. Myeloid dendritic cell numbers in the spleen were significantly decreased in *Btk*-deficient K/BxN, (*Btk*-sufficient=3.41e5±6.09e4, *Btk*-deficient=1.97e5±4.98e4 p=0.0064), as were plasmacytoid dendritic cells (*Btk*-sufficient=8.62e4±2.16e4, *Btk*-deficient=3.01e4±4.43e3 p<0.0001).

Btk-deficiency reduces mature B cell subsets in K/BxN mice.

Btk-deficiency results in a block in the late transitional (T2) stage of B cell development in NOD and C57Bl/6 mice (137, 248). To determine the developmental stage at which B cells are reduced in *Btk*-deficient K/BxN mice, we evaluated B cell subsets by expression of IgM, IgD, CD21, and CD23. Fig 2.3a shows representative samples of *Btk*-sufficient (left) and *Btk*-deficient (right)

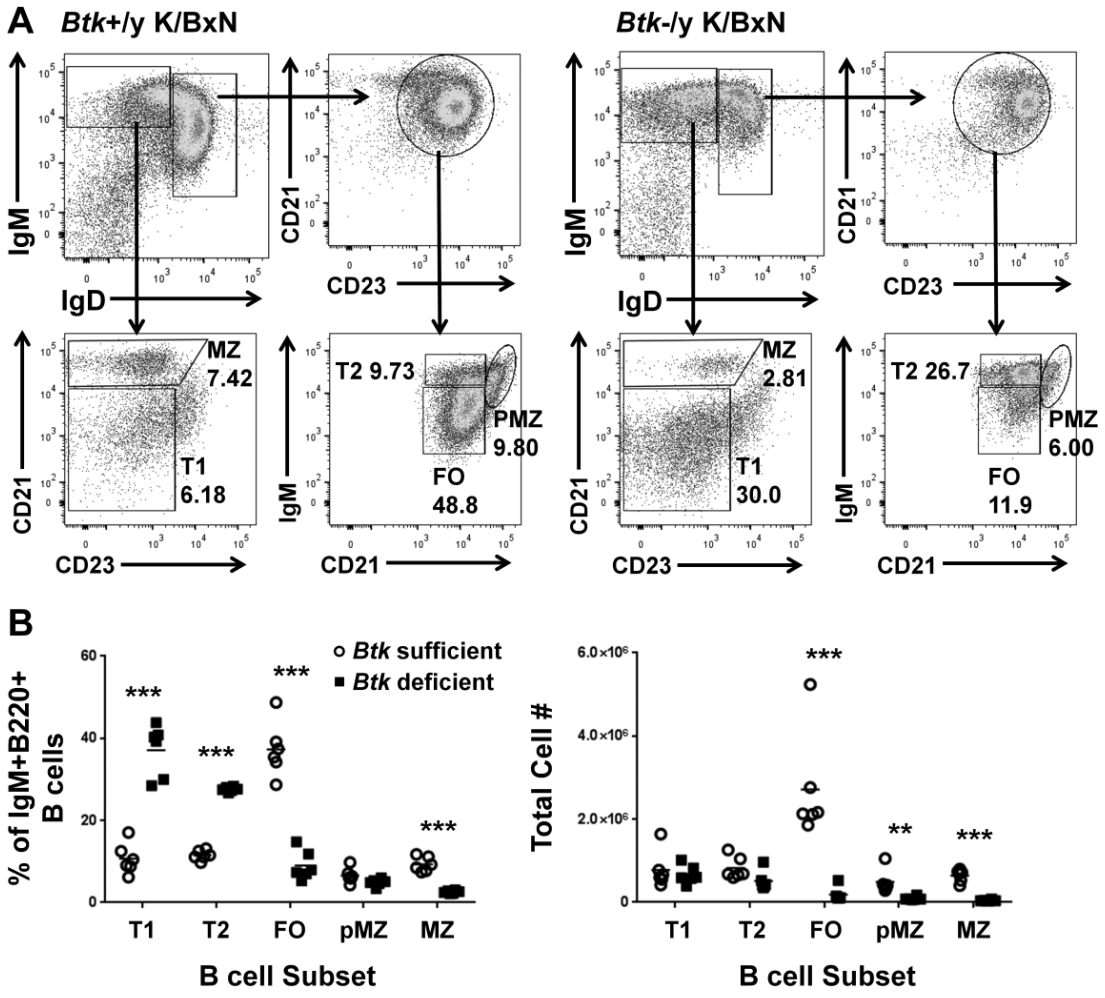


Figure 2.3: *Btk*-deficiency reduces mature B cell subsets in K/BxNs. A) Representative flow plots for *Btk*-sufficient (left) and *Btk*-deficient (right) K/BxN, showing gating scheme for B cell subsets. Cells are gated as single, live, $B220^{+}$ lymphocytes. B) Quantification of B cell subsets in *Btk*-sufficient (circles, n=6) or –deficient (squares, n=6) K/BxN by percentage of total $IgM^{+}IgD^{+}$ B cells (left) and by total cell number (right). **p≤0.01, ***p≤0.001, as calculated by the Holm-Sidak method of multiple T tests.

K/BxN B cells designated as early transitional (T1), late transitional (T2), follicular (FO), pre-marginal zone (PMZ), and marginal zone (MZ). Fig 2.3b shows quantification of B cell subsets by percentages (left) and total cell numbers (right). The percentages of T1 and T2 B cells are significantly higher in *Btk*-deficient K/BxNs (37.1±6.30, 27.6±0.571) compared to *Btk*-sufficient K/BxN (10.7±3.75, 11.5±1.205) ($p<0.0001$, $p<0.0001$). A corresponding significant decrease in the percentage of FO cells (*Btk*-sufficient=37.3±6.65, *Btk*-deficient=9.03±3.57, $p<0.0001$) points to a developmental block at T1 and T2 B cell stages. However, there is no increase in total T1 or T2 B cell numbers as seen in other *Btk*-deficient models, suggesting that B cells are lost at early and late transitional stages as well. Furthermore, the decrease in the number of FO (*Btk*-sufficient=2.71e6±1.27e6, *Btk*-deficient=1.85e5±1.69e5, $p=0.0007$), pre-MZ (*Btk*-sufficient=4.86e5±2.83e5, *Btk*-deficient=8.99e4±4.22e4, $p=.0069$) and MZ B cells (*Btk*-sufficient=6.47e5±1.50e5, *Btk*-deficient=4.67e4±1.73e4, $p<0.0001$) in *Btk*-deficient K/BxNs is severe, with substantially greater cell loss than is seen in *Btk*-deficient NOD or C57BL/6 mice, more typical of when BTK is removed from autoreactive B cells, as we have recently reported (187). Thus, B cells in K/BxN mice greatly rely on BTK-mediated signaling at all developmental stages. B1a B cells were also severely depleted, typical of *Btk*-deficient models (data not shown) (137, 140).

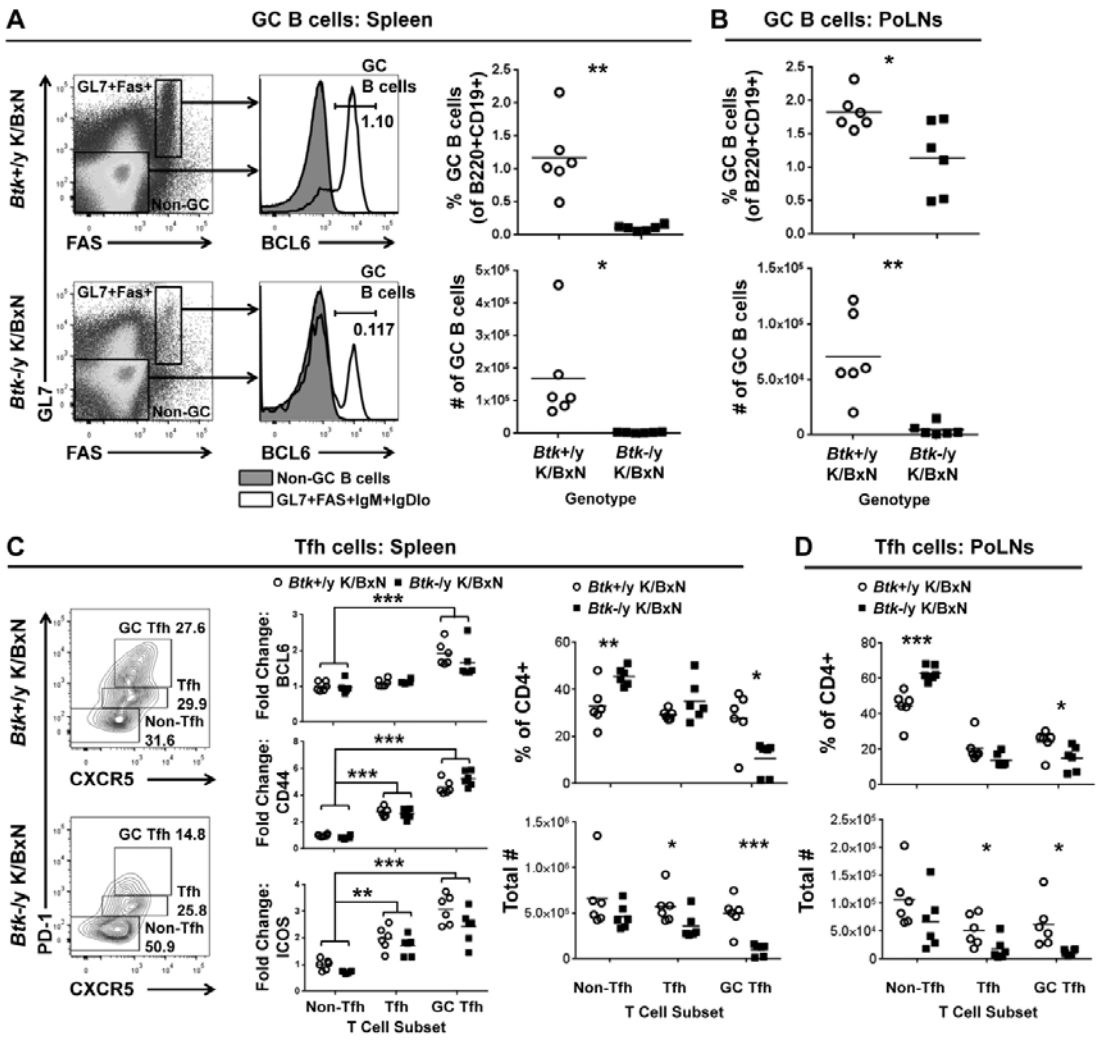


Figure 2.4: GC B cells and T follicular helper cells are decreased in *Btk*-deficient K/BxNs. A) Representative flow plots of *Btk*-sufficient and –deficient K/BxN B220^{+}CD19^{+} live splenocytes. IgM^{hi}IgD^{lo} gating was applied (not shown) and GL7^{+}Fas^{+} cells (white) or GL7^{-}Fas^{-} controls (gray) evaluated for GC marker BCL6. GC B cells (GL7^{+}Fas^{+}IgM^{hi}IgD^{lo}BCL6^{+}) were quantified as percent of B cells, and total number in spleen (A, right panel) and popliteal LNs (B). C) Representative plots of splenic CD4^{+} live lymphocytes stained for Tfh markers PD-1 and CXCR5. Expression levels of BCL6, CD44 and ICOS in GC Tfh (PD1^{hi}CXCR5^{hi}) and Tfh (PD1^{+}CXCR5^{+}), shown as fold change over *Btk*-sufficient K/BxN non-Tfh cells (middle panel). GC Tfh, Tfh and non-Tfh were quantified as percentage of CD4^{+} cells (top) or total cell number (bottom) for spleen (C, right) and popliteal LNs (D). *Btk*-sufficient, circles, n=6, *Btk*-deficient, squares, n=6. *p≤0.05, **p≤0.01, ***p≤0.001, calculated by unpaired T test with Welch's correction (A, B), the Holm-Sidak method of multiple T tests (C right, D) or by a multi-way AVOVA with Sidak correction (C middle).

Germinal center B cells are decreased in spleens and popliteal lymph nodes of Btk-deficient K/BxNs.

Formation of germinal centers (GCs) in spleen and draining lymph nodes is central to development of high-affinity anti-GPI IgG autoantibodies. GC formation was therefore assessed. $CD19^{+}/B220^{+}/IgM^{+}/IgD^{lo}$ live lymphocytes were defined as GC B cells using GL7, FAS, and BCL6 (Fig 2.4). Percentages of GC B cells in the spleens were significantly reduced in *Btk*-deficient K/BxN (0.1086±0.043) versus *Btk*-sufficient K/BxN (1.18±0.5524) (p=0.0051). This decreased percentage corresponded to dramatic reduction in cell number (*Btk*-sufficient=1.69e5±1.46e5, *Btk*-deficient=3.63e3±1.68e3, p=0.0393). GC B cells were similarly reduced in draining popliteal LNs of *Btk*-deficient K/BxN (1.14±0.544, 4.73e3±5.44e3) compared to *Btk*-sufficient counterparts (1.83±0.273, 7.08e4±3.80e4) (p=0.026, p=0.0076). Thus, BTK contributions to autoimmune arthritis include development or expansion of GC B cells.

Germinal center T follicular helper cells are decreased in spleens and popliteal lymph nodes of Btk-deficient K/BxN mice, while non-Tfh T cells are unchanged.

Though T cells do not rely on BTK for cell signaling, we found that their numbers were decreased in *Btk*-deficient K/BxN mice (Fig 2.2). B cell interactions with T cells drive T follicular helper (Tfh) cell formation and maintenance at several checkpoints, both at the T-B zone and within GCs (249). We therefore assessed Tfh cells in this model. Fig 2.4c (left) shows representative flow plots of *Btk*-sufficient K/BxN (top) and *Btk*-deficient K/BxN

(bottom) splenocytes, gated on live, $CD4^+$ lymphocytes. The Tfh markers PD-1 and CXCR5 were used for initial analysis. The double negative population is defined as non-Tfh, PD-$1^{mid}CXCR5^+$ as Tfh, and PD-$1^{hi}CXCR5^+$ cells as GC Tfh (250). These cell subsets were additionally characterized by their expression of Tfh markers BCL6, CD44, and ICOS. Expression levels were quantified by flow cytometry and shown in Fig 2.4c (middle) as fold change compared to the *Btk*-sufficient K/BxN non-Tfh. BCL6 (top), the transcription factor that is associated with GC B and Tfh cells, was significantly increased in the GC Tfh compartment compared to non-Tfh cells. CD44 and ICOS were also significantly increased in both the Tfh and GC Tfh compartments, further confirming the cells' classification. Fig 2.4c (right) shows each subset as a percentage of total $CD4^+$ (top) or as number of total cells (bottom). *Btk*-deficient K/BxNs had increased percentage of non-Tfh cells (45.5±3.76) over *Btk*-sufficient K/BxN (32.8±8.76) (p=0.0087); however, the total number of non-Tfh cells was not significantly different. Therefore, the loss of *Btk* does not impact non-Tfh cells. The most dramatic phenotype was the GC Tfhs, decreased in both percentage and number in *Btk*-deficient K/BxNs (10.5±7.04, 9.91e4±6.41e4) compared to *Btk*-sufficient controls (27.7±11.3, 4.98e5±1.82e5) (p=0.0103, p=0.0005). Popliteal LN Tfh were determined identically to splenocytes (Fig 2.4d), and also showed reduced GC Tfh cell numbers in *Btk*-deficient (1.22e4±5.22e3) versus *Btk*-sufficient K/BxNs (6.15e4±4.14e4) (p=0.0159). These data demonstrate that a defect in B cells, the loss of BTK, affects germinal center T cells in K/BxN arthritis.

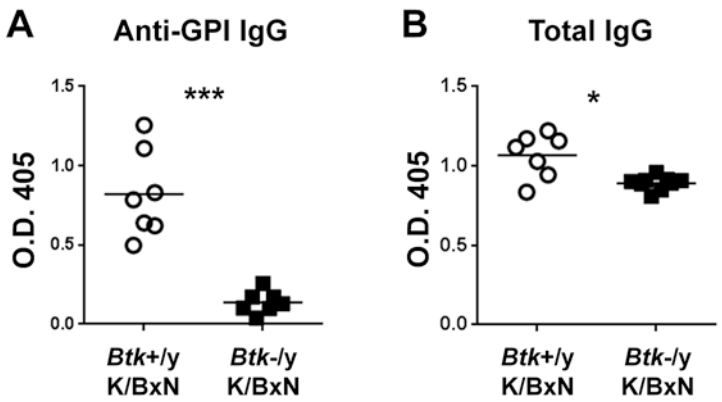


Figure 2.5: Anti-GPI IgG is severely reduced in *Btk*-deficient K/BxN, while total IgG is largely preserved. Serum anti-GPI IgG (A) and total IgG (B) were quantified by ELISA. *Btk*-sufficient, circles, n=7-8; *Btk*-deficient, squares, n=7-9. *p≤0.05, ***p≤0.001, calculated by unpaired T test with Welch's correction.

