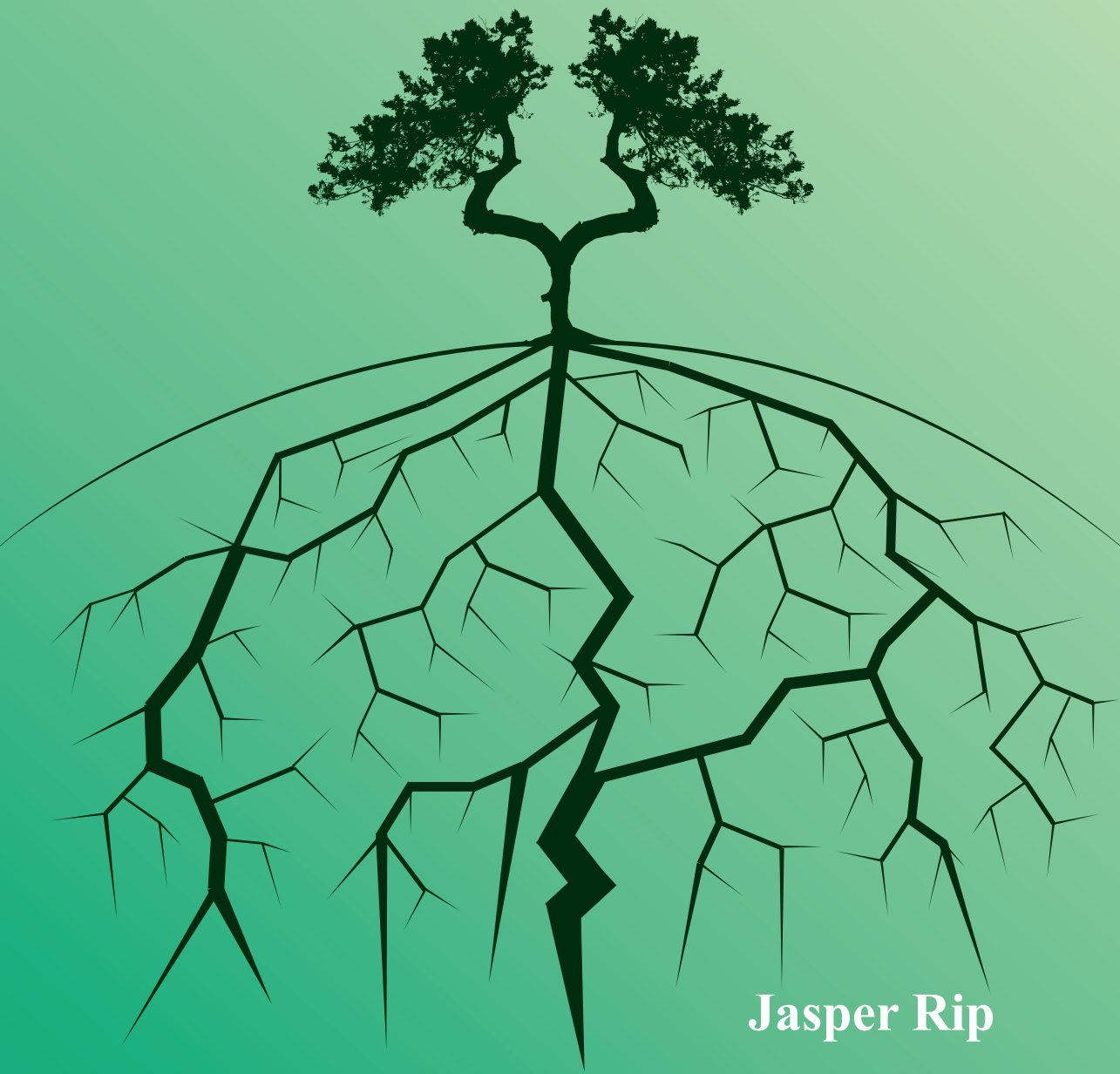
Bruton's tyrosine kinase signaling in health and disease:

a molecular perspective



Jasper Rip

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Bruton's Tyrosine Kinase Signaling in Health and Disease:
a molecular perspective.

Bruton's tyrosine kinase signalering in gezondheid en ziekte:
een moleculair perspectief.

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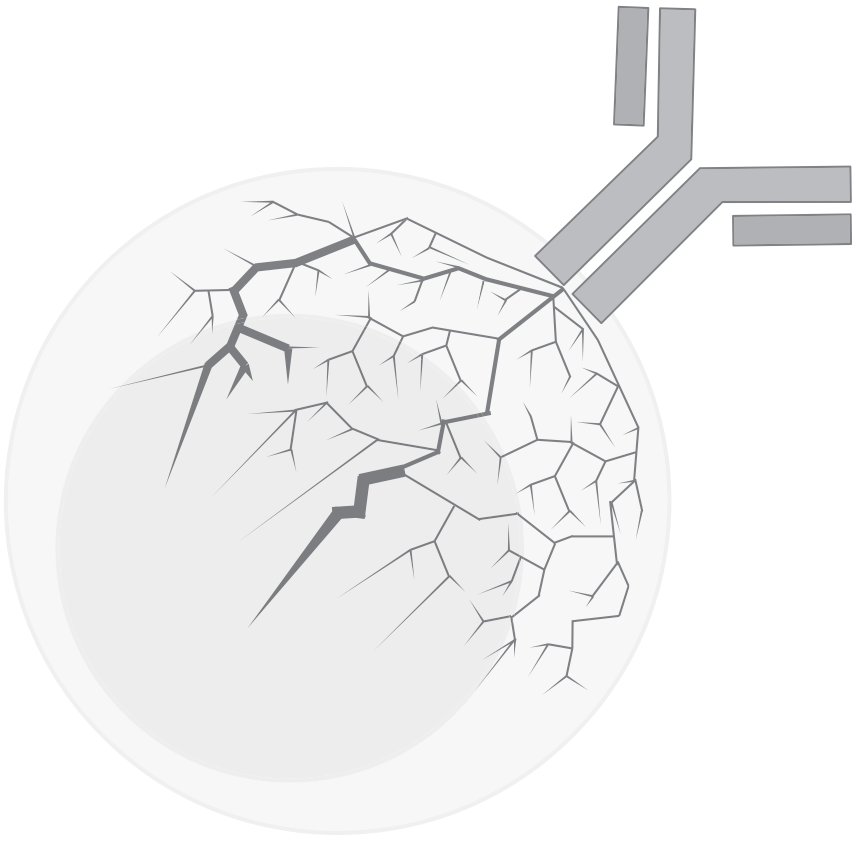