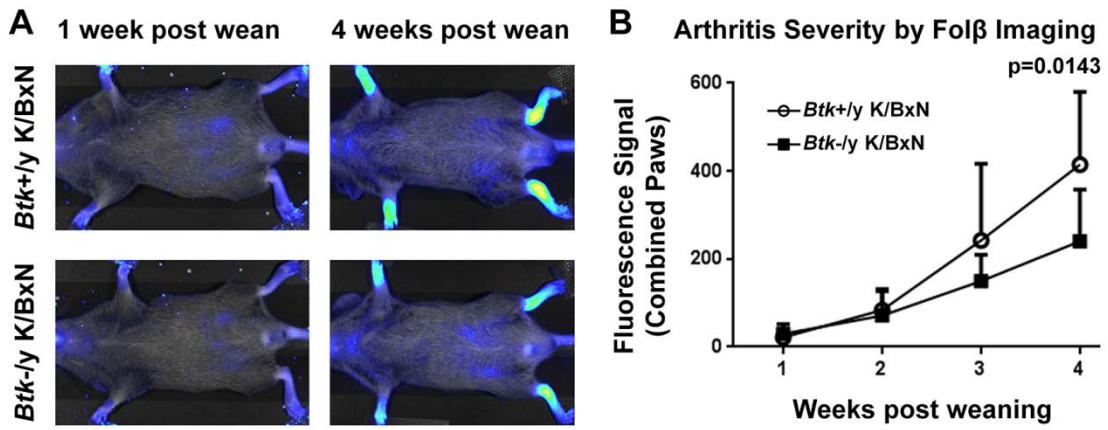


Figure 2.6: FolRβ imaging shows increased levels of activated macrophages in the paws of *Btk*-sufficient K/BxN compared to *Btk*-deficient counterparts. (A) Representative images of a *Btk*-sufficient (top) and *Btk*-deficient (bottom) K/BxN at 1 week post weaning and 4 weeks post weaning. Fluorescence in the paws was measured for quantification. (B) Macrophage infiltration into the paw is measured by FolRβ imaging for *Btk*-sufficient (circles, n=3) and –deficient (squares, n=4) Mean shown ± SD. p=0.0143 between genotypes, as calculated by a 2-way AVOVA with Sidak correction.

Btk-deficiency reduces anti-GPI IgG more severely than total IgG.

GCs are required for development of high affinity IgG antibodies, so loss of GC B and Tfh cells in *Btk*-deficient K/BxN indicates lack of support for production of anti-GPI autoantibodies. As GPI is an important autoantigen in both K/BxN and human rheumatoid arthritis (251), we next determined relative serum levels of anti-GPI autoantibody and found striking reduction in *Btk*-deficient K/BxN (Fig 2.5a, p=0.0004). In contrast, total IgG is only slightly decreased (Fig 2.5b, p=0.0146), which may reflect the loss of autoantibodies.

Btk-deficient K/BxN paws exhibit decreased macrophage infiltration.

Innate cell contributions to arthritis are well known, so we next used whole-body fluorescent imaging to assess activated macrophage recruitment to inflamed synovia of K/BxN mice. This technique utilizes a fluorescent probe that binds folate receptor beta (FolRβ), an activation marker on macrophages, and allows sequential, noninvasive, evaluation of mice as arthritis develops (247). From weaning to 7 weeks of age, *Btk*-sufficient and *Btk*-deficient K/BxN mice were imaged and fluorescence in each paw measured. Fig 2.6a shows a representative *Btk*-sufficient and -deficient K/BxN mouse at 1 and 4 weeks post-wean dates. Fig 2.6b shows fluorescence of *Btk*-sufficient and *Btk*-deficient combined paws from week 1 to week 4 post weaning. This method indicates that significantly more activated macrophages were recruited to the paws in *Btk*-sufficient mice (p=0.0143).

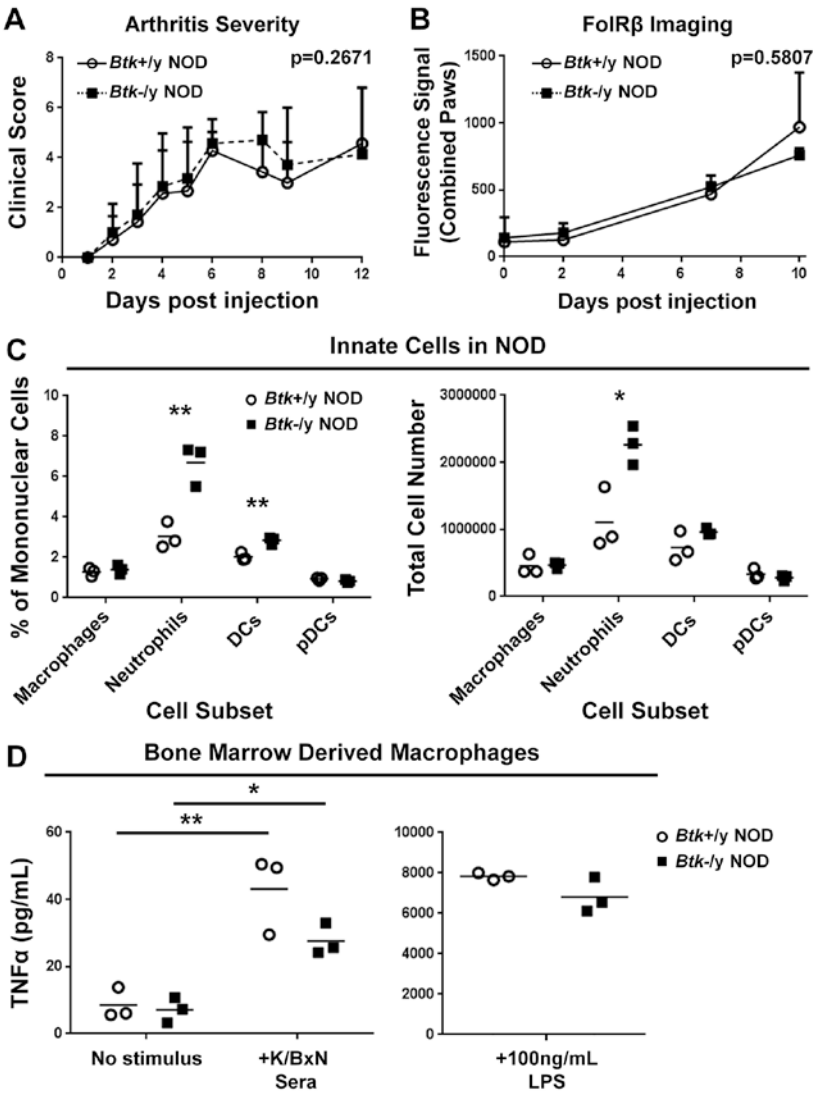


Figure 2.7: *Btk*-deficiency is not protective in the serum-transfer model of arthritis. (A) *Btk*-sufficient (circles, n=7) and *Btk*-deficient (squares, n=7) NOD mice were IP injected twice (day 0 and 2) with 200µL of pooled K/BxN sera. Clinical scores over 12 days post-injection are shown. (B) FolRβ imaging was performed on *Btk*-sufficient (n=3) and –deficient (n=3) NOD mice on day 0, 2, 7 and 10. Values are Mean ± SD. (C) Percentages (left) and total numbers (right) of *Btk*-sufficient (circles, n=3) and *Btk*-deficient (squares, n=3) age-matched male NOD. Cell subsets were gated as in Fig 2c. (D) Response of *Btk*-sufficient (circles, n=3) and *Btk*-deficient (squares, n=3) NOD BMDMs to incubation overnight with no stimulus, 1/20 K/BxN serum, or LPS. *p≤0.05, **p≤0.01, or as listed on graph, calculated by multi-way ANOVA with Sidak correction (A, D left), Holm-Sidak method of multiple T tests (C) or by unpaired T test with Welch's correction (D, right).

Btk-deficiency is not protective against development of serum transfer arthritis.

To directly determine contributions of innate cell intrinsic BTK-signaling to the development of arthritis, we bypassed adaptive immune requirements by transferring K/BxN serum into *Btk*-sufficient or *Btk*-deficient NOD mice. Assessment of recipients by clinical score showed that serum transfer arthritis (STA) was not reduced by *Btk*-deficiency, with *Btk*-sufficient NOD reaching a clinical score of 4.29±0.756 and –deficient NODs reaching 4.57±0.976 on day 6 (genotype factor p=0.2671) (Fig 2.7a). In addition, FolRβ imaging of activated macrophages showed no difference between *Btk*-sufficient and –deficient recipient mice (p=0.5807), indicating that loss of macrophage-intrinsic BTK-mediated signaling did not significantly affect activation and recruitment by transferred autoantibodies (Fig 2.7b). This finding contrasted previous studies showing that BTK-inhibitors prevent arthritis in serum transfer models, including one that used K/BxN serum (218, 219, 252). We therefore assessed the effects of BTK-deficiency on innate cell numbers and function in spleens from *Btk*-sufficient and *Btk*-deficient NOD that served as recipients for these studies. Cell subsets were determined by flow cytometry, and quantified by percent (Fig 2.7c, left) and total cell number (Fig 2.7c, right). We found that the percentages and numbers of splenic neutrophils were significantly increased in *Btk*-deficient mice (p=0.0063, p=0.0211). *Btk*-deficiency also resulted in a significant increase in percentage of DCs (p=0.0061), and a trend of higher DC numbers (p=0.1540). In addition, *Btk*-deficient DCs expressed significantly more CD11b than –sufficient controls (data not shown). Macrophage numbers did not differ, so we explored

the effects of BTK-deficiency on their function by generating bone marrow-derived macrophages (BMDMs) and testing their ability to produce TNFα in response to stimulation via FcγRs and TLR4. As shown in Fig 2.7d, *Btk*-deficient BMDMs treated with K/BxN serum were able to produce TNFα above baseline (unstimulated=7.103±3.737, stimulated=27.605±4.705, p=0.0297), although the amount of TNFα trended lower than that of *Btk*-sufficient BMDM (43.182±11.819, p=0.0992). In addition, both *Btk*-deficient and *Btk*-sufficient BMDMs responded robustly to LPS, and did not differ in their ability to produce large amounts of TNFα (*Btk*-sufficient=7838±176.5, *Btk*-deficient=6819±866.4, p=0.1744) (Fig 2.7d, right), Thus, *Btk*-deficiency in this model increases neutrophil numbers and causes a slight trend downward in TNFα production by macrophages in response to K/BxN that is not sufficient to protect mice against STA. Importantly, these imaging studies also suggest that BTK-deficiency does not interfere significantly with the ability of activated macrophages to invade target tissues in response to autoantibodies.

Discussion

BTK is a promising therapeutic target in autoimmune arthritis, but its mechanisms of action in this disease are not well-defined. My work presents the first detailed investigation of the role of BTK using genetic deletion in both spontaneous autoimmune and immune complex-mediated models of arthritis, and is summarized as a graphical abstract in Figure 2.8. These data demonstrate

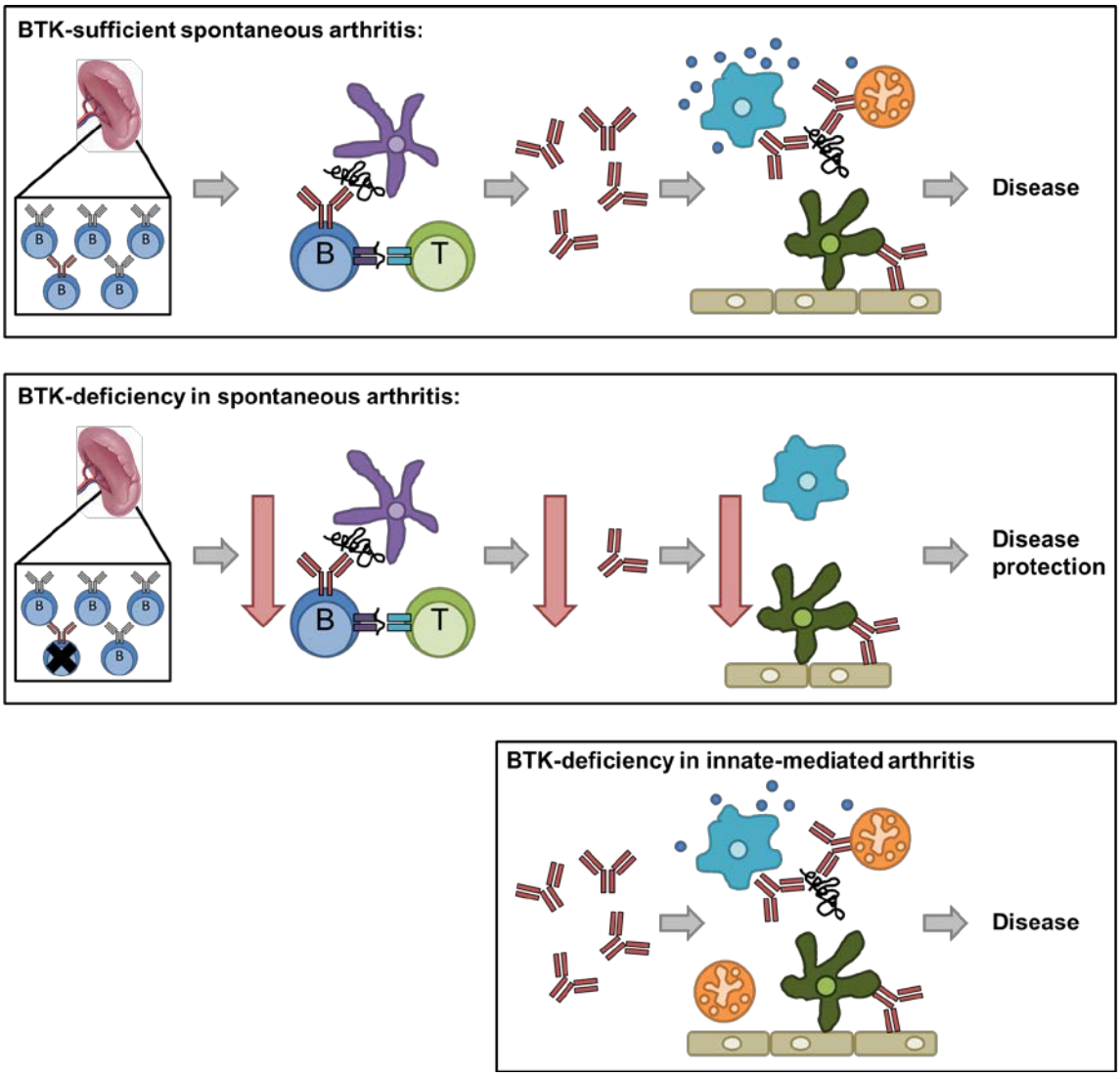


Figure 2.8: Graphical abstract for the role of Bruton's tyrosine kinase in autoimmune and immune complex-mediated arthritis.

that *Btk*-deficiency in the K/BxN model significantly inhibits development of spontaneous arthritis, which depends upon both innate and adaptive immunity (Fig 2.1). To understand the mechanisms responsible, I undertook a detailed study of immune cells in this model. K/BxN B cells are extremely sensitive to BTK loss, suffering a 74% reduction in numbers (Figures 2.2 and 2.3), and T cells show mild reductions, despite the fact that they do not express BTK (Figure 2.2). Dendritic cell numbers are also somewhat reduced, while other components of the innate system, particularly macrophages, are not (Fig 2.2). BTK-deficiency strongly inhibits GC development, with large reductions in GC B cells and milder effects on GC Tfh, resulting in loss of anti-GPI autoantibodies that initiate autoimmune arthritis (Fig 2.4). Therefore, BTK is clearly implicated as supporting adaptive immune drivers of autoimmune arthritis, possibly through its role in autoreactive B cell development.

The effect of BTK-deficiency on B cells in this model is profound, much more so than in any previous study using non-transgenic B cells. BTK is a well-defined cytosolic component of the signalosome that propagates signals from the BCR (140, 153, 160, 253-256). *Btk*-deficiency reduces B cell numbers by 50% in C57BL/6 mice and by 18% in NOD mice (137, 140). Therefore, the extreme 74% reduction in overall B cell numbers in *Btk*-deficient K/BxN is striking. Analysis of K/BxN B cell subsets shows increased percentages of transitional cells in *Btk*-deficient mice, indicating a block at both T1 and T2 stages. While the T2 block is classically found in other models, the T1 block is usually far less pronounced (137, 140). Furthermore, unlike *Btk*-deficient C57BL/6 or NOD, B cells fail to

accumulate at blocked stages in *Btk*-deficient K/BxN. This suggests significant cell loss at transitional stages, with further loss at the mature FO stage, resulting in very low FO numbers. This pattern is similar to transgenic models of autoimmunity, including an anti-insulin B cell model we reported to have 95% reduction in the absence of BTK (187, 257). Equally striking is the profound reduction of the MZ compartment, which begins even at the pMZ stage. Though in most models the MZ develops independently of BTK, my lab recently published that NOD MZ B cells rely in part on BTK signals (175). Even in NOD mice, however, loss of BTK causes a block at the pMZ stage, with an increase in numbers, and only partial reduction of MZ B cells (137). Again, this unusual reliance of the MZ compartment in *Btk*-deficient K/BxN mice mirrors anti-insulin B cells (187). The only other endogenous B cells known to rely so heavily on BTK-signaling are autoimmune-prone subsets such as B1a and anergic An1 (187). Further work is needed to determine the mechanism underlying this unusual pattern of B cell reduction in *Btk*-deficient K/BxN mice.

Germinal centers are critical to immune responses. GC B cells are primary responders in infection or autoimmunity, proliferating, undergoing somatic hypermutation and class switch to IgG, and then transforming into antibody-producing plasma cells. BTK is known to contribute to GC formation (9, 258), so reduction of GC B cells in *Btk*-deficient K/BxN is unsurprising. BTK is not present in T cells, and indeed, those that reached the Tfh and GC Tfh stages did not exhibit loss of activation marker or transcription factor expression, as shown by quantification of BCL6, CD44, and ICOS (Figure 2.4). However, GC Tfh and B

cells are reciprocally dependent. T-B interactions at multiple stages are necessary for Tfh development, including cognate interactions at the T-B border and non-cognate interactions that facilitate Tfh motility and follicular migration (249). Therefore we conclude that lack of available GC B cells in *Btk*-deficient mice removed cellular stimuli necessary for proper TfH development. GC failure in turn blocked development of anti-GPI autoantibodies (Figure 2.4), consistent with loss of autoantibodies in other *Btk*-deficient models (8, 137, 259). Anti-GPI antibody is preferentially targeted, as BTK-deficiency resulted in an 83% decrease in anti-GPI IgG, but only 16% in total IgG. This reflects similar findings in models of lupus and T1D, and further supports the conclusion that loss of BTK profoundly affects autoreactive B cells (8, 137, 210).