CHAPTER 1.1

General Introduction
BTK

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Historical Background

Bruton's tyrosine kinase (BTK), a member of the Tec family of non-receptor kinases, is expressed in all hematopoietic cells except T and NK cells and functions in many different signaling pathways (**Table 1**). It functions as a crucial signaling molecule downstream of many receptors, including the B cell receptor (BCR) on B lymphocytes. Loss-of-function mutations in the *Btk* gene were shown to drive X-linked agammaglobulinaemia (XLA), an inherited immunodeficiency disease marked by near absence of peripheral B cells and circulating immunoglobulins (Ig), first described by Dr. O.C. Bruton in 1952. Since this discovery, many striking findings have contributed to our understanding of the role of Btk in B cell development and function (**Figure 1**).

Similar to humans, mutations in the *Btk* gene also underlies the milder X-linked immunodeficiency (XID) phenotype in the CBA/N mouse strain. The effects of these mutations are largely limited to the B cell lineage, stressing the importance of Btk in B cell biology. Besides XLA and XID, a role for BTK has also been described in the context of oncogenic signaling and more recently in autoimmune disease. Several inhibitors of BTK have shown great efficacy in treatment of patients with various B cell malignancies, such as chronic lymphocytic leukemia (CLL) and mantle cell leukemia (MCL). In addition, mouse models have shown that a B cell intrinsic dysregulation of signaling can induce systemic autoimmune disease. These studies indicate that BTK expression levels and activity may be very relevant in B cell malignancies and systemic autoimmune disease.

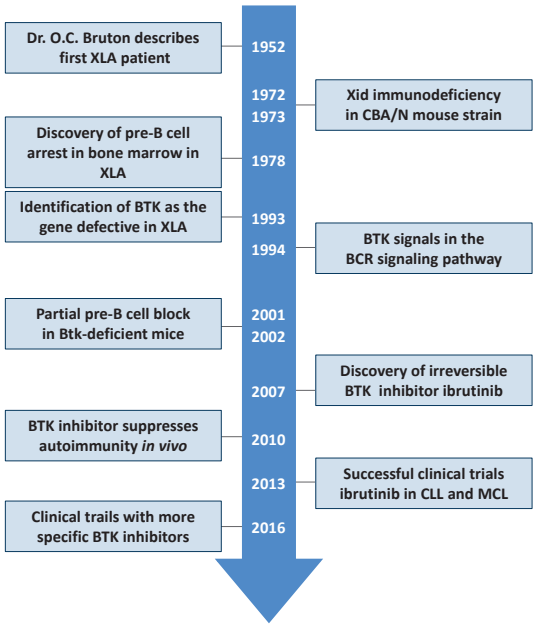

Figure 1. Key discoveries in XLA, XID and BTK research.

1. BTK in B cell receptor signaling

BTK is a cytoplasmic signaling molecule that is evolutionarily highly conserved and has a structure similar to SRC family kinases. The BTK protein consists of five domains (**Figure 2**) (Rawlings and Witte 1995). The pleckstrin homology (PH) domain is involved in recruitment of cytoplasmic BTK to the cell membrane upon receptor activation, and the Tec homology (TH) domain contains a zinc finger motif important for stability of the protein. The Src homology (SH) 2 and 3 domains are involved in binding of BTK to many other proteins, including the linker molecule SLP65. In addition, the SH3 domain contains the autophosphorylation site Y223. Finally, BTK has a kinase domain that harbors the catalytic capacity of BTK, containing the phosphorylation site Y551 that activates the protein. The kinase domain is also the target site of BTK inhibitors.

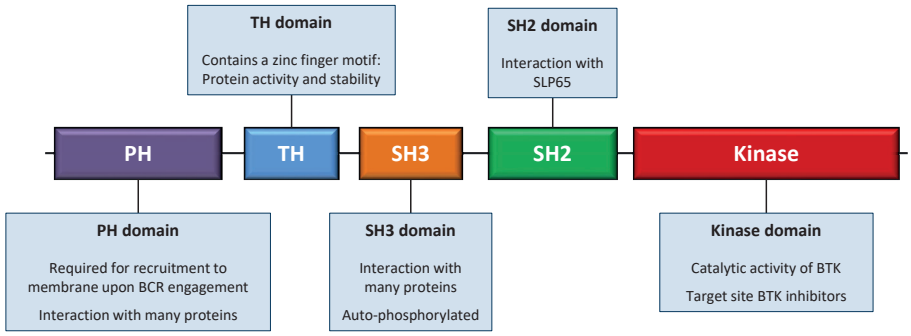

Figure 2. BTK protein structure.

BTK plays a key role in BCR signaling (**Figure 3**)(Aoki et al. 1994; de Weers et al. 1994), which provides crucial survival signals in circulating mature B cells and - upon antigen recognition - induces proliferation and terminal differentiation of B cells (Corneth et al. 2016). Moreover, BTK signals downstream of the pre-BCR, which is an immature form of the BCR that acts as a checkpoint during B cell development in the bone (Corneth et al. 2016). Upon triggering of the BCR, the Src family tyrosine kinase Lyn will phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR complex components CD79a/b, resulting in the recruitment of another tyrosine kinase called Syk. Lyn also phosphorylates the cytoplasmic tail of CD19, which is a co-receptor of the BCR. This will lead to the recruitment and activation of phosphoinositide 3-kinase (PI3K). Activated PI3K generates PIP_3, which can recruit BTK to the cell membrane by interacting with the PH domain. Subsequently, Lyn and activated Syk together can fully activate BTK by phosphorylation at Y551. In addition, activated Syk will phosphorylate the linker SLP65, which is crucial for the formation of a signaling complex. BTK and its downstream target phospholipase Cγ2 (PLCγ2) will then be able to bind phosphorylated SLP65 with their

SH2 domains, and BTK can phosphorylate PLCγ2. This multiprotein complex is involved in activation of many pathways, such as calcium mobilization, mitogen-activated protein kinases (MAPK) signaling, NF-κB translocation and actin remodeling. Furthermore, it has been shown that BTK can interact with other signaling molecules such as Akt, which can also be initiated by PI3K-mediated activation upon CD19 stimulation. Akt signaling induces survival and proliferation of B cells (Corneth et al. 2016).