In contrast to previous reports using pharmacologic inhibition (217-219), BTK-contributions to innate mediators of arthritis are not apparent in this genetically deficient model. While FolRβ imaging shows reduced synovial macrophage infiltration in the spontaneous model (Figure 2.5), this is likely secondary to reduced autoantibodies, since there is no difference in clinical score or FolRβ outcomes when autoantibodies are supplied exogenously in the serum transfer model (Figure 2.6). The most obvious way to interpret this difference in outcomes is to attribute the efficacy of BTK-inhibitors to off-target effects. Most recent studies regarding the role of BTK in autoimmune arthritis have focused on its role in FcγR stimulated phagocytosis and cytokine production by macrophages (138, 215-219, 260) and have relied solely on BTK inhibitors, rather than genetic deletion, to make their conclusions. However, kinase-specific

inhibition is difficult. For example, ibrutinib also binds many other kinases, including Tec, Jak3 and, importantly, the T cell signaling protein ITK, with known effects on T cell function (220). LFM-A13, used in well-cited macrophage studies, also interacts with Tec (138, 221). Our studies indicate that BTK-deficiency may blunt, but does not eliminate, macrophage inflammatory responses as measured by TNFα production by BMDCs in response to K/BxN serum autoantibodies (Figure 2.6D). The fact that macrophages also respond very dramatically to TLR4 stimulation, regardless of BTK status, further supports the idea that BTK may play only a minor role in macrophage driven inflammation. My findings are the first to use BTK-deficiency, rather than a small molecular inhibitor, to study the role of this protein in innate cell contributions to arthritis, and demonstrate the need for further studies, which will be discussed in Chapter IV of this dissertation.

Overall, macrophage and other innate cell numbers were mostly stable, with the exception of dendritic cells, which were decreased in *Btk*-deficient K/BxNs. This is not the case in *Btk*-deficient non-diabetic NODs (Figure 2.6C), suggesting that changes in dendritic cell numbers are not due to a developmental block. Rather, the ongoing immune reaction in *Btk*-sufficient K/BxN most likely drives expansion of DCs needed to facilitate antigen-presentation in the T cell zone. Alternatively, loss of BTK-signaling from innate receptors in DCs may contribute indirectly to failure of adaptive responses. Interestingly, previous studies using C57BL/6 mice have shown that *Btk*-deficient DCs have reduced IL-10 production, and exhibit increased T cell stimulatory activity (261). Thus, the role of BTK in DC contributions to autoimmune arthritis requires additional

investigation, and would benefit from studies using DC-targeted deletion in the future. Of note, the increase in neutrophil numbers found in *Btk*-deficient NOD recipients of K/BxN serum do not necessarily reflect increased functional contributions to inflammation in that model. *Btk*-deficient neutrophils in *xid* and C57BL/6 mouse models showed decreased E-selectin mediated recruitment (222) and decreased granules per cell (223). Interestingly, this contrasts neutrophils from human XLA patients that have showed no defects in effector function (262), and even had increased production of reactive oxygen species (263). Future studies are necessary to resolve these conflicts in the literature.

This work rigorously defines the contributions of BTK to autoimmune arthritis, using spontaneous and serum-transfer models to separate the function of BTK in B lymphocytes versus innate immune cells. It further supports the role of BTK in the development of autoreactive B cells and reveals that BTK may have both stimulatory and regulatory functions in the innate immune system. The findings support development of BTK-inhibitors for RA but demonstrate the need to more completely understand their effects on the immune system.

This chapter is adapted from previously published work (264).

CHAPTER III

THE ROLE OF BRUTON'S TYROSINE KINASE IN THE SURVIVAL AND FUNCTION OF AUTOREACTIVE B LYMPHOCYTES

Abstract

Bruton's tyrosine kinase (BTK) is a crucial regulator in the development of B cells, where it propagates signals from the B cell receptor (BCR). The innate-like autoreactive-prone B1 compartment, endogenous autoreactive An1s, and transgenic anti-insulin B cells are all dependent upon BTK for their development, and are absent in *Btk*-deficient mice. However, the requirement for BTK at specific stages of development, survival and function has not been defined. A loxP-flanked *Btk* mouse model was developed and paired with tamoxifen-inducible Cre-ER^{T2}, for studies of the role of BTK in the survival and function of mature B lymphocytes. Surprisingly, tamoxifen-induced excision of BTK in mature mice did not eliminate, or even reduce, B1 cell populations, indicating that it is not required for their survival once development has occurred. B1 cells are important for early, T-independent responses to pathogens and for ongoing production of natural IgM. Natural IgM remained present in serum up to five weeks after BTK deletion, indicating that this crucial function of B1 cells is BTK-independent. In contrast, BTK excision rendered mice unable to respond to T-independent type II immunization, mirroring phenotype of *Btk*^{null} mice that lack B1 cells. Additionally, inducible BTK knockdown revealed that transgenic anti-insulin B cells do not rely on BTK for their survival or internalization of antigen. These findings have implications for the use of BTK-inhibitors currently in clinical trials for treatment of autoimmunity.

Introduction

Bruton's tyrosine kinase (BTK) is a tec-family kinase expressed in B lymphocytes and in innate immune cells. BTK plays a role in signaling through the B cell receptor (BCR), as well as through innate receptors such as the Fcγ receptor (FcγR) and various toll like receptors (TLRs) (133-135, 137, 140, 160). The role of BTK has been mostly studied in B lymphocytes, where it is known to support the development of innate-like B1 cells, the anergic autoreactive An1 subset, and transgenic anti-insulin and anti-DNA B cells (140, 187, 265). The An1 and B1 cell subsets are endogenous autoreactive-prone B cells, while transgenic anti-insulin B cells allow the opportunity to study fixed autoreactive B cell specificity. Signaling through the B cell receptor (BCR) is regulated differently in autoreactive-prone B cells as compared to their non-autoreactive, naïve counterparts. B1 and An1 cells do not mobilize calcium or proliferate in response to BCR crosslinking, but have higher basal levels of cytoplasmic free calcium (188, 192, 193). These cells also exhibit constitutive ERK (extracellular signal-related kinase) phosphorylation (194, 195). An1 cells and B1s show an increase in negative mediators of BCR signaling, implying that though positive signals like calcium levels and ERK phosphorylation are increased, these signals are controlled by negative regulation (82, 196). Less is known about BCR signaling in anti-insulin B cells, but their anergic phenotype and dependence on BTK leads to the conclusion that BCR signaling in this model may be dysregulated similarly to An1 B cells and other anergic models (53, 187).

Despite their similarities in BCR signaling, it is clear that An1 and B1 are discrete B cell subsets. An1 cells express CD23, which is the low affinity IgE receptor and a marker for B cell maturity, but also express the immaturity marker CD93 and low levels of surface IgM (43). They are continually generated from the bone marrow, and are short-lived (82, 83). In contrast, B1 cells are initially generated in fetal liver and found primarily in peritoneal and pleural cavities (266). They exhibit slow turnover, are self-renewing, and produce polyreactive natural IgM that is germline-configured to recognize bacterial antigens but can also cross-react with autoantigens (94). B1 cells quickly respond to antigen and are therefore well-suited for early, T-independent responses to infection (105, 112). B1 cells are absent in BTK deficient mice, which also lack natural IgM (267) and are unable to respond to T-independent immunization due to the loss of this B cell population (268-270). Humans are reported to have B cell subsets that are similar to both An1 and B1 cells. An1s in humans are reported to be increased in autoimmune disease (41, 84). Polyreactive IgM-producing B cells similar to B1 cells are found in umbilical cord and adult peripheral blood and characterized by expression of CD20, CD27, and CD43 (126-128). As BTK inhibition becomes a more common treatment for B cell lymphomas and a proposed target in autoimmune disease, more information is needed on the impact of BTK loss on the survival and function of mature B cell subsets.

The reliance of autoreactive-prone subsets such as An1, B1 and anti-insulin B cells upon BTK for development has been well studied, but the lack of an inducible knockout model has rendered BTK's role in their survival and

function unclear. One study used an inhibitor to study BTK's role in mature B cell subsets (271); however, this inhibitor also inhibits TEC and BMX and has a half-life of only five hours. Discrete study of the specific role of BTK requires a genetic knock-down model. Our lab has developed the first *Btk^{flox}* model, which we have paired with a tamoxifen-inducible Cre (CreER^{T2}), achieving 95% knockdown of the protein. This model shows that BTK is not required for the survival of the B1 subset or the production of natural IgM, but is required for their response to a T-independent polysaccharide antigen. Additionally, the loss of BTK from mature anti-insulin B cells does not result in B cell loss and does not affect the ability to internalize antigen. In contrast, An1 B cells are swiftly lost after BTK knockdown, though this effect may be due to their short half-life. These data demonstrate that BTK has differing roles in autoreactive B cell development, survival, and function, and indicates that B1 and anti-insulin B cells undergo a positive selection step in development for which BTK is required.

Materials and Methods

Mice and Cre-ER^{T2} induction.

Btk^{flox} mice were developed in the lab of Wasif Khan (University of Miami, Department of Microbiology and Immunology). *Cre-ER^{T2}* mice were purchased from the Jackson Laboratory (B6.Cg-Tg(UBC-cre/ERT2)1Ejb/1J). *Btk^{null}* B6 mice were generated as previously described (140). BCR transgenic mice express anti-insulin VDJ$_H$-125, targeted to J$_H$ loci, as described in (234), and a non-

targeted Vκ125 described in (53). Mice were bred and maintained under specific pathogen free conditions. To induce Cre activation, mice were injected interperitoneally (I.P.) on days -2, -1, and 0 with 3mg of tamoxifen-free base (Sigma) in 200μL of safflower oil, or vehicle alone. All studies have been approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Flow cytometry and antibodies.

Single-cell suspensions of spleen, bone marrow, and peritoneal cavity were obtained as previously described (137) and stained using fluorochrome or biotin-conjugated antibodies against B220 (RA3-6B2), IgM (μ-chain, Life Technology), IgMb (AF6-78), IgD (11-26c.2a), CD5 (53-7.3), CD11b (M1/70), CD11c (HL3), F4/80 (BM8, eBioscience), Ly6G (IA8), CD19 (ID3), CD21 (7G6), CD23 (B3B4), CD93 (AA4.1), CD86 (GL1), CD44 (IM7), CD9 (KMC8), CD43 (S7) and/or CD138 (281-2). Unless otherwise stated, antibodies were procured from BD Biosciences. Biotin-conjugated antibodies were secondarily stained with streptavidin-conjugated fluorochromes and dead cells were excluded using fixable viability dye 455UV or eFluor 450 (eBioscience) or Alexa Fluor 700-conjugated succinimidyl ester (Life Technologies). For intracellular staining, cells were fixed using 1.6% paraformaldehyde (Electron Microscopy Sciences), then permeabilized with a solution of 0.05% Triton-X-100 (SigmaUltra) and stained with rabbit anti-mouse BTK (D3H5, Cell Signaling), followed by a fluorochrome-conjugated anti-Rabbit IgG (F'ab2) secondary (Cell Signaling). Samples were

collected on an LSRII flow cytometer (BD Biosciences) and data analyzed using FlowJo software (TreeStar).

B cell proliferation.

Splenocytes were stained with CFSE (Life Technologies) or CellTrace Violet (Life Technologies) according to manufacturer's instructions, and then cultured at $1x10^6$ cells/mL for three days in cRPMI alone, stimulated with 5μg/mL goat anti-mouse IgM (μ-chain specific, Jackson Immunoresearch) or stimulated with 1μg/mL lipopolysaccharide (LPS, Dibco). Following incubation, cells were harvested and analyzed by flow cytometry.

Immunization studies.

Five days after tamoxifen injections, mice were immunized I.P. with 50μg of TNP_{37}-Ficoll (Biosearch Technologies) diluted in 200μL sterile PBS or mock-immunized with PBS alone. Blood for serum Ab analysis was collected one day pre- and five days post-immunization. For TNP-Ficoll specific B cell analysis, cells were isolated from spleen or peritoneal lavage five days post-immunization and incubated with 20μg/mL of TNP_{65}-Ficoll-Fluorescein in PBS containing 2% fetal calf serum, then subsequently stained for analysis by flow cytometry.

ELISAs.

Serum anti-phosphoryl-choline (PC) IgM, anti-TNP-Ficoll IgM, and anti-TNP-Ficoll IgG were measured. 96-well flat-bottom NUNC plates were coated with

1µg/mL of PC-BSA (Biosearch Technologies) or TNP_{37}-Ficoll (Biosearch Technologies) in borate-buffered saline or carbonate buffer overnight at 4ºC. Plates were blocked with 1% BSA in PBS+0.05% Tween-20 (PBST). For anti-PC IgM ELISA, samples were serially diluted starting at 1:10. For anti-TNP IgM and IgG, samples were diluted at 1:5000. IgM and IgG antibodies were detected using goat anti-mouse IgM or IgG conjugated to alkaline phosphatase (AP) (Southern Biotech). p-Nitorphenyl phosphate (PNPP) was added and the plate read on a Microplate Autoreader (Bio-Tek Instruments) at O.D. 405nm. Plates were washed in between steps using PBST.

BCR-internalization assay.

Antigen internalization assay was performed as previously described (51). Briefly, freshly isolated splenocytes were incubated on ice for 30min with biotinylated insulin, and then washed to remove excess. Then, cells were incubated in complete RPMI (Gibco) for 0-30 minutes, at which point the reaction was stopped with cold buffer containing 0.1% azide. Cells were then stained with streptavidin-fluorochrome and appropriate antibodies, and the relative surface level of biotinylated insulin quantified by the division of mean fluorescent intensity (MFI) at each time point by the MFI at t=0.

Statistical Analysis.

Statistics were performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA). P-values were calculated using

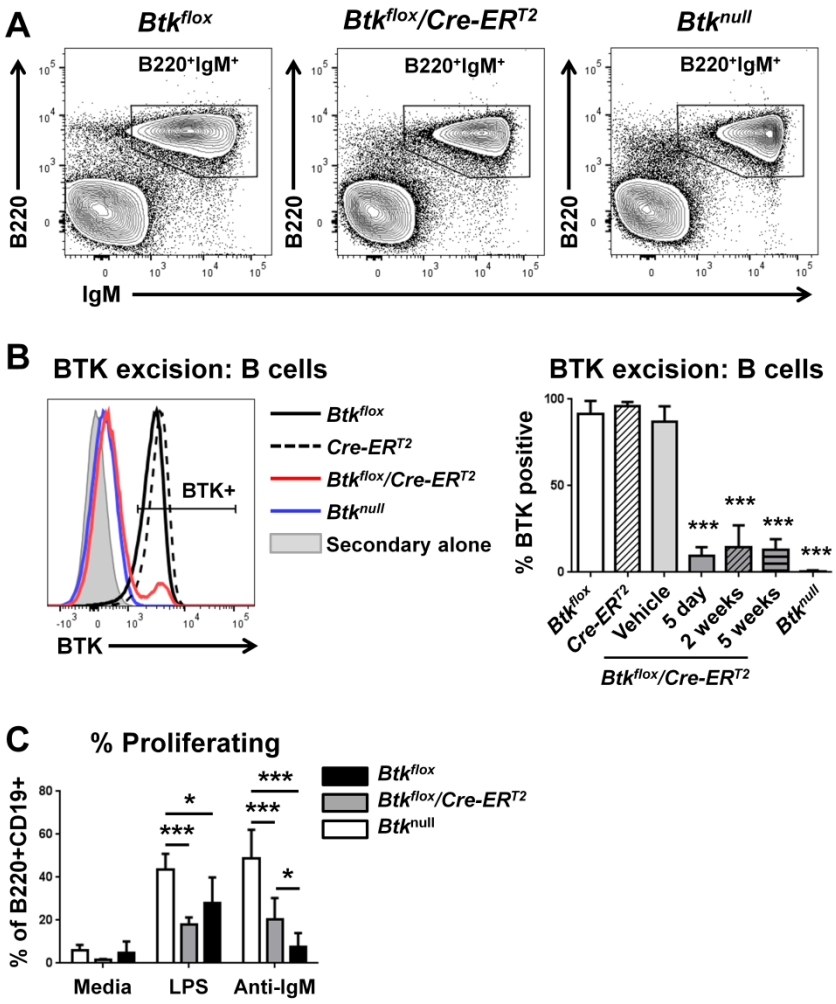


Figure 3.1: Inducible knockdown of BTK in *Btk^{flox}/Cre-ER^{T2}* is stably achieved in splenic B cells. (A) Representative flow plots for *Btk^{flox}* (left), *Btk^{flox}/Cre-ER^{T2}* (middle) and *Btk*^{null} (right) showing splenic B cells five days after tamoxifen injections. Splenic B cells are designated as B220^{+}IgM^{+}. Cells are pre-gated as single live lymphocytes. (B, left) Representative histogram of B cell BTK expression five days after treatment. Shown are a representative *Btk^{flox}* (black, solid), *Cre-ER^{T2}* (black, dashed), *Btk^{flox}/Cre-ER^{T2}* (red), *Btk*^{null} (blue), and isotype control (gray). (B, right) Percent Btk positive splenic B cells. Shown in white are *Btk^{flox}* (solid, n=24), *Cre-ER^{T2}* (diagonal pattern, n=13), in light gray vehicle control (n=5), in gray *Btk^{flox}/Cre-ER^{T2}* after five days (solid, n=13), two weeks (diagonal pattern, n=5), or five weeks (horizontal pattern, n=6) and in black, *Btk*^{null} (n=15). (C) Unstimulated and response to 1μg/mL LPS (left) or 5μg/mL of anti-IgM are shown for live CD19+B220+ B cells of *Btk^{flox}* (white, n=4), *Btk^{flox}/Cre-ER^{T2}* (gray, n=6), and *Btk^{null}* (black, n=4), quantified by percent proliferating of CD19+B220+. *p<0.05, ***p<0.001 as calculated by one-way or two-way ANOVA compared to *Btk^{flox}*.

one-way or two-way ANOVAs, or Kruskal-Wallice with Dunn's multiple comparison test, as appropriate.

Results

Cre activation in mature Btk^{flox}/Cre-ER^{T2} mice depletes BTK at all stages of B cell development.

To determine the role of BTK in mature cells, I employed a novel LoxP-flanked *Btk* (*Btk^{flox}*) model in tandem with a tamoxifen-inducible Cre (*Cre-ER^{T2}*). Induction of the *Cre-ER^{T2}* by administration of tamoxifen in *Btk^{flox}/Cre-ER^{T2}* mice resulted in successful knockdown of BTK within five days (90.39%±4.9% splenic B cells were BTK-deficient, $p<0.001$, Figure 3.1B). This knockdown was stable, as B cells from tamoxifen-treated *Btk^{flox}/Cre-ER^{T2}* mice remained BTK-deficient five weeks later (86.82%±5.96% BTK-negative B cells, $p<0.001$, Figure 3.1B). Analysis of bone marrow showed successful protein deletion begins at the earliest stages of B cell development (Figure 3.2A, 3.2B). BTK was successfully knocked down in pre- and pro- (91.03%±12.68%, $p<0.001$) and immature B cells (88.79%±12.38%, $p<0.001$), as well as in mature recirculating B cells (89.20%±4.66%, $p<0.001$), and all bone marrow B cell subsets remained largely BTK-negative even up to five weeks after injection (Figure 3.2B). As expected, treated *Btk^{flox}/Cre-ER^{T2}* mice also exhibited stable knockdown in macrophages and conventional dendritic cells in the spleen (Figure 3.3). These data demonstrate the efficacy and the stability of the inducible BTK knockdown. Of

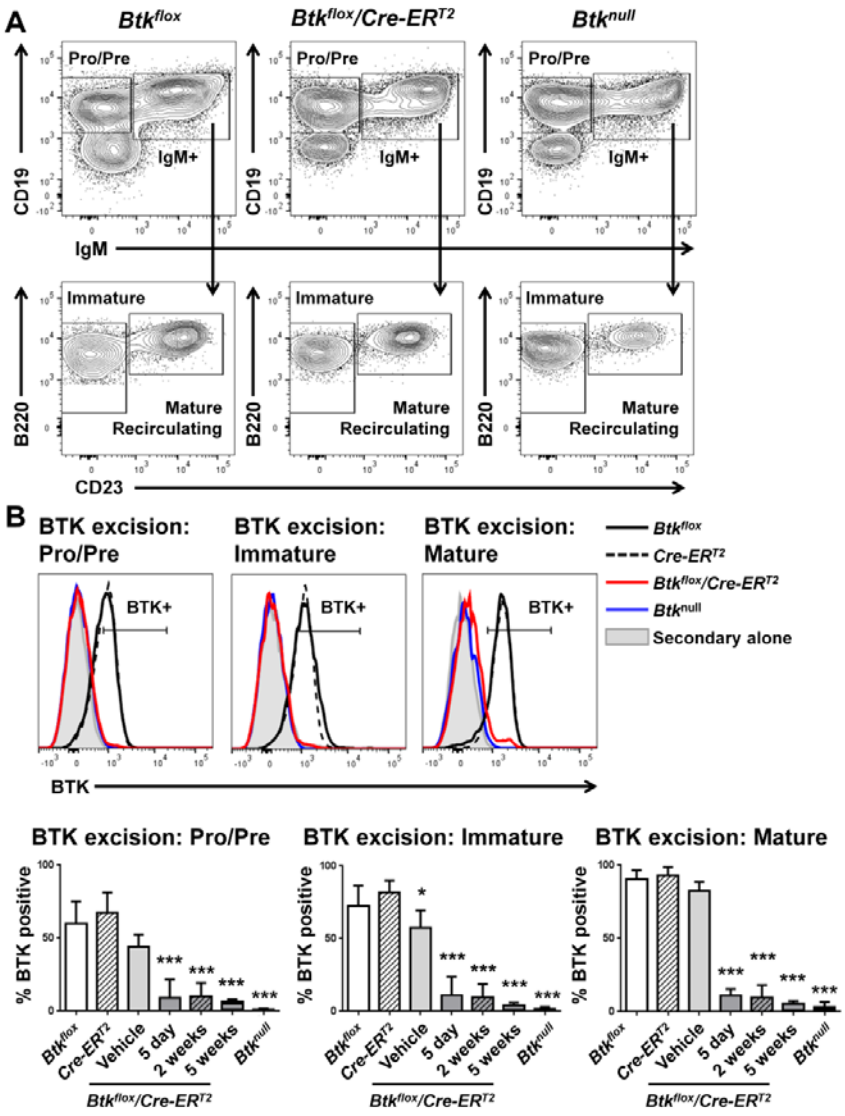


Figure 3.2: Inducible knockdown of BTK in *Btk^{flox}/Cre-ER^{T2}* is stably achieved in bone marrow B cells. (A) Representative flow plots for *Btk^{flox}* (left), *Btk^{flox}/Cre-ER^{T2}* (middle) and *Btk*^{null} (right) showing bone marrow B cells, five days after tamoxifen injections. Pro/pre-B, immature, and mature recirculating bone marrow B cells are gated by expression of CD19, IgM, and CD23. Cells are pre-gated as single live lymphocytes. (B, top) Representative histograms of B cell BTK expression five days after treatment. Shown are a representative *Btk^{flox}* (black, solid), *Cre-ER^{T2}* (black, dashed), *Btk^{flox}/Cre-ER^{T2}* (red), *Btk*^{null} (blue), and isotype control (gray). (B, bottom) Percent BTK positive pro/pre B cells (B, left), immature B cells (B, middle) or mature recirculating B cells (B, right). Shown in white are *Btk^{flox}* (solid, n=16), *Cre-ER^{T2}* (diagonal pattern, n=9), in light gray vehicle control (n=4), in gray *Btk^{flox}/Cre-ER^{T2}* after five days (solid, n=8), two weeks (diagonal pattern, n=5), or five weeks (horizontal pattern, n=3) and in black, *Btk*^{null} (n=11). ***p<0.001 as calculated by one-way or two-way ANOVA compared to *Btk^{flox}*.

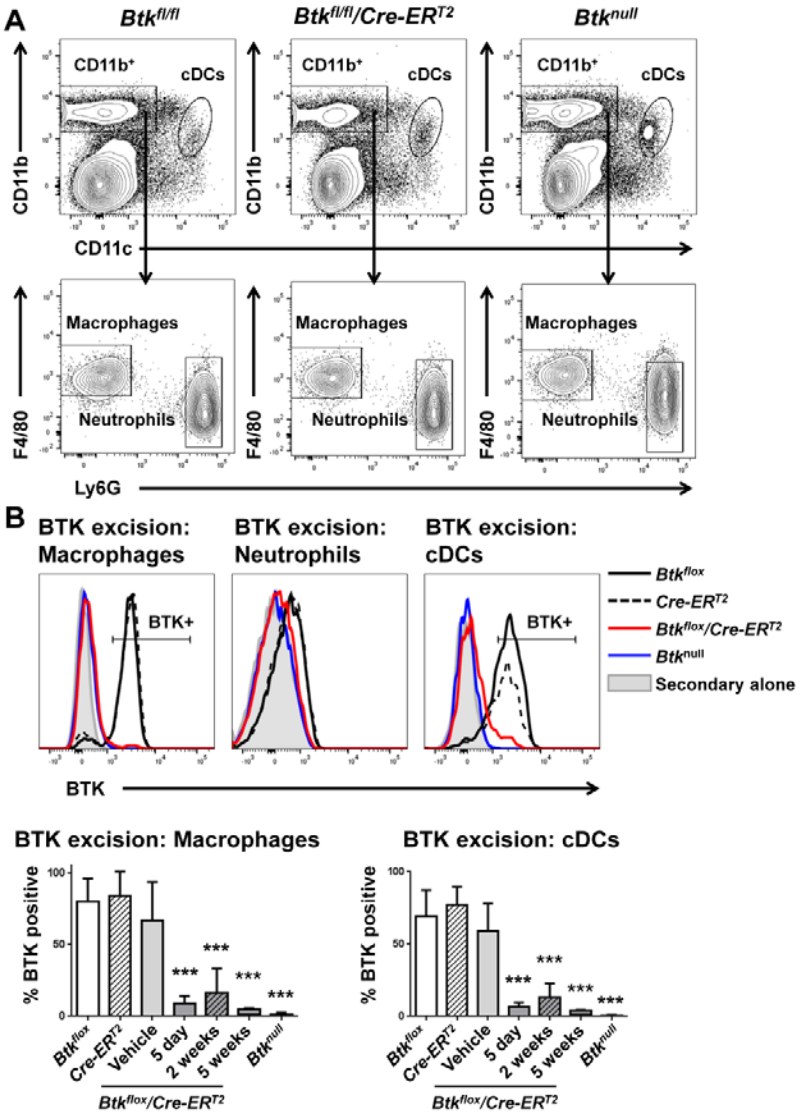


Figure 3.3: Inducible knockdown of BTK in *Btk^{flox}/Cre-ER^{T2}* is stably achieved in splenic innate cells. (A) Representative flow plots for *Btk^{flox}* (left), *Btk^{flox}/Cre-ER^{T2}* (middle) and *Btk*^{null} (right) showing splenic macrophages (CD11b+CD11c-F4/80+) , neutrophils (CD11b+CD11c-Ly6G+), and conventional dendritic cells (cDCs) (CD11b+CD11c+), five days after tamoxifen injections. Cells are pre-gated as single live cells. (B, top) Representative histograms of macrophage (left), neutrophil (middle) and cDC (right) BTK expression five days after treatment. Shown are a representative *Btk^{flox}* (black, solid), *Cre-ER^{T2}* (black, dashed), *Btk^{flox}/Cre-ER^{T2}* (red), *Btk*^{null} (blue), and isotype control (gray). (B, bottom) Percent BTK positive macrophages (left) and cDCs (right). Shown in white are *Btk^{flox}* (solid, n=18), and *Cre-ER^{T2}* (diagonal pattern, n=8), in light gray *Btk^{flox}/Cre-ER^{T2}* vehicle controls (horizontal pattern, n=3), in gray *Btk^{flox}/Cre-ER^{T2}* after five days (solid, n=12), two weeks (diagonal pattern, n=5), or five weeks (horizontal pattern, n=3) and in black, *Btk*^{null} (n=13). ***p<0.001 as calculated by one-way ANOVA compared to *Btk^{flox}.*

note, one out of four vehicle treated female control mice did exhibit a BTK-negative B cell population in the bone marrow, resulting in the appearance of a slight, but significant, loss of BTK in immature B cells (42.75%±12.36% BTK negative) (p=0.049). This mouse also exhibited a slight loss of BTK in pro- and pre- B cells, but the trend was less evident in the spleen. This confirms previous findings of others that endogenous estrogen can induce some degree of nonspecific activation in the CreER^{T2} system.

Proliferation is decreased in splenic B cells after BTK knockdown.

To confirm that loss of BTK results in a defective B cell response to stimuli, I harvested spleens from *Btk^{flox}, Btk^{flox}/Cre-ER^{T2},* and *Btk^{null}* five days after tamoxifen injection and stimulated B cells with anti-IgM or LPS (Figure 3.1C). *Btk^{flox}/Cre-ER^{T2}* B cells showed blunted proliferation after BTK deletion compared with *Btk^{flox}* control B cells in response to LPS (17.95±3.39% proliferation vs. 43.68%±7.28% proliferation, p<0.001) or anti-IgM (20.49%±9.82% proliferation vs. 48.78%±13.34% proliferation, p<0.001). In fact, BTK knockdown was functionally equivalent to *Btk*-deficiency in *Btk^{null}* B cells in response to LPS (27.95%±11.98% proliferation, p=0.14), though still slightly increased as compared to *Btk^{null}* B cells in response to anti-IgM (7.77%±6.34% proliferation, p=0.04). These data confirm that BTK deletion after cellular maturation results in a functional defect in proliferation response to LPS and anti-IgM that is similar to lifelong *Btk*-deficiency.

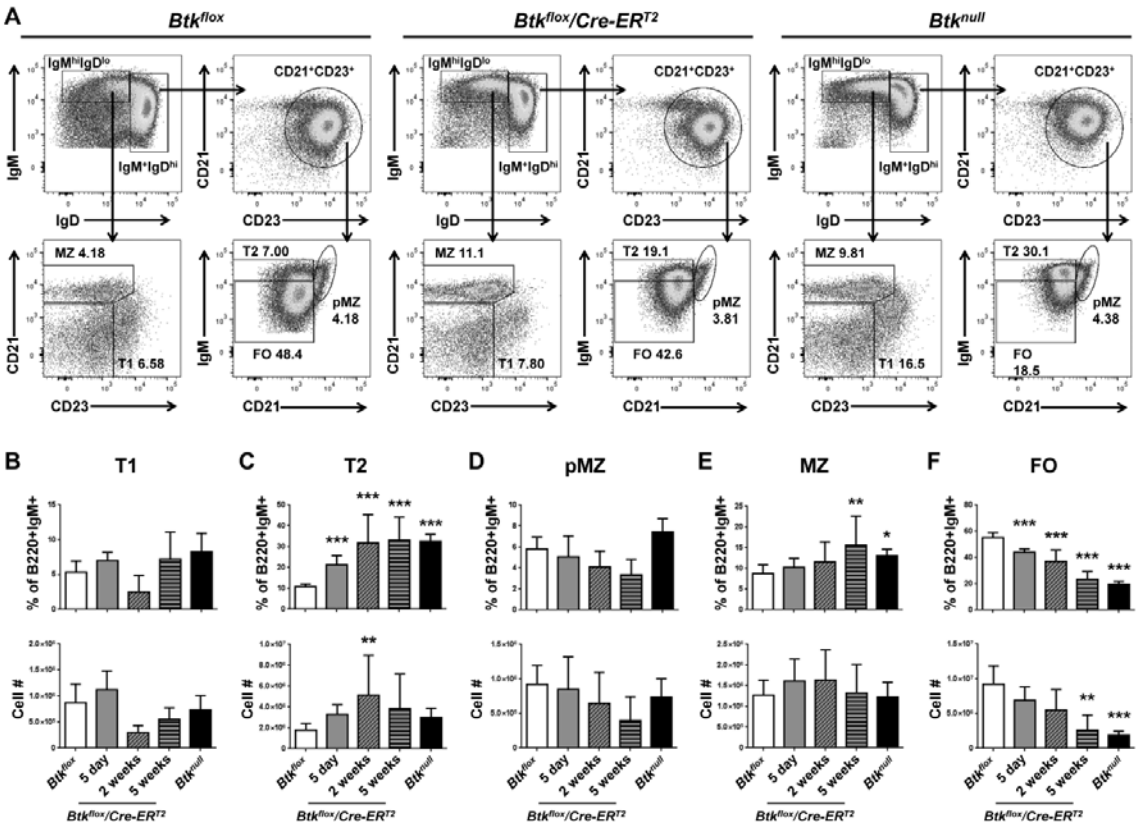


Figure 3.4: Induced BTK knockdown results in transitional 2 developmental block. (A) Representative flow plots for *Btk*^{flox} (left), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (right) splenic B cells, pre-gated as B220^{+}IgM^{+} single live lymphocytes, five days after tamoxifen injection. Transitional 1 (T1), transitional 2 (T2), pre-marginal zone (pMZ), marginal zone (MZ) and follicular (FO) B cell subsets are determined by expression of IgM, IgD, CD21 and CD23. (B-F) B cell subsets are quantified by percent of B220^{+}IgM^{+} (top) and total cell number (bottom) for *Btk*^{flox} (white, n=17)*, Btk*^{flox}*/Cre-ER*^{T2} 5 days (gray, n=8), 2 weeks (diagonal pattern, n=5), or 5 weeks (horizontal pattern, n=6) post tamoxifen injection, or *Btk*^{null} (black, n=12). *p<0.05, **p<0.01, ***p<0.001 as calculated by one-way ANOVA compared to *Btk*^{flox}*.*

BTK knockdown results in immediate developmental block at the late transitional (T2) stage, but requires weeks to reduce the follicular compartment.