Upon BCR signaling, BTK protein levels in B cells are increased. It is not fully understood how BTK levels are regulated, but it is clear that BTK can induce its own transcription in an NFκB dependent way, and that microRNA-185 is involved in post-translational regulation (Corneth et al. 2016). Regulation of BTK levels is vital for normal B cell function. Sub-physiological expression levels of BTK cannot restore the BTK-deficient phenotype in mice, whereas physiological levels can. Furthermore, enhanced expression of BTK leads to enhanced activation of B cells and the development of autoimmunity in mice.

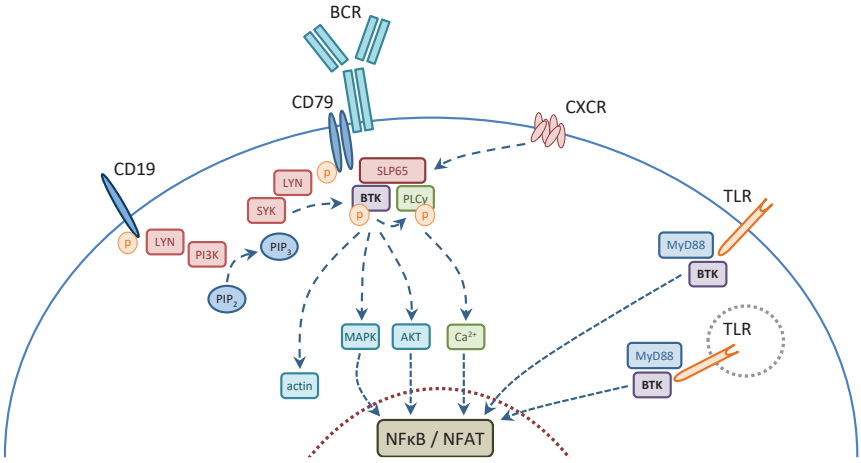

Figure 3. **BTK in BCR signaling.**

2. BTK in other signaling pathways

In addition to BCR signaling, BTK plays a major role in many other signaling pathways (**Table 1**). Toll-like receptor (TLR) function has been described to depend on the expression of BTK (Rawlings et al. 2012). For example, Btk deficient B cells show decreased activation following TLR4 stimulation with LPS compared to normal controls. Upon triggering, TLRs recruit adaptor molecules such as Myeloid differentiation primary response gene 88 (MyD88) or TIR domain-containing adaptor protein inducing interferon-β (TRIF). Signaling via these adaptor proteins leads to activation of interferon regulatory factor 3 (IRF3) and translocation of NF-κB, providing proliferation and survival signals. BTK is able to interact directly with TIR domains of the TLRs, but also with adaptor molecules MyD88 and TRIF

and other downstream signaling molecules, although the domain of BTK that interacts with these molecules is still unknown. Furthermore, BTK has been shown to mediate synergistic signaling between the BCR and TLR9, which is an endosomal TLR that recognizes nuclear material. Synergistic signaling of the BCR with TLRs provides a strong survival signal for B cells and has been linked to development of autoimmune diseases (Rawlings et al. 2012).

Table 1. Btk is involved in signaling pathways downstreamof various receptors on different immune cell types.

Receptor pathway	Cell type(s)
Pre-BCR	Pre-B cells
BCR	B cells
CXCR4	Pre-B, B cells
CD38	Activated B cells
Epo-R	Erythrocytes
TRAIL-R1	Erythrocytes
FcεR	Mast cells, basophils
FCγR	Myeloid cells
GPVI	Platelets
IL-5R	B cells, eosinophils, basophils
IL-6R	Activated B cells, plasma cells
TLR	Myeloid cells, B cells
M-CSFR	Macrophages
CD303 (BDCA-2)	Plasmacytoid dendritic cells
HGF/c-MET	Dendritic cells
fMLFR	Neutrophils

R: receptor; Epo: Erythropoietin; GPVI: collagen receptor glycoprotein VI; IL: Interleukin; TLR: Toll like receptor; TRAIL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand; HGF; hepatocyte growth factor; fMLFR: formyl-methionyl-leucyl-phenylalanine receptor.

BTK is also involved in chemokine receptor signaling (de Gorter et al. 2007), in particular downstream of CXCR4 and CXCR5. Chemokine receptors belong to the family of G protein-coupled receptors and signal via G protein subunits, which can be bound by the PH and TH domain of BTK (Tsukada et al. 1994). Homing of B cells to lymph nodes was hampered in Btk-deficient mice, a process in which chemokine receptors play a key role. Furthermore, BTK inhibitors induce lymphocytosis in CLL patients by drawing malignant cells out of their (survival) niche in the lymph nodes and into the circulation (Hendriks et al. 2014).

BTK-mediated signaling has been described in Fc receptor signaling, which is not limited to B cells, but also affects monocytes and macrophages. Depending on the nature of the Fc receptor, signaling through BTK may activate a B cell, or induce its apoptosis. In addition, BTK may also play a role CD38 and CD40 signaling, however, the exact role of BTK in these pathways is less well defined (Corneth et al. 2016).

3. BTK in immunodeficiency

Mutations in the *BTK* gene in humans are the underlying cause of the severe primary immunodeficiency disease, X-linked aggamaglobulinemia (XLA)(Tsukada et al. 1993; Vetrie et al. 1993). XLA, which was first described in 1952 by Dr. O.C. Bruton (Bruton 1952), is the most common primary immunodeficiency with a reported incidence of 1/380,000 live births in the U.S. BTK has a crucial function in pre-BCR signaling and therefore in the associated checkpoint during B cell development (Corneth et al. 2016). Early in B cell development (**Figure 4**), functional gene recombination events within the Ig heavy chain gene locus result in surface expression of the heavy chain, which marks a crucial checkpoint. In the pre-BCR complex the Ig heavy chain protein is associated with the invariant surrogate light chain proteins that have homology to Ig light chains. Expression of the pre-BCR induces clonal proliferation of large pre-B cells and subsequently their developmental progression to the stage of resting, small pre-B cells. In human pre-B cells, the pre-BCR-mediated expansion and differentiation is crucially dependent on BTK (Corneth et al., 2016). In the bone marrow of boys with XLA the number of pre-B cells expressing intracellular Ig heavy chain is is rather variable, but generally reduced. These pre-B cells are significantly smaller in XLA patients than in healthy controls, which is in agreement with a crucial function of BTK in the induction of proliferative expansion of pre-B cells that Ig heavy chain in their cytoplasm. But even those pre-B cells present in XLA patients appear to have a developmental block, since very few pre-B cells undergo Ig light chain recombination. In healthy individuals, the transition from large cycling to small resting pre-B cells is marked the initiation of Ig κ and λ light chain rearrangement. Following successful Ig light chain rearrangement, the pre-B cells progress to the immature B cell compartment, in which the BCR is checked for autoreactivity. If these immature B cells do not recognize antigen, they leave the bone marrow (**Figure 4**). Taken together, BTK deficiency leads to a severe block in early B cell development in the bone marrow at the pro- to pre-B cell stage, resulting in an almost complete absence (<1%) of mature B cells in the circulation (Pearl et al. 1978) (**Figure 4**). As a consequence, there are no plasma cells and very low levels of immunoglobulins in the periphery. Those few B cells that do remain have an immature IgM^{hi} phenotype and harbor BCRs that are auto- or poly-reactive, however, autoimmune diseases in these patients are relatively rare.

The gene encoding BTK is located on the X-chromosome. Therefore, heterozygous female carriers of a BTK mutation are healthy, whereas affected males present with recurrent infections of the airways, the gastrointestinal tract and the skin caused by parasites and encapsulated bacteria. B cells of female carriers all express the unaffected X-chromosome and have inactivated the affected chromosome. This is explained by the phenomenon of random X chromosome inactivation that takes place in every female somatic cell early in embryogenesis, whereby in female XLA carriers developing B cells that harbor the defective *BTK* gene on their active X chromosome have a selective disadvantage. This is not the case for other cell types that express BTK, indicating that the defect in XLA is B cell-intrinsic. Furthermore, because T cells and NK cells do not express BTK and are therefore unaffected, viral infections do not cause severe problems in patients (Corneth et al. 2016).

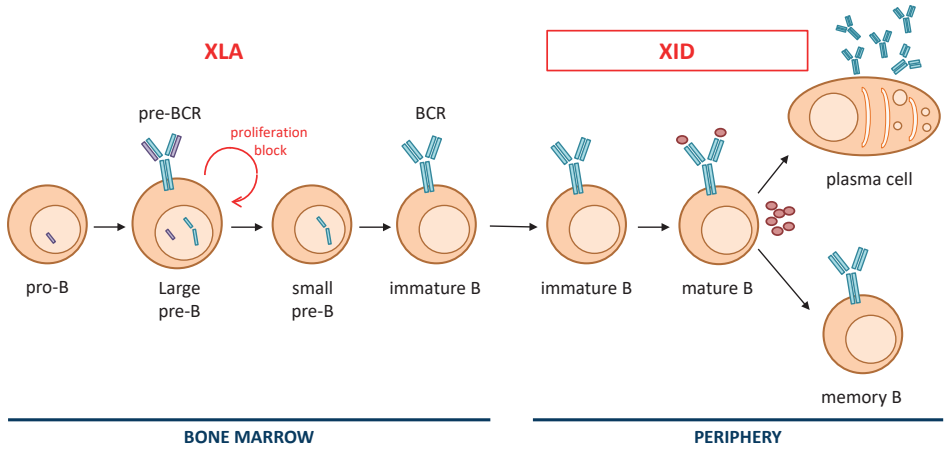

Figure 4. **B cell development. Defects in XLA and Xid are indicated.**

XLA is a very heterogeneous disease. Many mutations that cause loss of function of BTK have been described in all domains, except the SH3 domain containing the autophosphorylation Y223 tyrosine residue. In addition, no correlations have so far been made between specific mutations and clinical or immunological symptoms. XLA patients require life-long treatment with intravenous Ig and antibiotics, but when on sufficient treatment they are relatively healthy, indicating that the effects of loss of BTK are mostly restricted to humoral immunity (Corneth et al. 2016). It has been proposed that – based on promising findings in animal models - that XLA forms a good candidate for gene therapy replacing current non-curative treatment.

In contrast to human XLA patients, *Xid* CBA/N mice, which harbor a mutation in the Btk gene, present with the milder X-linked immunodeficiency (XID) phenotype (Amsbaugh et al. 1972). Homozygous Btk deficient mice show normal B cell development in the bone marrow. However, heterozygous females show loss of Btk-deficient B cells beyond the pre-B cell stage, indicating a selective advantage of Btk-sufficient B cells similar to human B cells (Hendriks et al. 1996). Transition through the pre-B cell stage is delayed in Btk-deficient compared to wild type B cells, consistent with the role for Btk at the pre-BCR checkpoint. Furthermore, Btk is actively involved in light chain rearrangement, and κ and λ immunoglobulin light chain locus rearrangement are reduced in Btk deficient B cells (Corneth et al. 2016).

B cells are present in Btk-deficient mice in the circulation, although they are ~50% reduced in number compared to normal control mice (**Figure 4**). They retain an immature $IgM^{hi}IgD^{lo}$ phenotype and show impaired activation and differentiation *in vitro*. They fail to proliferate upon IgM or IgD stimulation, and cannot obtain an activated phenotype upon IgM stimulation. BCR-mediated survival signals are decreased in Btk-deficient B cells, and

they are more sensitive to apoptosis due to lower expression of the survival proteins BCL2 and BCL-XL. However, they do respond normally to Phorbol myristate acetate / ionomycin stimulation, which bypasses the BCR (Corneth et al. 2016).

Follicular and marginal zone B cell numbers in the spleen are reduced in Btk deficient mice, although the proportions of these populations are relatively normal. In contrast, B1 B cells, which are a specific subset of self-renewing B cells of mainly fetal origin with a specific BCR repertoire are completely absent in the spleen and peritoneal and pleural cavities. As a consequence, IgM and IgG3 isotype antibody levels are decreased in Btk deficient mice, which can be explained by the lack of natural antibodies produced by B1 cells, but other isotypes are normally present. Btk-deficient mice fail to show a B cell response to thymus independent TI antigens, which is thought to be dependent on B1 cells. In addition, Btk-deficient mice have reduced antigen-specific antibody levels upon primary immunization with thymus dependent TII antigens. However, secondary immunization mounts a normal memory response, suggesting that Btk is not crucial for germinal center, memory B cells or plasma cell formation. In contrast with the observed normal T cell-dependent responses to model antigens in adjuvants, Btk-deficient mice have reduced numbers of GC B cells in their draining lymph nodes following pulmonary infection with influenza virus (Corneth et al. 2016).