It is well established that the conventional *Btk*^{null} genetic mouse models have an increased percentage of B cells at the transitional 2 (T2) stage of development, with a concurrent loss of follicular (FO) B cells (132, 140). However, it is possible that deletion of BTK from fully mature B cells would lead to a different outcome. Therefore B cell subsets were assessed five days, two weeks, and five weeks after BTK knockdown. Transitional 1 (T1), T2, pre-marginal zone (pMZ), marginal zone (MZ) and FO B cells were determined by expression of IgM, IgD, CD21, and CD23, as shown in Figure 3.4A. The most immediate effect of BTK knockdown was increased surface IgM expression in *Btk*^{flox}*/Cre-ER*^{T2} five days post injection, leading to a significant increase in the percentage of T2 B cells (21.35%±5.25%) as compared to *Btk*^{flox} controls (10.71%±2.78%) ($p=0.001$). This was accompanied by a reciprocal trend toward decreased FO B cell proportions, but did not reduce their numbers. The T2 developmental block with concurrent loss of follicular B cells continued to emerge over the next five weeks, finally resulting in significantly decreased FO B cell numbers (2.64e6±2.07e6) compared to *Btk*^{flox} controls (9.20e6±5.13e6) ($p<0.001$). As in the *Btk*^{null} B6 mouse, there were no changes in cell numbers of T1 (Figure 3.4B), pMZ (Figure 3.4D) or MZ B cells (Figure 3.4E). Interestingly, this model shows no immediate loss of FO B cells at the time of BTK knockdown. Rather, follicular B cell similarity to *Btk*^{null} models emerges five weeks later, after B cell turnover has occurred. This shows for the first time that the phenotype

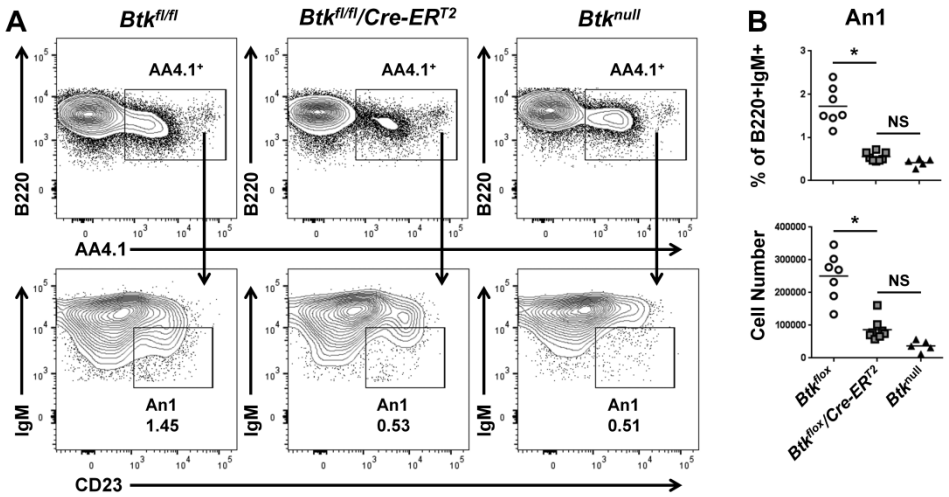


Figure 3.5: The autoreactive, anergic An1 B cell subset is depleted in *Btk^{flox}/Cre-ER^{T2}* mice five days after tamoxifen treatment. (A) Representative flow plots for *Btk^{flox}* (left), *Btk^{flox}/Cre-ER^{T2}* (middle) and *Btk^{null}* (right) spleen B cells, pre-gated as B220^{+}IgM^{+} single live lymphocytes, five days after tamoxifen treatment. Anergic An1 B cells are determined by expression of AA4.1, IgM, and CD23. (B) An1 B cells are quantified by % of B220^{+}IgM^{+} (top) and total cell number (bottom) for *Btk^{flox}* (circles, n=7), *Btk^{flox}/Cre-ER^{T2}* 5 days after tamoxifen injection (squares, n=8), or *Btk^{null}* (triangles, n=5). *p<0.05 as calculated by Kruskal-Wallace with Dunn's multiple comparison test.

seen in BTK-deficient B cells is due to developmental factors, and supports the idea that murine B cells require BTK to mature through the transitional stages, but is not required for survival of mature FO B cells.

An1 B cells are depleted following BTK knockdown.

An1 B cells are an anergic, autoreactive B cell subset (82) that my lab and others have found to be strikingly reduced in *Btk*-deficient mice (43, 187). Btk^{flox}, Btk^{flox}/*Cre-ER*^{T2}, and Btk^{null} An1 B cells were assessed in the spleen five days after tamoxifen treatment, by expression of B220, AA4.1, IgM, and CD23 (Figure 3.5A). As shown in Figure 3.5B, An1 B cells in Btk^{flox}/*Cre-ER*^{T2} animals were significantly decreased in both percentage (0.56%±0.10%) and number (8.67e4 ±3.29e4) as compared to Btk^{flox} controls' percentage (1.72%±0.437%)(p=0.012) and number (2.5e5±7.2e4)(p=0.036). An1 B cells are known to have a short life cycle (43). Therefore, it is unclear if this loss of cell numbers is due to a block in development or a reliance on BTK for An1 B cell survival. Regardless, these data show that An1 B cells are rapidly depleted following BTK knockdown.

B1 cells in the peritoneal cavity do not require BTK for survival.

The innate-like, autoreactive-prone B1 cell subset is known to be important for the production of natural IgM (94, 95) and response to polysaccharide antigens (105, 112, 113) and is absent in Btk^{null} models (267). To determine if BTK is required for development or survival of the B1 cell subset, I induced BTK knockdown and assessed B1a and B1b cells in the peritoneal

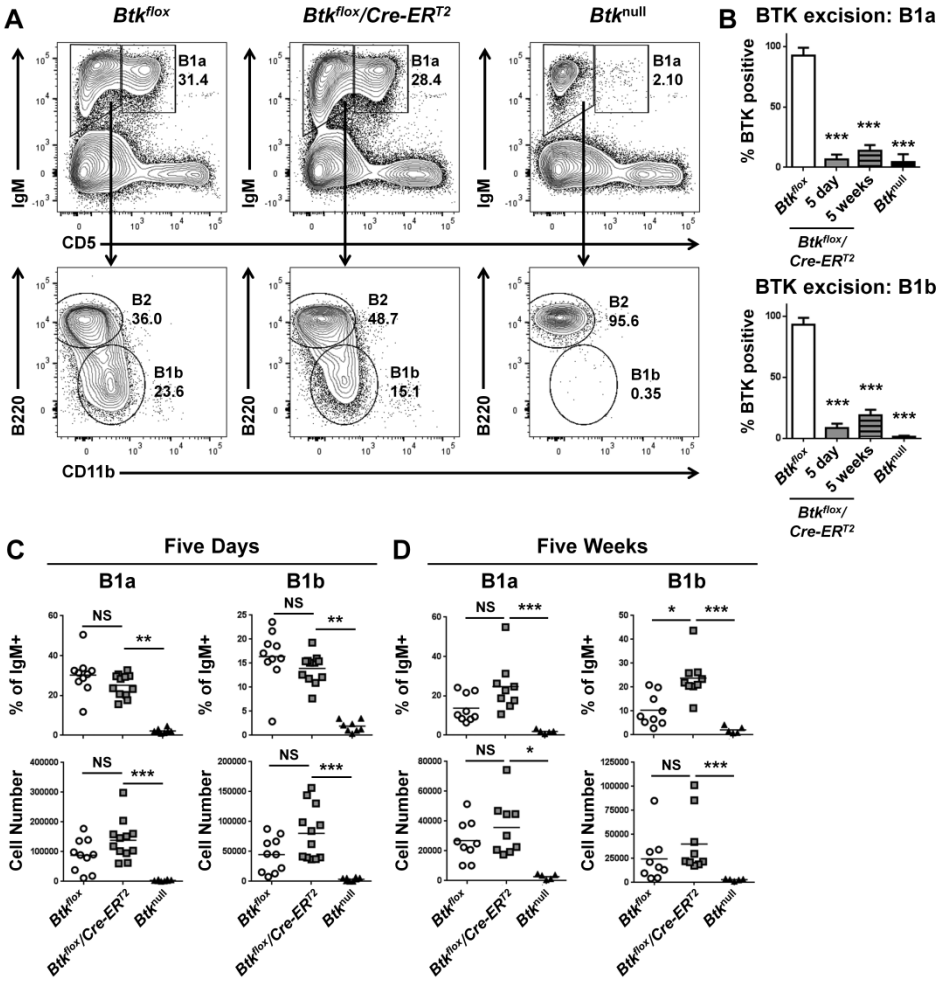


Figure 3.6: The survival of B1a and B1b cells does not depend upon BTK. (A) Representative flow plots for *Btk*^{flox} (left), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (right) peritoneal cells, pre-gated as Ly6G^{-}, single live lymphocytes. B1a, B1b, and B2 cells are determined by expression of IgM, CD5, B220, and CD11b. (B) Btk knockdown is reported as % Btk positive for B1a (top) and B1b (bottom) cells of genotypes *Btk*^{flox} (white), *Btk*^{flox}*/Cre-ER*^{T2} 5 days (gray) or 5 weeks (horizontal pattern) after tamoxifen injection, or *Btk*^{null} (black). (C-D) B1a (left) and B1b (right) cells of *Btk*^{flox} (circles, n=9-10), *Btk*^{flox}*/Cre-ER*^{T2} (squares, n=9-12) and *Btk*^{null} (triangles, n=5-8), five days (C) or five weeks (D) post injection are quantified by % of IgM^{+} (top) and total cell number (bottom). *p<0.05, **p<0.01, ***p<0.001 as calculated by Kruskal-Wallace with Dunn's multiple comparison test.

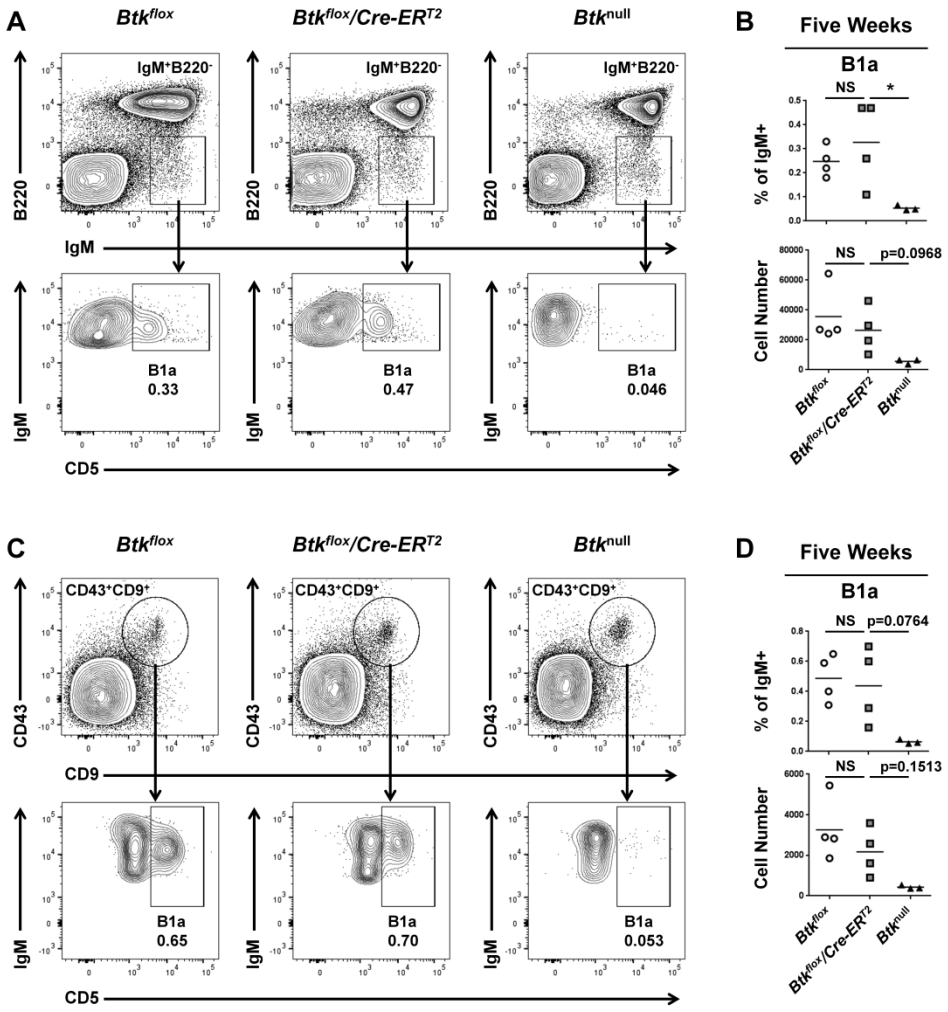


Figure 3.7: B1a cells are present in the spleen and bone marrow five weeks after BTK knockdown. (A, C) Representative gating strategy for *Btk*^{flox} (left), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (right) splenic (A) or bone marrow (C) B1a cells. Cells are gated as single live lymphocytes. Splenic B1a (A) are identified by expression of IgM and CD5, and by low B220 expression. Bone marrow B1a (C) are pre-gated as IgM^{+}CD19^{+}, then further identified by expression of CD43, CD9, and CD5. (B, D) B1a cells of *Btk*^{flox} (circles, n=4), *Btk*^{flox}*/Cre-ER*^{T2} (squares, n=4) and *Btk*^{null} (triangles, n=3) are quantified by percent of IgM+ (top) or total cell number (bottom) in the spleen (B) and bone marrow (D) five weeks after tamoxifen injection. p values are calculated by Kruskal-Wallace with Dunn's multiple comparison test.

lavage by expression of IgM, CD5, B220, and CD11b (Figure 3.6A). Knockdown was successful and stable up to five weeks after injections in both subsets (Figure 3.6B). Five days after tamoxifen treatment, B1a cell percentages were not significantly changed, forming 25.13%±5.68% of total IgM^+ cells in treated *Btk^{flox}/Cre-ER^{T2}*, and 30.27%±9.58% in *Btk^{flox}* controls (p=0.38). B1b cell percentages also remained unchanged, forming 13.91%±3.03% of IgM^+ cells in *Btk^{flox}/Cre-ER^{T2}*, and 16.38%±5.61% in *Btk^{flox}* controls (p=0.33) (Figure 3.6C). To determine if this effect persisted over time, I assessed B1a and B1b cells in the peritoneal lavage five weeks after injection (Figure 3.6D). Even at this later timepoint, *Btk^{flox}/Cre-ER^{T2}* animals retained similar B1a cell numbers (3.57e4±1.87e4) in comparison to *Btk^{flox}* controls (2.69e4±1.35e4) (p>0.999). B1b numbers were also maintained, as *Btk^{flox}/Cre-ER^{T2}* lavages contained 4.01e4±3.14e4 B1b cells and *Btk^{flox}* control lavages contained 2.45e4±2.51e4 (p>0.397). Though numbers of B1a and B1b cells were decreased at five weeks compared to five days after injection, this decrease was irrespective of genotype and most likely can be traced to the effects of the injection itself. These data show that B1a and B1b cells do not depend on the presence of BTK for survival, despite its crucial developmental contributions.

B1 cells persist in the spleen and bone marrow after long-term BTK knockdown.

Though B1s are the major B cell subset present in the peritoneal cavity, it is B1as in the spleen and bone marrow that are theorized to produce the majority of natural IgM (95, 96). Therefore, I assessed splenic and bone marrow B1as five

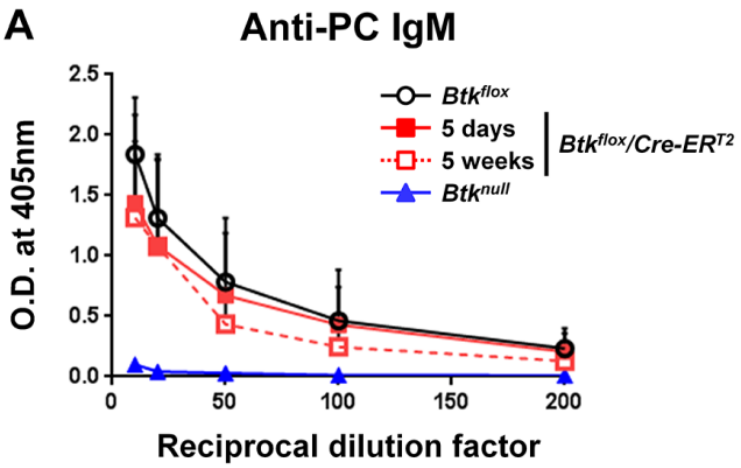


Figure 3.8: Production of natural IgM is independent of BTK. (A) Anti-phosphocholine (PC) IgM is measured by ELISA for *Btk*^{flox} (black, n-7), *Btk*^{flox}*/Cre-ER*^{T2} five days after treatment (red, solid line, n=4), *Btk*^{flox}*/Cre-ER*^{T2} five weeks after treatment (red, dashed line, n=3) and *Btk*^{null} (blue, n=4). Statistics were performed using a 2-way ANOVA and listed in Table 3.1.

		Btk^{flox}	*Btk*^{flox}/*Cre-ER*^{T2} 5 day	*Btk*^{flox}/*Cre-ER*^{T2} 5 week	*Btk*^{null}
1:10	*Btk*^{flox}	X	NS	NS	<0.001
	Btk^{flox}/*Cre-ER*^{T2} 5 day	X	X	NS	<0.001
	Btk^{flox}/*Cre-ER*^{T2} 5 week	X	X	X	0.0012
1:20	*Btk*^{flox}	X	NS	NS	<0.001
	Btk^{flox}/*Cre-ER*^{T2} 5 day	X	X	NS	0.0031
	Btk^{flox}/*Cre-ER*^{T2} 5 week	X	X	X	0.0073
1:50	*Btk*^{flox}	X	NS	NS	0.0215
	Btk^{flox}/*Cre-ER*^{T2} 5 day	X	X	NS	NS
	Btk^{flox}/*Cre-ER*^{T2} 5 week	X	X	X	NS

Table 3.1: Statistics for Figure 3.8.

weeks after BTK knockdown to determine if these crucial subsets were preserved after BTK loss. B1a cells in the spleen were identified by high expression of IgM, low expression of B220, and as CD5-positive (Figure 3.7A). B1a cells were not significantly reduced in number in *Btk^{flox}/Cre-ER^{T2}* (2.64e4±1.54e4) as compared to *Btk^{flox}* controls (3.56e4±1.92e4) (p>0.999). B1a cells were also maintained in the bone marrow, where they were identified as IgM^{+}CD19^{+}, then by expression of CD43, CD9, and CD5, all reported to be markers of B1a cells in the bone marrow niche (Figure 3.7C) (95). *Btk^{flox}/Cre-ER^{T2}* maintained 2.17e3±1.17e3 B1a cells, as compared to *Btk^{flox}* controls at 3.26e3±1.54e3 (p=0.788). These data further confirm that B1a cells do not require BTK for survival, and indicate that these producers of natural IgM may continue to function.

Anti-phosphoryl-choline antibody production is not decreased by loss of BTK.