As in humans, the defect in Btk deficient mice is restricted to humoral immunity. Infections with pathogens which require the presence of natural antibodies will lead to more severe disease. However, Btk deficient mice will develop less severe disease upon infections with pathogens that induce the production of harmful antibodies, or primarily infect B1 cells (Corneth et al. 2016).

4. BTK in cancer

Btk has been implicated in both murine and human leukemia and lymphoma (Hendriks et al. 2014). Murine Btk-deficient pre-B cells show increased proliferation *in vitro*. Although Btk deficiency alone does not lead to tumor formation *in vivo*, combined deficiency with SLP65 enhances pre-B cell leukemia in mice compared to SLP65 single mutants, showing tumor suppressive capacity of Btk in pre-B cells which was independent of its kinase function. Mutations in BTK have been found in human childhood pre-B cell acute lymphoblastic leukemia (pre-B ALL), but these were all mutations affecting kinase functions of BTK; a single XLA patient with pre-B ALL has been described. On the other hand, overexpression of Btk in murine B cells leads to decreased susceptibility to apoptosis. Again overexpression of Btk alone did not lead to tumor development, but did increase the incidence and mortality rate of mice in a chronic lymphatic leukemia (CLL) mouse model. Interestingly, in this model, deficiency of Btk prevented tumor development, clearly illustrating the differential roles for Btk in pre-B cells and mature B cells. Although it is still unclear whether mutations in BTK can cause B cell tumors in humans, these data show the importance of correct regulation of expression levels of BTK.

BTK protein expression is enhanced in several B cell malignancies, including CLL and mantle cell lymphoma (MCL), and in some patients, phosphorylated BTK is also highly expressed (Hendriks et al. 2014). Because BTK is crucial for B cell survival and proliferation, great effort has been undertaken to develop specific inhibitors targeting BTK. Several of these inhibitors have already shown impressive efficacy in human B cell malignancies *in vitro* and *in vivo*. Treatment with ibrutinib, the first FDA approved small molecule inhibitor of BTK approved in the clinic, significantly reduced survival and proliferation of primary tumor cells and tumor cell lines *in vitro*. Phosphorylation of PLCγ2, Akt and ERK, important downstream targets of BTK, was reduced in these cultures. Importantly, not only viability of the cells was affected, but also adhesion and migration, through inhibition of BTK dependent chemokine receptor signaling. In CLL and MCL, this is considered the main mode of action of BTK inhibition (Byrd et al. 2013; Wang et al. 2013). Upon BTK treatment, patients exhibit lymphocytosis, caused by an egress of malignant cells from the lymph nodes. Upon leaving the lymph nodes, tumor cells lose important survival signals provided by stromal cells, rendering them susceptible to apoptosis. In addition, tumor cells lose the cell intrinsic proliferation signals mediated through BCR signaling induced phosphorylation of PLCγ2 and Akt, which may also contribute to the successful elimination of cancer cells (Hendriks et al. 2014).

BTK inhibition may also affect TLR signaling or the interaction between BCR and TLR signaling in tumors (Hendriks et al. 2014). In Waldenström's macroglobulinaemia patients, who frequently harbor an activating mutation in MyD88, BTK is often constitutively active. BTK inhibition was shown to limit interaction of BTK and MyD88 in these patients and to induce apoptosis *in vitro*. However, *in vivo*, it is unclear whether BTK inhibition works primarily through inhibition of the TLR signaling pathway, or whether inhibition of the BCR and chemokine receptors is more important.

Apart from affecting tumor cells, BTK inhibition also limits tumor development by targeting the tumor cell survival niche (Hendriks et al. 2014). In multiple myeloma (MM), a plasma cell derived tumor, BTK inhibition crucially affects osteoclasts in the bone marrow that provide essential survival signals to MM cells, including CCL3, an important marker for disease progression. Ectopic expression of BTK was found in non-hematological tumors, including prostate cancer and breast cancer cell lines. Inhibition of BTK in these tumors shows promising results, suggesting that the role of BTK in aberrant cell proliferation is not limited to the hematopoietic lineage.

Although BTK inhibition has shown impressive efficacy in lymphoma patients, not all patients respond well to this therapy. In some tumors, mutations in BTK or other genes were shown to promote resistance to BTK inhibitors (Chiron et al. 2014). These mutations may be present before onset of treatment, but mutations have been shown to arise during treatment, although it is unclear whether treatment itself may promote these mutations. To overcome this therapy resistance, new treatment strategies are being developed, including novel more selective inhibitors, including Acalabrutinib (Byrd et al. 2016) for treatment of CLL, that are specifically designed to improve on the safety and efficacy of BTK inhibition. Moreover,

inhibitors of multiple pathways are combined and now being tested in the clinic. Indeed combinations of BTK inhibitors with PI3K inhibitors or inhibitors of the Akt pathway have shown better results than mono-treatment (Woyach et al. 2014).

5. BTK in autoimmunity

B cells are involved in many autoimmune diseases, and B cell intrinsic defects have been shown to be sufficient to induce autoimmunity in mice (Corneth et al. 2016). The discovery that BTK plays a crucial role in the selection of pre-B cells during development and in activation of mature B cells in the periphery prompted studies into the role of BTK in autoimmune diseases. Early studies in mice showed an important role for Btk in the formation of auto-reactive antibodies. When the XID mutation was crossed into the lupus-prone NZWxNZB or MRL.lpr/lpr background, spontaneous autoantibody formation and kidney damage were dramatically reduced. Interestingly, stimulation of B cells from these mice with TLR ligands did induce the production of non-autoreactive antibodies. Similarly, Btk deficiency in the NOD mouse model of diabetes prevented the development of autoantibodies without affecting total antibody levels in serum of mice (Corneth et al. 2016).

Studies with NOD mice harboring an insulin-reactive BCR transgene showed that BTK deficiency affects only mature cells in the periphery, as insulin specific pre-B cells in the bone marrow were unaffected. Similarly, expression of low levels of the constitutively active E41K-BTK mutant, which allows for B cells survival past the pre-B cell stage, enhances B cells survival and activation, leading to a rapid enhanced formation of IgM plasma cells producing autoreactive antibodies. These data indicate that BTK expression and activation levels affect mature B cells and may be involved in peripheral B cell selection (Corneth et al. 2016).

Overexpression of human BTK specifically in B cells in mice leads to a spontaneous autoimmune phenotype resembling systemic lupus erythematosus (SLE) and Sjögren's syndrome. Mice first develop spontaneous germinal centers and plasma cells in the spleen, followed by an increase in memory B cells and plasma cells in the bone marrow. Plasma cells produce autoreactive antibodies leading to antibody deposition in the kidneys and immune infiltrates of the kidneys, salivary glands and lungs. This phenotype depends strongly on T cells, as crosses with CD40 ligand deficient mice, inhibiting B-T cell interaction, abrogated the disease. However, these mice did still develop IgM autoreactive antibodies, suggesting that BTK may be involved in a two-step induction of autoreactivity, by enhancing survival of autoreactive B cells and subsequent induction of the germinal center response. Importantly, the phenotype depended on BTK kinase activity, as a kinase inactive BTK mutant did not develop autoimmunity, and inhibition of BTK kinase activity by ibrutinib prevented the formation of spontaneous germinal centers (Corneth et al. 2016).

Because these studies show the involvement of Btk in B cell mediated autoimmunity, Btk inhibition has been studied extensively in mouse autoimmune models. In collagen-induced arthritis, a mouse model for rheumatoid arthritis (RA), Btk inhibition before onset

of disease completely prevented arthritis development, and treatment after onset greatly decreased disease severity. Similar to Btk-deficient mice in autoimmune models, inhibition of Btk affected the formation of autoantibodies, but non-autoimmune antibodies in serum remained present. Of note, the efficacy of Btk inhibition in this model may be partly due to the important role for Fc-mediated signaling in monocytes and macrophages, which is also dependent on Btk. In addition, in several models of murine lupus, Btk inhibition limits the formations of autoantibodies and prevents or decreases levels of kidney damage, significantly improving survival of mice (Honigberg et al. 2010; Corneth et al. 2016).

BTK expression in human autoimmune patients has not yet been extensively studied, although some studies indicate a pathogenic role for BTK. In RA patients, phosphorylated BTK levels correlate with rheumatoid factor (RF) titers and are increased in anti-citrullinated-protein-antibody (ACPA) positive patients, indicating a link between activation of BTK and autoantibody production. In addition, BTK signaling was required for IL-21 expression by B cells, which is important for the maintenance of tertiary lymphoid follicles involved in autoantibody production. Furthermore, upstream signaling molecule SYK was more highly expressed in blood of RA patients. In SLE patients, expression of downstream target ARID3A was shown to be correlated with disease severity. The promising results of BTK inhibition in mouse autoimmunity studies and the finding that BTK inhibitors are very well tolerated by leukemia patients with limited side effects have prompted several clinical trials of BTK inhibitors in human autoimmune diseases that are currently underway (www.clinicaltrials.gov).

Summary

BTK is a signaling molecule expressed in many hematopoietic cells, but most crucially involved in B cell development in the bone marrow and activation and terminal differentiation of peripheral B cells. As it is involved in many signaling pathways, deregulated BTK expression can lead to a number of clinical diseases. BTK deficiency in humans leads to XLA, a severe X-linked immunodeficiency affecting humoral immunity. Male with this defect suffer from severe recurrent infection due to loss of peripheral B cells. In mice, Btk deficiency leads to a milder phenotype with decreased numbers of B cells and impaired humoral immunity. BTK signaling is crucially involved in the proliferation, migration and adhesion of leukemic cells in several B cell derived malignancies. Inhibition of BTK in these patients leads to expulsion of cells from their survival niche and discontinuation of intrinsic survival signals, and is now a very successful new therapeutic approach in the clinic. In addition, increased expression of Btk in mice can induce an autoimmune phenotype, and BTK has been implicated in human autoimmune diseases. Ongoing clinical trials will reveal the potential of BTK inhibitors in autoimmune patients.

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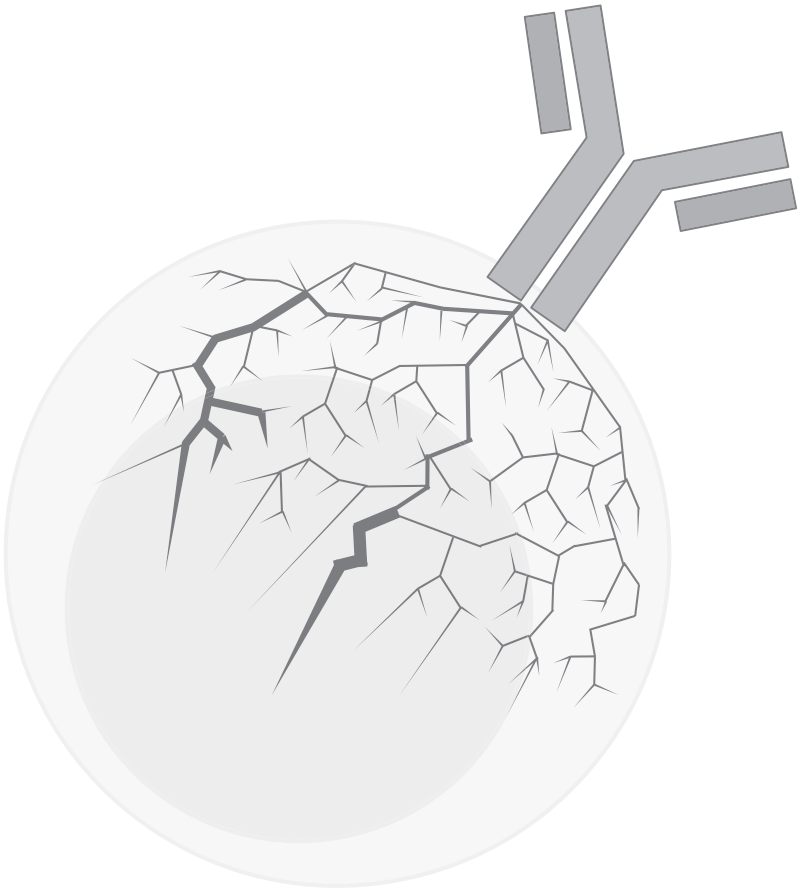

CHAPTER 1.2

Aims and outline of this thesis

AIMS AND OUTLINE OF THIS THESIS

Taken together, Bruton's tyrosine kinase is a crucial signaling molecule in the BCR pathway. Regulation of Btk protein expression levels is essential for appropriate B cell function, because lack of Btk causes immunodeficiency and overexpression of Btk leads to development of autoimmune disease[1-4].