B1 cells are thought to be responsible for up to 80% of natural IgM (94). Some of these are germline-encoded to recognize phosphoryl-choline, and are present in serum even in germ-free conditions (272-274). To determine whether B1 cells require BTK to produce natural IgM, ELISA was used to measure anti-phosphoryl-choline (anti-PC) antibodies in serum after BTK knockdown (Figure 3.8). There was no significant difference between anti-PC antibody levels in *Btk^{flox}* serum (1.840±0.469) and *Btk^{flox}/Cre-ER^{T2}* five days (1.436±0.732) or five weeks (1.315±0.631) after injections (p=0.4041, p=0.5257). As expected anti-PC antibody levels in *Btk^{null}* animals were nearly undetectable (0.102±0.038) due to

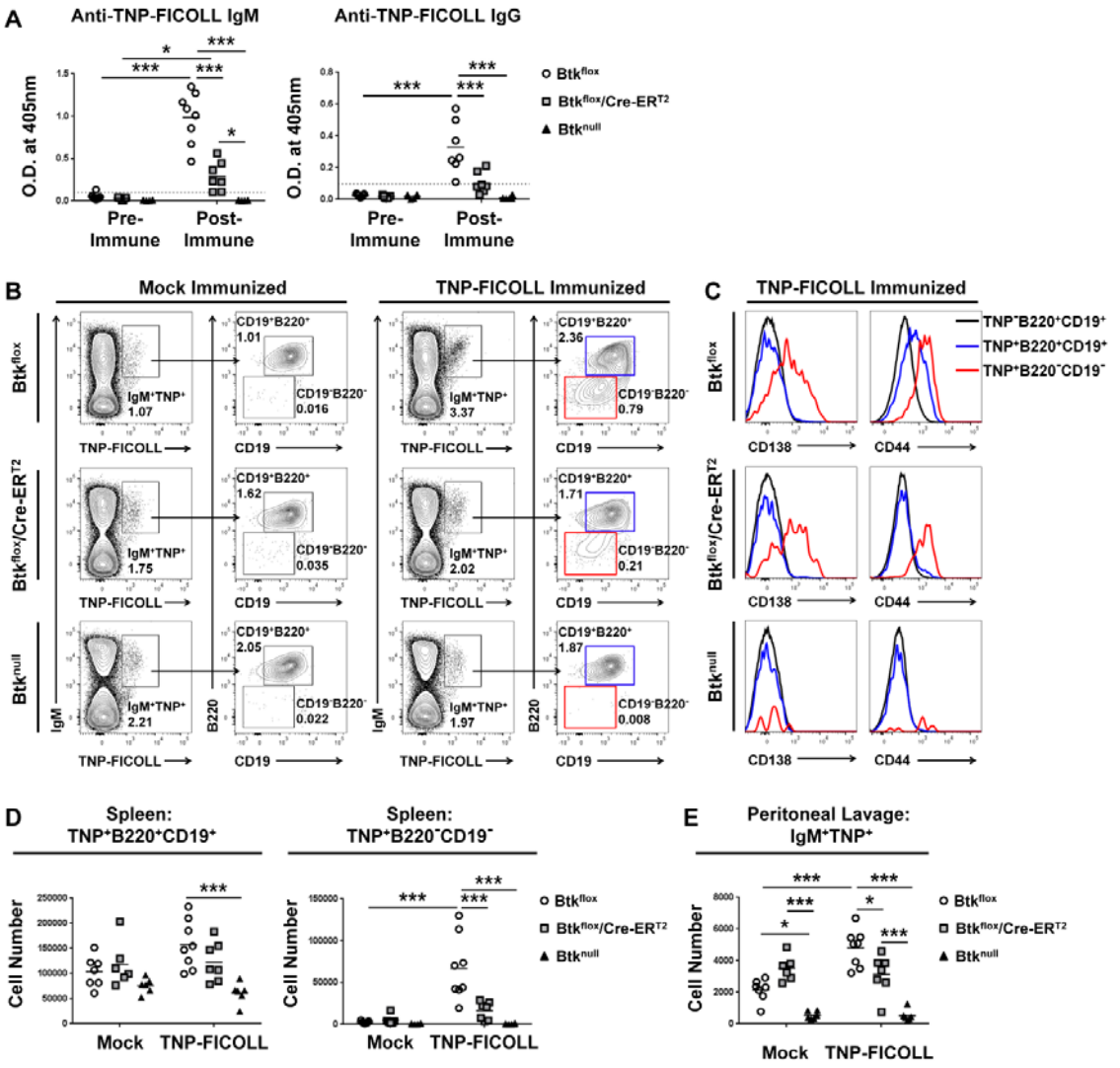


Figure 3.9: The response to T-independent type II immunization is reliant upon BTK. (A) Anti-TNP IgM (left) and IgG (right) is measured pre- and post-immunization by ELISA for *Btk*^{flox} (circles, n=7-8), *Btk*^{flox}*/Cre-ER*^{T2} (squares, n=7) and *Btk*^{null} (triangles, n=6) animals. (B) Representative flow plots of anti-TNP B cells, gated by expression of IgM and binding to TNP-Ficoll-FITC, then by expression of B220 and CD19. Cells are pre-gated as single live lymphocytes. Mock (left) and TNP-Ficoll (right) immunized are shown of *Btk*^{flox} (top), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (bottom). (C) Representative expression of CD138 (left) and CD44 (right) on TNP^{-}B220^{+}CD19^{+} (black), TNP^{+}B220^{+}CD19^{+} (blue), and TNP^{+}B220^{-}CD19^{-} (red) from TNP-ficoll immunized *Btk*^{flox} (top), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (bottom). Cells are pre-gated as IgM^{+}. (D) Total splenic cell numbers of TNP^{+}B220^{+}CD19^{+} (left), and TNP^{+}B220^{-}CD19^{-} (right) from mock or TNP-Ficoll immunized *Btk*^{flox} (circles, n=7-8), *Btk*^{flox}*/Cre-ER*^{T2} (squares, n=6-7) and *Btk*^{null} (triangles, n=6). (E) Total cell numbers of IgM^{+}TNP^{+} cells in the peritoneal lavage of mock and TNP-Ficoll immunized mice. *p<0.05, **p<0.01, ***p<0.001 as calculated by two-way ANOVA.

their lack of B1 cells. As the half-life of IgM in serum is estimated at 2 days (275), the continued level of anti-PC IgM shows that B1 cells continue to produce natural IgM even after BTK loss.

Mice have reduced responses to T-independent type II immunization after BTK deletion.

Btk^{null} mice have long been known to have deficient T-independent type II (TI-II) immunization responses (268-270). In part, this deficiency is due to a lack of B1 cells, known to be critical for TI-II responses (105, 113). In addition, the increased frequency of immature T2 and loss of FO B cells in the spleen could play a role in the response of *Btk*^{null} animals to T-independent immunization. Therefore, I injected *Btk*^{flox}, *Btk*^{flox}*/Cre-ER*^{T2}, and *Btk*^{null} mice five days after BTK knockdown with a mock injection of PBS or a TNP-Ficoll immunization. Figure 3.9A shows anti-TNP IgM (left) and IgG (right) from serum before and after immunization. Though immunized *Btk*^{flox}*/Cre-ER*^{T2} mice did exhibit a significant IgM response to TNP-Ficoll immunization, with an O.D. of 0.292±0.172 after immunization compared to an O.D. of 0.028±0.014 before immunization ($p=0.037$), this post-immunization response was significantly decreased compared to the response of *Btk*^{flox} control mice, which reached an anti-TNP-Ficoll IgM O.D. of 0.986±0.304 ($p<0.001$). In addition, the *Btk*^{flox}*/Cre-ER*^{T2} mice did not produce anti-TNP-ficoll IgG (0.104±0.066) after immunization, as compared to pre-immune control sera (0.017±0.008) ($p=0.4134$). B1 cells are known to be important contributors in the response to TNP-Ficoll immunization

(113). After BTK knockdown, *Btk*^{flox}*/Cre-ER*^{T2} mice have significantly reduced ability to produce anti-TNP-Ficoll IgM as compared to *Btk*-sufficient controls, and cannot produce significant anti-TNP-Ficoll IgG, data which points to a loss of function in B1 cells after BTK loss.

To further characterize the TI-II immunization response, I used FITC conjugated TNP-Ficoll to track antigen specific B cells in the spleen and peritoneal lavage. Figure 3.9B shows representative *Btk*^{flox} (top), *Btk*^{flox}*/Cre-ER*^{T2} (middle), and *Btk*^{null} (bottom) splenic anti-TNP-Ficoll B cells in mock-immunized (left) or TNP-Ficoll immunized (right) animals. In immunized *Btk*^{flox} controls, I observed two TNP-Ficoll-specific IgM+ populations, one of which was CD19+B220+ and the other CD19-B220-. The IgM+TNP-Ficoll+CD19-B220- also exhibited higher levels of CD138 and CD44 (Figure 3.9C), leading to the conclusion that this population is most likely expanding plasmablasts. These TNP-Ficoll-specific plasmablasts were significantly increased in number in the spleens of immunized *Btk*^{flox} mice (6.71e4±3.84e4) compared to mock-immunized controls (2.61e3±1.38e3) ($p<0.001$). This contrasts *Btk*^{flox}*/Cre-ER*^{T2} mice after BTK deletion, in which the number of TNP-Ficoll-specific plasmablasts was not significantly increased in TNP-Ficoll immunized mice (1.64e4±1.08e4) compared to mock-immunized controls (5.33e3±5.67e3) ($p=0.9925$). Furthermore, these numbers were significantly reduced compared to immunized *Btk*^{flox} control mice ($p<0.001$), indicating that BTK contributes to development of antigen-specific plasmablasts. Non-plasmablast anti-TNP-Ficoll B cells (IgM+TNP-Ficoll+CD19+B220+) (Figure 3.9D, left) were not different in mock-

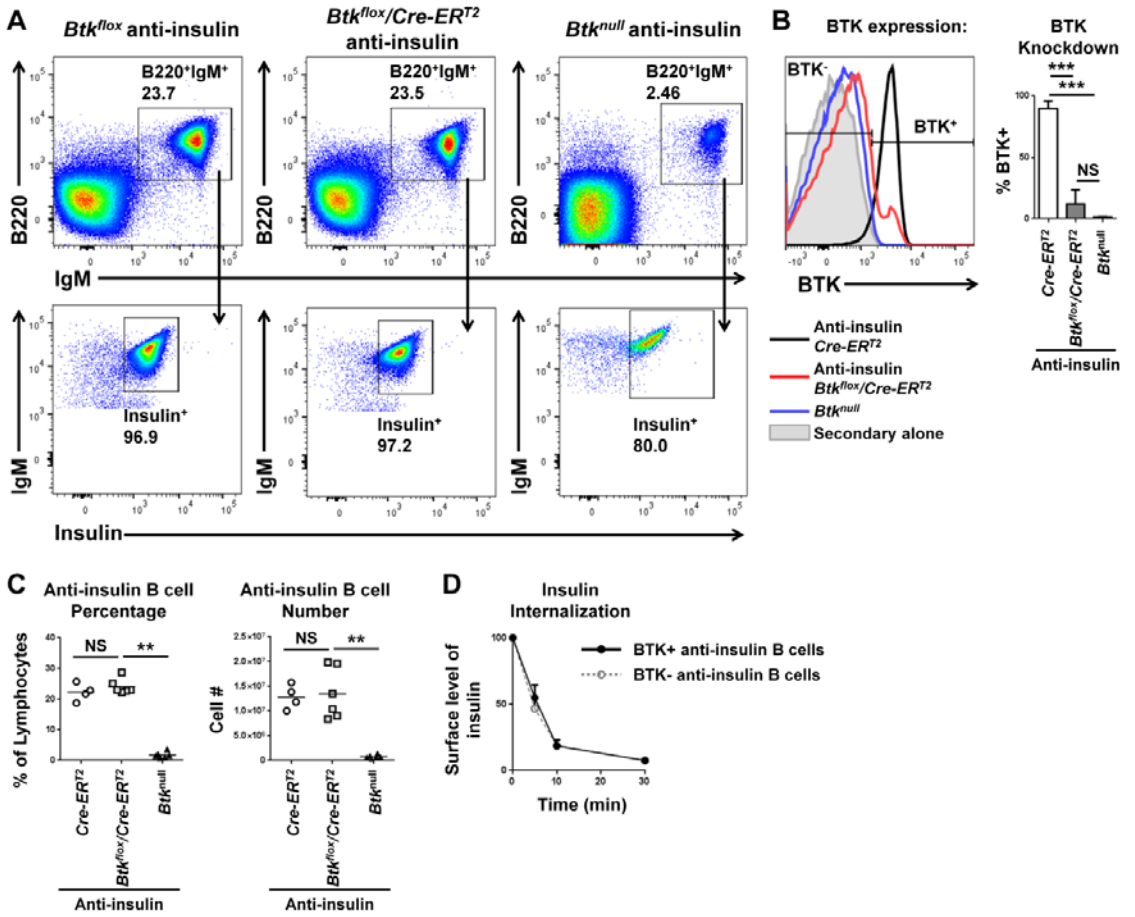


Figure 3.10: Anti-insulin B cell survival and antigen internalization are independent of BTK. (A) Representative gating of anti-insulin B cells from transgenic *Btk*^{flox} (left), *Btk*^{flox}*/Cre-ER*^{T2} (middle) and *Btk*^{null} (right) mice. Anti-insulin B cells are identified by expression of B220 and IgM, and by binding to fluorescent insulin. Cells are pre-gated as single live lymphocytes. (B, left) Representative histograms of BTK expression in B cells from transgenic *Cre-ER*^{T2} (black) and *Btk*^{flox}*/Cre-ER*^{T2} (red), a non-transgenic *Btk*^{null} (blue), and an isotype control (gray). (B, right) The percent of B cells that are BTK positive in transgenic *Cre-ER*^{T2} (white, n=4), *Btk*^{flox}*/Cre-ER*^{T2} (gray, n=6) and *Btk*^{null} (black, n=6) mice, five days after tamoxifen treatment. (C) Anti-insulin B cells are quantified by % of total lymphocytes (left) or total cell number (right) in transgenic *Cre-ER*^{T2} (circles, n=4), *Btk*^{flox}*/Cre-ER*^{T2} (squares, n=6) and *Btk*^{null} (triangles, n=6) mice. D) The surface level of insulin is shown for BTK-positive (black, n=4) or BTK-negative (gray, n=4) anti-insulin B cells after 5, 10, or 30 minutes at 37°C, in comparison to the level of binding at t=0. ***p<0.001 as calculated by one-way ANOVA (B), **p<0.01, as calculated by Kruskal-Wallace with Dunn's multiple comparison test (C).

immunized *Btk*^{flox} (1.04e5±3.08e4), *Btk*^{flox}*/Cre-ER*^{T2} (1.19e5±4.55e4), and *Btk*^{null} (7.59e5±1.50e4), and none of the genotypes showed significantly increased numbers of this subset after TNP-Ficoll immunization.

I also assessed IgM+TNP-Ficoll+ cells in the peritoneal lavage to directly evaluate contributions by B1 cells (Figure 3.9E). After TNP-ficoll immunization, *Btk*^{flox} controls had significantly higher numbers of IgM+TNP-Ficoll+ cells (4.80e3±1.17e3) than *Btk*^{flox}*/Cre-ER*^{T2} (3.12e3±1.25e3) (p=0.011). IgM+TNP-Ficoll+ cell number in TNP-immunized *Btk*^{flox}*/Cre-ER*^{T2} (3.12e3±1.25e3) was not different from that of mock-immunized *Btk*^{flox}*/Cre-ER*^{T2} (3.513e3±8.038e2) (p=0.9998). Due to the significantly decreased IgM response, a lack of IgG responses, a loss of plasmablasts in the spleen, and a failure to increase numbers of TNP-specific B cells in the peritoneal lavage, I conclude that though B1 cells do not require BTK for their survival (Figure 3.6), BTK is required for TI-II responses by both B1 and B2 cells.

Anti-insulin B cells do not require BTK for survival or internalization of antigen.

Autoreactive B cells are critically important to the pathogenesis of type 1 diabetes, where they drive disease by presenting antigen and activating autoreactive T cells (3-5). The anti-insulin transgenic model, in which 95% of the B cells bind insulin, allows us to study fixed autoreactive BCR specificity. These anti-insulin B cells are known to present antigen and drive disease, even while remaining otherwise anergic (51). To determine if these B cells would be depleted by loss of BTK, I injected transgenic *Btk*^{flox}, *Btk*^{flox}*/Cre-ER*^{T2} and *Btk*^{null}

animals with tamoxifen to knock down BTK. Five days after the last injection, I assessed the percentages and numbers of insulin-binding splenic B cells (Figure 3.10). BTK knockdown was successful, as 87.92%±11.68% of B cells in *Btk^{flox}/Cre-ER^{T2}* animals were BTK negative. There was no depletion of anti-insulin B cells in *Btk^{flox}/Cre-ER^{T2}* mice, despite the loss of BTK. B cell numbers were unchanged after BTK loss, with 1.35e7±5.16e6 anti-insulin B cells remaining in the *Btk^{flox}/Cre-ER^{T2}* compared to 1.29e7±2.48e6 anti-insulin B cells in *Btk^{flox}* mice ($p>0.999$). The *Btk^{flox}/Cre-ER^{T2}* model did not recapitulate the conventional *Btk^{null}* model, which exhibited a 94% decrease in anti-insulin B cell numbers (8.18e5±3.76e5) ($p=0.009$). In addition, I assessed if BTK-negative anti-insulin B cells remain able to function by internalizing insulin. Figure 3.10D shows insulin internalization by both BTK-positive and BTK-negative B cells from *Btk^{flox}/Cre-ER^{T2}* animals. BTK-negative B cells remained able to internalize antigen at the same rate as BTK-positive anti-insulin B cells. These data show that though anti-insulin B cell development is dependent upon BTK, mature anti-insulin B cell survival is not. In addition, anti-insulin B cells that are BTK-negative remain able to internalize antigen.

Discussion

The phenotype of *Btk^{null}* mice has been extensively reported, by our own and other's work (132, 137, 140). It is well known that *Btk^{null}* mice lack B cell subsets such as anergic An1s and B1s, and fail to respond to T-independent

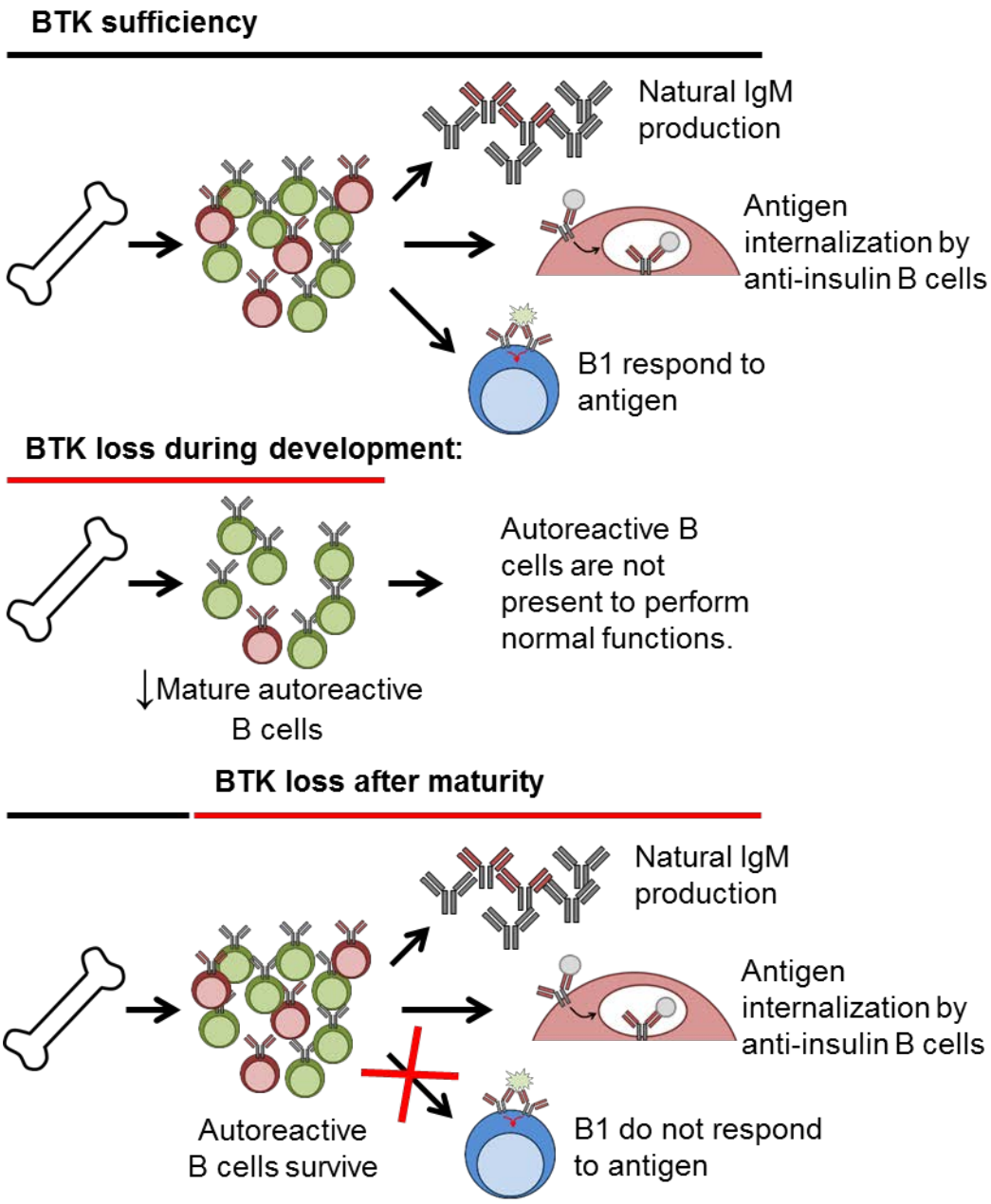


Figure 3.11: Graphical abstract for the role of Bruton's tyrosine kinase in the survival and function of autoreactive B lymphocytes.

antigens. In addition, BTK-deficiency in the transgenic anti-insulin model reduces B cell numbers by 95%. However, the lack of an inducible knockout has resulted in a gap in our understanding of how mature B cell subsets rely on BTK. As BTK inhibitors are considered for use in more human diseases, it is vital to understand these effects. In this chapter, I detail the first use of *Btk*^{flox}*/Cre-ER*^{T2} inducible knockdown to study the effect of loss of BTK on the survival and function of mature B cell subsets. Figure 3.11 is a graphical abstract summarizing my findings.

First, I established the efficacy of the *Btk*^{flox}*/Cre-ER*^{T2} system (Figure 3.1, 3.2, 3.3). Treatment of *Btk*^{flox}*/Cre-ER*^{T2} with tamoxifen resulted in efficient knockdown in B cells at all subsets and developmental stages, as well as in splenic macrophages and dendritic cells. This knockdown was stable up to five weeks after injection. I then analyzed splenic B cell subsets to assess the effect of BTK knockdown on mature B cells (Figure 3.4). *Btk*^{null} mice exhibit a block in transition from T2 to mature FO B cells (42, 140). T2 and FO B cells both express IgD, CD21, and CD23, and are differentiated by expression of IgM, which is high on T2 and lower on FO B cells. Because IgM surface expression is generally higher in the absence of BTK, it was possible that FO B cells would shift to a more T2-like appearance immediately. However, though there was an immediate increase in IgM after BTK loss, the treated *Btk*^{flox}*/Cre-ER*^{T2} did not fully mimic the phenotype of *Btk*^{null} mice until five weeks after knockdown, when cell turnover would essentially recapitulate a *Btk*^{null} B cell repertoire. In contrast to this delay in developmental phenotype, the effect of *Btk*-excision immediately

results in loss of function related to cell signaling, as *Btk^{flox}/Cre-ER^{T2}* B cells proliferated significantly less in response to LPS or anti-IgM stimulation within days of tamoxifen treatment (Figure 3.1C). Thus, this model reveals the split role of BTK in cellular development versus function in B cells, and shows that the impaired proliferation in B cells lacking BTK is not simply due to maturational defect.

The lack of B1s in *Btk^{null}* models correlates with a loss of natural IgM and deficient responses to T-independent immunization. However, the lack of a genetic knockdown model has prevented the study of BTK's role in the survival and function of this important B cell subset. Surprisingly, I found normal numbers of BTK-negative B1a and B1b cells present in the peritoneal lavage of *Btk^{flox}/Cre-ER^{T2}* mice even five weeks after BTK knockdown (Figure 3.6). B1a cells in the spleen and bone marrow were also not reduced by BTK loss (Figure 3.7).These are the first data that show that B1a and B1b cells do not depend on the presence of BTK for survival, but instead require it for development.

B1 cells, particularly those in the spleen and bone marrow, produce natural IgM. Natural IgM serves several important roles. These antibodies serve as a first defense against many pathogens, such as *Streptococcus pneumoniae* (105), *Listeria monocytogenes* (107), influenza virus (106, 109) and others (104, 108). In addition, natural IgM contributes to tissue homeostasis through the binding of self-antigens (97, 98) and has been shown to be atheroprotective (99, 100). It is known that *Btk^{null}* mice lack natural IgM; however, this lack cannot be separated from their lack of B1 cells. In Figure 3.8, I have shown that the natural

antibody anti-phosphoryl-choline IgM remains present in *Btk^{flox}/Cre-ER^{T2}* serum five days and even five weeks after BTK knockdown. The half-life of IgM *in vivo* is estimated at 2 days (275). Therefore, B1 B cells continue to produce natural anti-PC antibody even after the loss of BTK, contrasting its absence in *Btk^{null}* controls. Thus, the production of natural IgM by B1 cells is independent of continued signaling through BTK. Small molecular BTK inhibitors are now in use for B cell lymphomas, and are currently in clinical trials for autoimmune disease. Therefore preservation of natural IgM, and its associated functions, in the absence of BTK is a clinically relevant finding that should prompt further study in patients who receive these drugs.

Another role of B1 cells is in the initial response to infection. B1b cells are known to rapidly produce protective IgM in response to pathogens such as *Borrelia hermsii* (112, 276) and *Streptococcus pneumoniae* (105). B1b, as well as B1a and marginal zone B2 cells are known to be the main contributors to T-independent (TI) antibody production (200, 277-279). I immunized *Btk^{flox}/Cre-ER^{T2}* mice with TNP-Ficoll after BTK knockdown, to determine if the surviving B1 cells could respond to a model TI-type II (TI-2) antigen (Figure 3.9). Both B1 cells in the peritoneal lavage and B2 cells in the spleen were unable to respond to antigen *in vivo*. *Btk^{flox}/Cre-ER^{T2}* animals exhibited significantly reduced anti-TNP IgM and very little anti-TNP IgG post immunization, compared to *Btk^{flox}* controls. This finding shows that though mature B1 cells survive the loss of BTK, and retain their ability to produce natural IgM, they are unable to respond to TI-2 immunization. These data could also have implications for the use of BTK

inhibitors in human disease, particularly in long term use. BTK inhibitors may decrease the efficacy of immunizations such as the pneumococcal conjugate vaccine (PCV13), which are particularly targeted towards adults 65 years or older (https://www.cdc.gov/vaccines/vpd-vac/pneumo/). This could be of particular importance in their proposed use for rheumatoid arthritis (RA), as the onset of RA is highest among adults in their sixties (280).

Systemic autoimmune disorders like RA are mediated by autoantibody production by autoreactive B cell subsets. An1 B cells are an endogenous autoreactive B cell subset (82). A similar subset is present in humans and is increased in autoimmunity (41, 84). Our lab and others have found that the development of An1 B cells is dependent upon BTK (43, 187). Therefore, I assessed the An1 subset in *Btk^{flox}/Cre-ER^{T2}* animals five days after BTK knockdown. An1 cells were swiftly depleted, and were significantly reduced after BTK loss (Figure 3.5). However, it remains unclear as to whether this is due to a survival defect or a loss at development, as An1 B cells are known to have a life cycle of only five days (43). Nevertheless, the swift depletion of this subset implies that short courses of BTK inhibition may impact similar autoreactive anergic populations in humans, without greatly impacting non-autoreactive B2 subsets.

Though An1s are an important B cell subset in both mice and humans (41, 128), it is useful to study an autoreactive population with a fixed BCR specificity. Therefore, I used a transgenic mouse model, in which 95% of the B cells bind insulin, to determine the role of BTK in the survival of mature anti-insulin B cells.

Autoreactive B cells, particularly anti-insulin B cells, drive T1D by presenting antigen to autoreactive T cells (3-5, 281). Anti-insulin B cells are also drastically reduced in *Btk*^{null} models. Therefore, one proposed strategy for the treatment of T1D is treatment with BTK inhibitors, with the goal of depleting anti-insulin B cells. However, in Figure 3.10, I show that anti-insulin B cell survival is independent of BTK. Also, BTK-negative anti-insulin B cells remain competent to internalize antigen. Therefore, the depletion of anti-insulin B cells by targeting BTK may take longer than originally expected and may be difficult to achieve. This data informs how we might attempt to use BTK inhibition in T1D in the future.

This work is the first to use an inducible genetic knockdown of BTK to rigorously study its role in the survival and function of mature B cells. With rising use of BTK inhibitors, as well as the pursuit of more specific BTK inhibition, the basic mechanism of BTK's role in mature B cell subsets will only become more important. The loss of BTK greatly impacts the ability to respond to polysaccharide antigens. However, these findings show that important immune functions, such as the production of natural IgM, are intact following the loss of BTK. In addition, An1 B cells are swiftly depleted after BTK loss, which may have implications for BTK inhibition as short-term therapy for autoimmune disorders.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The prevalence of B lymphocyte-mediated autoimmune diseases, and their negative impact upon those who suffer them, often results in the question: why do autoreactive B cells develop? Indeed, the fact that autoreactive B cells are evolutionarily conserved in mice and humans indicates that perhaps they are present for a reason or are simply an inevitable side effect of an immune system that is as balanced as possible. Some autoreactive subsets may serve an important purpose, such as the production of autoreactive natural IgM by fetal-derived B1 cells, but autoreactivity in the B2 compartment often results in chronic autoimmunity. Autoimmunity results when tolerance mechanisms, both central and peripheral, are breached. As I have previously discussed, autoreactive B cells form the majority of developing B cells in the bone marrow, estimated at 70-80% (25). Though most of these are deleted by central tolerance, some escape to the peripheral organs where peripheral tolerance mechanisms such as anergy are to hold them in check (43, 82). However, tolerance mechanisms can be leaky, allowing autoreactive B cells to present antigen to and activate autoreactive T cells, or can be broken, resulting in autoreactive B cell activation and production of autoantibody. These autoantibodies can then bind to autoantigen and activate innate immune cells through Fc receptors, resulting in destruction and disease.

Understanding the regulation of these processes is critical to our understanding of autoimmune disease. One process that regulates each step of tolerance is that of B cell receptor (BCR) signaling. During B cell development, tonic signaling through the BCR must be strong enough to signal the successful rearrangement of the receptor. However, if signaling through the BCR is too strong, it indicates autoreactivity and initiates central tolerance mechanisms. In the periphery, mature B cells signal through the BCR in response to antigen, and lack of response to antigen is a key determinant of anergy. Finally, the strength of BCR signaling regulates the germinal center reaction and is theorized to determine which germinal center B (GCB) cells are selected to become memory B cells or plasma cells. The BCR signaling protein Bruton's Tyrosine Kinase (BTK) is the specific focus of this research, as it affects B cell receptor signaling at every stage of the B cell life cycle, and also plays a role in the innate response to antibody. My research, detailed in this dissertation, has touched on the role of BTK in autoreactive B cells at every stage throughout this cycle.

Differential contribution of BTK to development and survival of autoreactive B cells has implications for the treatment of autoimmunity.

Previous to my work, it was established that autoreactive B cells are more severely impacted by loss of BTK as compared to non-autoreactive B cells (43, 140, 187). In Chapter II, I showed that *Btk*-deficient K/BxN mice exhibited a severe developmental block in the B cell compartment, resulting in a 74%

reduction of B cell numbers. This pattern of B cell depletion, with a block at transitional stage 1 (T1) and a loss of marginal zone and follicular B cells, is characteristic of the anti-insulin transgenic model. In addition, I documented severe depletion of autoreactive anti-GPI IgG, even while total IgG remained relatively intact. Therefore, I concluded that autoreactive B cells were successfully depleted at development in this model, which subsequently led to protection from spontaneous arthritis. The pronounced depletion of anti-GPI autoantibody, compared to the relatively small decrease in total IgG, indicates that autoreactive B cells are yet again more impacted by the loss of BTK than non-autoreactive B cells.

Before the development of an inducible knockdown model, it was impossible to separate the role of BTK in autoreactive B cell survival from its role in autoreactive B cell development. Autoreactive B cell populations are known to have dysregulated signaling compared to non-autoreactive B cells (188, 192), including increased positive signals such as high basal calcium levels, and increased negative signaling mediators such as SHIP-1 and Siglec-G (189, 196). Originally, I hypothesized that the loss of a positive signaling mediator, BTK, would result in increased negative signaling and a loss of mature B cells. I theorized that autoreactive B cells would depend upon BTK for not just development, but survival, and this dependence could be used to deplete autoreactive B cells and treat disease. However, in chapter III, I show that the autoreactive-prone B1 cell subset and anti-insulin transgenic B cells do not require BTK for survival. An1 B cells were swiftly depleted by BTK knockdown,

but that may be due to their short lifespan and dependence on BTK for new An1 B cell development. The fact that autoreactive B cells require BTK to progress through development, but not to survive once they have reached maturity, suggests that autoreactive B cells must require BTK for a positive selective step during development. Additionally, this step is somehow less necessary for non-autoreactive B cells.

My findings may also have implications for the treatment of autoimmune arthritis with BTK inhibitors. The protection seen in the spontaneous arthritis model was due to the loss of mature naïve B cells, a loss that in turn decreased the number available to become GCB cells, resulting in decreased autoantibody. If autoreactive B cells in the K/BxN model are similar to B1 or anti-insulin B cells, and do not require BTK for their survival, their numbers may be unaffected by the loss of BTK after they have reached maturity. This is of particular importance, because if the relevant subsets remain, they may interact with T cells, overcome BTK-deficiency, and continue to produce autoantibody. It has long been known that *Btk*-deficient mice are able to respond to T-dependent immunization, though the response remains blunted (140, 147, 199). This serves as more evidence that it is a loss of autoreactive B cells that provides protection in the *Btk*-deficient K/BxN model. While it is possible that autoreactive B cells respond differently to T cell help, it is more likely that the autoreactive B cells are simply not present to interact with the T cells at all, resulting in a differing outcome as compared to T-independent immunization. Therefore, if BTK knockdown does not result in autoreactive B cell depletion, protection from disease may be lost. Alternatively, if

anti-GPI B cells are located in the An1 compartment, they may be depleted only a few days after BTK loss. If this is the case, early enough intervention may result in disease protection.

Future directions regarding differential contribution of BTK to development and survival of autoreactive B cells:

Assessing BTK's role in ongoing autoreactive immune reactions:

I have established that BTK supports autoreactive B cells during development, and the loss of BTK results in protection from autoimmune arthritis in the K/BxN model. However, it is yet to be determined if targeting BTK-mediated signaling after the development of the mature B cell repertoire will ameliorate arthritis. BTK loss could prove effective in two possible ways. If anti-GPI B cells are anergic and possess a short lifespan, similar to the An1 compartment, I hypothesize that the loss of BTK will result in swift depletion and protection from disease. However, even if anti-GPI B cells persist, I hypothesize that BTK loss will disrupt antibody production after germinal center reactions are already underway. These hypotheses can be tested by applying the *Btk*^{flox}/tamoxifen-inducible Cre system to K/BxN arthritis.