In this thesis, we aimed to investigate the role of Btk in BCR signaling and other pathways that contribute to B cell activation and survival. In the current chapter (**Chapter 1.1**), we introduced the molecular characteristics of Btk and its role in health and disease. In **Chapter 2**, we discuss the important role of Btk in autoimmunity, in the context of the increasing evidence that dysregulated Btk activity might contribute to the development of autoimmune disease.

BCR signaling is crucial for development, proliferation, differentiation and survival of B cells[5]. During the past years, major methodological advancements have improved the sensitivity and depth of cell signaling analysis. Traditional western blotting methods are increasingly being replaced by phosphoflow, a flow cytometry-based technique to detect protein phosphorylation with high sensitivity at the single-cell level. In order to study Btk function and BCR signaling kinetics, we aimed to further improve existing protocols. In particular, our key objective was to reliably measure phosphorylation of various signaling molecules in different B cell subpopulations from several anatomical compartments without pre-sorting of cells. A detailed description of procedures to measure phosphorylation using phosphoflow is provided in **Chapter 3**.

Although the role of Btk in B cells has been extensively studied, the role of close family members such as Tec kinase remain largely unclear. It was previously shown that Tec kinase has the ability to partly compensate for loss of Btk activity in B cell differentiation[6], but the underlying mechanisms are largely unknown. It is crucial to better study Tec function, also because some Btk inhibitors that are currently used in the clinic are thought to impair the kinase function of Tec as well. Therefore, in **Chapter 4**, we investigate whether Tec is required for BCR signaling and how Tec regulates BCR signaling in the presence and absence of Btk kinase function in mice. Furthermore, we evaluated whether absence of Tec impairs B cell function and activation using *in vitro* and *in vivo* models.

Activation of downstream mediators involved in B cell survival were previously shown to be dampened in absence of Btk protein[7, 8]. Indeed, Btk inhibition is an effective therapy in various diseases. However, the consequences of direct and indirect effects of Btk-kinase inhibition on the BCR signaling pathway in the light of positive and negative feedback mechanisms of healthy and malignant B cells remained elusive. In **Chapter 5**, we investigate the role of Btk protein and effects of inhibition of Btk-kinase activity in major components of the BCR signaling pathway in B cell subpopulations, both in humans and in mice. We measured phosphorylation upon BCR engagement using phosphoflow cytometry and investigated whether absence of Btk or lack of kinase function resulted in altered responsiveness of BCR signaling molecules expressed up- and downstream of Btk protein.

In addition to its role in BCR signaling, Btk protein is also involved in other signaling routes related to B cell activation. It was previously shown that Btk protein is upregulated upon stimulation with Toll-like receptor (TLR) ligands and CD40 co-stimulation mimicking T cell-help[3]. In **Chapter 6**, we aim to the identify the importance of CD40:CD40L interaction for the development of Btk-mediated autoimmunity. To this end we evaluated the onset of autoimmune disease in CD40L-deficient Btk-overexpressing mice. As TLR signaling was previously shown to be crucial for the development of autoimmune disease in several mouse models[9-13], we investigate in **Chapter 7** whether TLR signaling is required for the Btk-mediated autoimmune phenotype using mice deficient for MyD88, which is a critical linker molecule downstream of TLRs.

The importance of Btk and BCR signaling is evident in chronic lymphocytic leukemia (CLL), as inhibition of Btk and other BCR signaling molecules provides very effective treatment in these patients[14-17]. Because the involvement of signaling routes such as CD40 and TLR signaling to CLL B cell activation is not well established, we aim to better define the contribution of BCR, CD40 and TLR signaling in CLL pathogenesis in **Chapter 8**. We therefore performed genome-wide RNA sequencing on murine CLL B cells and compared the CLL signature genes with differentially expressed genes upon stimulation with anti-IgM, CD40 or TLR4 as well as Btk overexpression.

The BCR mutational status is a crucial marker for disease progression as CLL patients with unmutated BCRs (U-CLL) have a worse prognosis compared to patients with mutated BCRs (M-CLL)[18, 19]. Although some differences in BCR signaling between U-CLL and M-CLL have been described [20-22], our understanding of the connection between BCR signaling molecules and BCR responsiveness in the two forms of CLL remains limited. In **Chapter 9**, we therefore compared basal phosphorylation and responsiveness of the major pathways involved in BCR signaling between circulating malignant B cells from U-CLL and M-CLL patients.

Finally, in **Chapter 10** an integrated overview on our knowledge on the regulation of Btk activity in health and disease is provided and the implications of our findings for the field of signal transduction in B cell biology are discussed.