First, the dependence of anti-GPI B cells on BTK can be examined in the *Btk*^{flox}*/Cre-ER*^{T2} K/BxN model. Anti-GPI B cells can be difficult to detect, but do expand during arthritis progression (282). Using a GPI-PE tetramer, specific anti-GPI B cells can be assessed during early and late arthritis, and I can determine if

mature anti-GPI B cells require BTK. This method can also identify if anti-GPI B cells are located in the An1 compartment. If endogenous anti-GPI B cells are difficult to detect, their reliance upon BTK can be assessed in a transgenic model (283). The transgenic anti-GPI model features a higher-affinity BCR, generated by paired site-directed anti-GPI heavy and light chains, or lower-affinity BCRs that are generated by the pairing of the anti-GPI heavy chain with endogenous light chains. Analysis of this model would allow specific tracing of anti-GPI B cells, and the identification of their dependence upon BTK.

The second way that BTK loss from the mature compartment could provide protection from arthritis is by the disruption of antibody production after germinal center reactions have already begun. It is unknown if BTK is necessary for the survival and continued propagation of GCB cells, memory B cells (B_{mem}), and plasma cells. We do have some clues on how exactly BTK may affect GCB, B_{mem} and plasma cells. First of all, BCR signaling is vitally important to the formation of the germinal center, and to the affinity maturation process. Affinity maturation works by ensuring that the highest affinity B cells are the cells that receive survival signals from T follicular helper (Tfh) cells and follicular dendritic cells (66). GC B cells acquire Tfh help by capturing antigen on their BCR and then processing and presenting it. A higher affinity B cell captures more antigen and has a higher density of peptide-MHC complexes on its surface, resulting in more Tfh help (284). Due to the importance of BCR signaling in this process, I hypothesize that BTK loss will decrease signaling through the BCR and disrupt affinity maturation, resulting in lower antibody affinity. In contrast, plasma cell

survival may be unaffected by the loss of BTK. Plasma cells downregulate the BCR and exhibit low expression of BTK (213). The relatively preserved IgG levels in *Btk*-deficient mice also point to the ability of plasma cells to form and survive even without BTK. In an autoantibody mediated disease, such as rheumatoid arthritis, autoreactive plasma cells may continue to produce damaging autoantibody even after BTK loss. These hypotheses can be tested by knocking down BTK after arthritis has already begun, and assessing GC B cells, plasma cells, and anti-GPI autoantibody.

Identifying the role of BTK in non-autoreactive immune reactions:

BTK inhibitors are targeted at aberrant immune reactions, such as B cell cancers and autoimmunity. However, these conditions do not occur in a vacuum, and it is important to understand the effect of BTK loss on immune responses to exogenous antigen. One tool to help answer this question is the use of T-dependent immunization models. In addition, an immunization model has the advantage of allowing control over when the germinal center forms. *Btk*^{flox}*/Cre-ER*^{T2} animals can be tamoxifen-treated before or after initial immunization, or after boosting. This allows the assessment of BTK's function in the initiation and survival of GCB cells and plasma cells. Furthermore, the use of a model antigen allows assessment of both the affinity of the resulting antibody and direct assessment of responding B cells through the use of fluorochrome-conjugated antigen. The use of an immunization model enables clear assessment of the role of BTK in GC B, B_{mem}, and plasma cells. It also informs us of the effect of BTK

inhibition on infection or vaccination, which are likely to occur during the course of treatment.

The use of the *Btk^{flox}/Cre-ER^{T2}* model, applied to both autoimmunity and a model immunization, allows for the assessment of BTK's role in both autoreactive and non-autoreactive immune reactions. Autoimmunity is treated after disease has already progressed, and any treatment targeting the immune system has the risk of resulting in immune-deficiency. Therefore, the best possible method of treatment would be to quickly deplete autoreactive B cells, hopefully before plasma cell formation, while leaving non-autoreactive B cells intact. If BTK inhibition cannot accomplish this, it may be necessary to pursue other avenues of treatment.

BTK as a potential regulator of innate immunity.

My work with the serum-transfer model of arthritis in Chapter II represents the first use of a genetic model to study the contribution of BTK in innate mediated arthritis. Surprisingly, I found that the loss of BTK was not protective against serum-transfer arthritis. This data contrasts work done using BTK inhibitors, which focused primarily on the role of BTK in macrophages (215-219, 237). My findings have several important implications. First of all, if treatment for autoimmune arthritis commences after autoantibody has formed, truly specific BTK inhibition may not be effective in reducing them. Plasma cells may continue to produce damaging antibody, and my data shows that the innate immune

system can still respond to that antibody and mediate immune destruction. Secondly, the contrast between my work using a genetic deletion model, and the field's use of BTK inhibition highlights that it may be the off-target effects by BTK inhibitors that lead to protection from serum transfer arthritis. These previous studies used inhibitors such as ibrutinib. Ibrutinib is known to bind the T cell analogue of BTK, interleukin-2-inducible T cell kinase (ITK), as well as the tyrosine-protein kinase TEC, which is known to be important for both macrophage and mast cell function (220). The revelation that it may be these effects, rather than binding to BTK, that mediates disease protection, is a question that deserves further study. It raises the possibility that perhaps specific inhibition of other kinases such as TEC and ITK should be sought after to treat autoimmune disease. My final conclusion from this data set is that it highlights how little we really know about the role of BTK in innate cells. The available data paints a picture of conflicting stimulatory and regulatory roles. Previous work has found that BTK-loss in dendritic cells may lead to an inflammatory, T cell stimulatory, phenotype (261), whereas data conflicts on BTK's role in neutrophils. Neutrophils from human XLA patients are not defective in effector function (262) and may even produce increased reactive oxygen species (263), whereas *Btk*-deficient neutrophils in mice were found to have decreased granules per cell and reduced E-selectin-mediated recruitment (222, 223). My work in the serum-transfer model of arthritis does not indicate that innate cells are completely unimpaired by BTK loss, but that BTK loss was not enough to impact disease

progression. Exactly how BTK functions in innate immune cells, and how those functions impact arthritis, is an area that requires further work.

Future directions regarding *BTK as a potential regulator of innate immunity*:

BMX and TEC kinases as alternative targets in autoimmune arthritis:

If specific BTK inhibition proves ineffective in the treatment of autoimmune arthritis, it becomes necessary to seek other targets that may be more efficacious. Two possible candidates for inhibition are BMX and TEC, Tec-family kinases that function in innate immune cells. BMX is expressed in macrophages and neutrophils (285, 286), and TEC is expressed in macrophages, neutrophils, mast cells and T cells (287-289). Total genetic deletion of BMX has been shown to be effective against K/BxN serum transfer arthritis. However, when BMX was present, but its kinase function inactivated, serum transfer arthritis was the same as in BMX-competent mice (290). Therefore, a kinase-specific inhibitor would most likely prove ineffective for arthritis treatment. BMX, like BTK, may also function as an adaptor, and targeting its pleckstrin-homology or adaptor domains for inhibition could be protective in autoimmune arthritis. There has been no study of the role in TEC in autoimmune arthritis, but *Btk*-deficient macrophages exhibit an increase in TEC expression as they mature (227), and both BMX and TEC are theorized to compensate for the loss of BTK in myeloid cells (170). TEC-knockout mice have no obvious B cell phenotype (170) and should be used

in both K/BxN spontaneous and serum-transfer arthritis to determine if targeting TEC may be protective. The redundant functions of BTK, BMX, and TEC may mean that targeting only one will never be an effective disease treatment. However, we should collect as much detail from genetic models as possible, to inform us as to how to develop inhibitors and treat autoimmunity while preserving normal immune function.

Cell-specific deletion of BTK will reveal its role in discrete immune subsets:

The *Btk*^{flox}*/Cre-ER*^{T2} model is invaluable for its ability to knockdown BTK in mature cells. *Btk*^{flox} is not only useful for the study of temporal deletion, of course, but can also be paired with a cell-specific Cre to study the role of BTK in specific immune subsets. Expressed under the CD11c promoter, Cre can be used to knockdown BTK in conventional dendritic cells. Under the LysM promotor, Cre can specifically knockout BTK in macrophages and granulocytes. While the serum-transfer model proved that BTK's contribution to innate immunity was not enough to protect from arthritis, the use of cell-specific knockdown in tandem with the K/BxN model will provide important information on the roles of specific cells in this disease. There is evidence that BTK has both stimulatory and regulatory roles in the innate immune system, and cell-specific deletion of BTK will help determine which role BTK plays in specific cell subsets. Finally, while the use of the K/BxN spontaneous and serum transfer models does allow study of both innate and adaptive arms of the immune system, it bypasses a key player: dendritic cell antigen presentation to autoreactive T cells. *Btk*-deficient

dendritic cells have been reported to be more stimulatory to T cells, due to their inability to self-regulate with production of IL-10 (261). *Btk*^{flox}/CD11c-Cre and the K/BxN model will determine if BTK supports antigen presentation by dendritic cells in autoimmune arthritis, and provide more information as to its role in this bridge between innate and adaptive immunity.

The role of BTK in B1 cell function: novel stimuli vs established function:

I have established that the autoreactive-prone B1 cell subset and transgenic anti-insulin B cells do not require BTK for survival. However, though this fact alone is novel and implies that autoreactive B cells require BTK for a selection step during development, it was also critical to determine if these cells still perform their various functions. In chapter III, I determined that B1 cells continue to produce natural IgM, even after BTK loss, but are unable to respond to T-independent immunization.

Natural IgM has many important functions, including acting as an initial barrier to infections (104-109), contributing to tissue homeostasis (97, 98), and is atheroprotective (99, 100). B1 cells in the spleen and bone marrow produce the majority of natural IgM. Like plasma cells, they secrete antibody, are long-lived, and express CD43. However, in contrast to plasma cells, they retain high expression of the BCR and co-receptors such as CD19 (35). Like antibody production by plasma cells, natural IgM production by B1 cells may be enforced by a transcriptional program. I found that these cells persisted even after the loss

of BTK, and their continued production of natural IgM shows that this immune function may be preserved in BTK inhibitor-treated patients. Indeed, in clinical trials using the BTK inhibitor Ibrutinib for mantle cell lymphoma, chronic lymphocytic leukemia, or small lymphocytic lymphoma, patients exhibited no loss of serum IgM levels. In one of these studies, serum IgM levels actually increased (291-293). Of course, the immune system in leukemia or lymphoma is already dysregulated, but the fact that the use of a BTK inhibitor did not recapitulate the phenotype of XLA is both encouraging for their use and implies that my findings in the role of BTK in mature B cells may be applicable to humans.

Contrasting natural IgM production, immunization involves providing a new stimulus to which the B cell must respond. It has long been established that global BTK deletion in mouse models results in a lack of T-independent B cell responses. These mouse models completely lack B1 cells, though, and the inability to respond to antigen because of a lack of signal through the BCR could not be separated from the absence of any B cells able to respond. Because of my finding that mature B1 cells survive even after BTK loss, I was able to determine if these B cells could respond to T-independent antigen in the absence of BTK. The loss of BTK leads to reduced responses to T-independent type II immunization, as detailed in Chapter III. The inability of BTK-deficient B cells to respond to T-independent immunization may inform the use of BTK inhibitors in humans. The B1 cell response is important for certain immunizations and also the reaction to infections such as *Streptococcus pneumoniae* and *Borrelia hermsii*, so patients treated with BTK inhibitors may be especially at risk for these

diseases. These data, and my work on natural IgM, have produced clinically relevant data for the use of BTK inhibitors.

Future directions regarding the role of BTK in B1 cell function: novel stimuli vs established function:

Identification of BTK's role in the development of B1 cells.

The work of this dissertation makes it clear that BTK plays a role in the development, but not survival, of B1 cells. However, the exact nature of that role remains unknown. B1 cells may undergo a positive selection step during development that relies on signaling through the BCR. The B1 cell subset is known to contain anti-Thy-1 B cells. These autoreactive B cells require binding to Thy-1 to develop, because mice lacking Thy-1 expression also lack anti-Thy-1 B1 cells (93). It is possible that BTK mediates this positive selection step through its function in BCR signaling. A second possibility is that BTK mediates expression of developmental factors on which B1 cells rely. Interestingly, mice deficient in the atypical inhibitory protein IκBNS exhibit a similar phenotype to *Btk*-deficient animals, with a lack of B1a and B1b cells but relatively normal B2 cell numbers (294). It would be interesting to determine if overexpression of BTK rescued anti-Thy-1 B cell numbers in Thy-1 knockout mice, or if induced expression of IκBNS would rescue B1 cell numbers in *Btk*-deficient models. If these methods fail, transcriptional profiling of neonatal B1 cells may provide clues to the developmental requirements of this critical cell subset.

BTK-independence of anti-insulin B cells is significant in treatment of Type 1 Diabetes.

B1 cells are not the only autoreactive-prone B cells that lack a BTK requirement for their survival. Transgenic anti-insulin B cells are not depleted after BTK loss, and still internalize antigen even without BTK. The preservation of insulin internalization may have important consequences. This function and the survival of insulin-specific B cells indicate that targeting BTK to treat Type 1 Diabetes (T1D) may be ineffective. BTK has been an exciting target in T1D research, because *Btk*-deficiency is protective in the non-obese diabetic (NOD) mouse model of T1D. This protection is most likely due to the depletion of anti-insulin B cells, a conclusion which is supported by the finding that anti-insulin IgG is drastically reduced in this model while total IgG remains unchanged. If even a small number of anti-insulin B cells reach maturity, disease is restored. The provision of a transgenic anti-insulin heavy chain (HC) restored disease, because though *Btk*-deficiency greatly reduces anti-insulin B cells in the anti-insulin HC model, it does not eliminate them, and the remaining B cells can still instigate disease (137). Anti-insulin B cells drive T1D by presenting antigen to autoreactive T cells (3, 4). This fact, and my lab's previous work, implies that *Btk*-deficient anti-insulin B cells, if they are present, can still present antigen to and activate destructive autoreactive T cells. Therefore, my finding that mature anti-insulin B cells are not depleted by BTK loss implies that BTK inhibitor treatment will not result in disease protection.

I have also shown that BTK-negative mature anti-insulin B cells remain competent to internalize antigen. This finding contrasts work by Song *et al*, who found that BTK was required for internalization of crosslinking anti-IgM by naïve B cells (197). This difference may be due to the difference in signaling between a crosslinking antigen like anti-IgM, and a non-crosslinking antigen such as insulin. BTK is reported to regulate actin remodeling, which is required for the internalization of a cross-linking stimulus (197, 295). In contrast, insulin internalization may be due to passive internalization of the BCR, which is independent of actin remodeling (296). Anti-insulin B cells are anergic, and B cells in the anti-HEL and the 3H9/Vκ8 models of anergy also rapidly internalize antigen, indicating that rapid internalization may be a feature of anergy (297, 298). The exact mechanism by which these autoreactive B cells internalize antigen, and whether insulin-internalization can be inhibited, is an area that requires further study. These data will inform our approach on B cell targeting in the treatment of T1D.

Future directions regarding BTK-independence of anti-insulin B cells is significant in treatment of Type 1 Diabetes:

Turnover kinetics and antigen presentation of anti-insulin B cells:

My work on BTK and anti-insulin B cells raises certain immediate questions which must be answered. First of all, though what we currently know implies that anti-insulin B cells are able to present antigen and activate cognate T

cells, this fact has not yet been tested. In the conventional *Btk*-deficient anti-insulin mouse model, anti-insulin B cells are few and those that remain are mostly found in the Transitional 1 compartment. Therefore, performing antigen presentation assays proved difficult. Use of the $Btk^{flox}/Cre\text{-}ER^{T2}$ model will allow the harvest of mature BTK-negative anti-insulin B cells, which will finally answer this important question. Secondly, it still may be possible to deplete anti-insulin B cells, as BTK-negative B cells turnover in the spleen. We would expect newly developing anti-insulin B cells to be blocked in development similarly to conventional *Btk*-deficient models. The kinetics of this turnover can be determined using long-term knockdown and BrdU labeling, and determine the duration that NOD mice in relevant disease studies must receive BTK inhibition to achieve protection.

Development of multiple transgenic models for further study of anti-insulin B cell survival factors:

The current double-transgenic anti-insulin mouse model provides critical insight into the behavior of anti-insulin B cells. NOD mice do possess anti-insulin B cells, evidenced by the production of anti-insulin antibody during the disease process. However, anti-insulin B cells are rare, and difficult to identify and study in wild-type mice, so the use of a transgenic model is incredibly important. The double-transgenic is only one model of insulin-reactivity. In the HC-only transgenic model, the anti-insulin HC is paired with endogenous light chains. Of these light chains, two result in insulin-binding: Vκ4-74 and Vκ5-57-1 (299). The

development of transgenic models featuring the insulin-specific heavy chain and these separate light chains would provide increased insight into how anti-insulin B cells are regulated and what their contributions to disease may be. Anti-insulin B cells would be analyzed to identify their antigen presentation capacity, dependence upon BTK, and other survival factors, to inform how best to deplete autoreactive B cells in an autoimmune setting.

Summary

The purpose of this project was to further define the role of BTK in the development, survival, and function of autoreactive B cells, and how those roles may impact autoimmune disease. I found that BTK regulated the development, but not survival, of various autoreactive B cell subsets, and that BTK impacted certain autoreactive B cell functions but not others. My work represents the first use of genetic deletion to identify the role of BTK in both spontaneous and serum-transfer arthritis, revealing that specific inhibition of BTK may be ineffective in treating autoimmune arthritis. More work remains, particularly on the role of BTK in germinal center B cells, in innate immune cells, and on the function of anti-insulin B cells in T1D.

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