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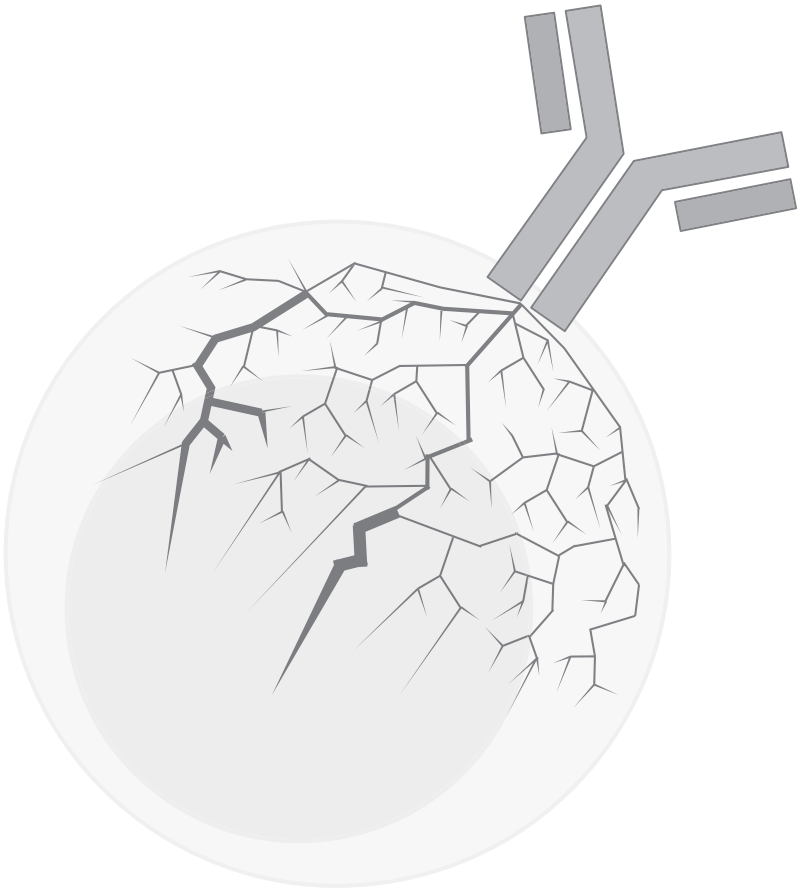

CHAPTER 2

The role of Bruton's Tyrosine Kinase in immune cell signaling and systemic autoimmunity

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ABSTRACT

Bruton's tyrosine kinase (BTK) is an intracellular signaling molecule first identified as the molecule affected in X-linked agammaglobulinemia (XLA) patients, who almost completely lack peripheral B cells and serum immunoglobulins. BTK is crucial for B cell development and various B cell functions, including cytokine and natural antibody production. Importantly, it is also expressed in numerous other cells, including monocytes, macrophages, granulocytes, dendritic cells and osteoclasts. A few rare cases of autoimmune disease in XLA patients have been described. Interestingly, increased BTK protein expression in patients with systemic autoimmune disease appears to be correlated with autoantibody production. In addition, BTK may promote autoimmunity as an important driver of an imbalance in B-T cell interaction. Because of this overwhelming evidence of a pathogenic role of BTK in autoimmunity, several clinical trials in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients with BTK inhibitors are currently running. Here, we review BTK function in different signaling pathways and in different cell lineages, focusing on the growing body of literature indicating a critical role for BTK in autoimmunity. We will also discuss BTK and the promising results of BTK inhibition in animal models of autoimmune disease.

INTRODUCTION

Systemic autoimmunity is a condition characterized by an aberrant immune response against self-antigens. Rheumatoid arthritis (RA), Sjögren's syndrome (SjS), inflammatory bowel disease (IBD) and diabetes mellitus type I (T1D) are among the most common autoimmune diseases.[1] Around 23.5 million people in the USA have an autoimmune disorder (www.niehs.nih.gov), and the incidence is slowly rising with an estimated 7.6-9.4% of people suffering from autoimmunity worldwide.[1] Autoimmune diseases are chronic and although symptoms can be suppressed by medication, they cannot be cured, placing a heavy burden on society. Understanding the pathogenesis of these diseases is essential to improve current treatment strategies and to identify new therapeutic targets. A breakdown of B cell tolerance, resulting in autoantibody production, is a major pathogenic event in systemic autoimmunity, and B cell depletion therapy was shown to be effective in several autoimmune diseases.[2-4] The importance of B cell receptor (BCR) signaling in this process is underlined by genetic studies showing that B cell-intrinsic effects are central to autoimmune disease development.[5] Among the risk loci identified by genetic studies are genes that encode intracellular signaling molecules downstream of the BCR or molecules that regulate BCR signaling, including BLK, Lyn, BANK1 and PTPN22.[6-12]

A crucial signaling molecule downstream of the BCR is Bruton's tyrosine kinase (BTK). Located on the X chromosome, BTK was first identified as the gene mutated in the primary immunodeficiency X-linked agammaglobulinemia (XLA).[13, 14] In XLA patients, the absence of functional BTK protein in B cells leads to an almost complete arrest in early B cell development in the bone marrow at the pre-B cell stage.[15] As a consequence, hardly any circulating B cells are present and antibody levels are very low in the serum of these patients, who suffer from recurring infections from an early age.[16, 17] BTK also plays an important role in BCR signaling in mature peripheral B cells. Mouse studies have shown that BTK is crucial for peripheral B cell differentiation and survival, as well as for their activation upon BCR ligation.[18-21] Furthermore, BTK inhibition completely blocks BCR signaling in human primary B cells.[22] Increases in BTK expression or activation has been shown in chronic lymphocytic leukemia (CLL) patients and treatment with BTK inhibitors is very effective in this disease.[22-24] Increasing evidence shows that dysregulation of BTK may also be involved in systemic autoimmunity. Increased expression of BTK in B cells in transgenic mice induces a spontaneous autoimmune phenotype, and in RA and SjS patients BTK levels in peripheral B cells are increased.[25, 26] These findings make BTK an interesting new target in the treatment of autoimmunity.

In this review, we will discuss BTK function as a critical tyrosine kinase downstream of the BCR, as well as its involvement in other signaling pathways in various cell lineages of the immune system. Hereby, we will highlight the evidence for involvement of BTK in autoimmune disorders and discuss opportunities for BTK targeting as a therapeutic strategy.

I. BTK PROTEIN AND BCR SIGNALING

A. BTK protein structure and its role in B cell development

Bruton's tyrosine kinase (BTK) is a protein kinase consisting of 659 amino acids. It belongs to the strongly conserved family of TEC non-receptor kinases, which includes four additional members: tyrosine kinase expressed in hepatocellular carcinoma (TEC), inducible T cell kinase (Itk), bone marrow-expressed kinase (Bmx) and resting lymphocyte kinase (Rlk). BTK is most similar to Itk and TEC, containing five different domains: a plekstrin homology (PH), TEC homology (TH), Src homology (SH) 2 and 3 and kinase domain.[27, 28] BTK is expressed in all hematopoietic cells except for T cells and is shown to be especially crucial for B lymphocytes.[29]

Signaling via the BCR is vital for B cell survival during B cell development, as BCR affinity - and thereby signaling strength - determines B cell selection and survival. As mentioned above, XLA patients with mutations in the *BTK* gene show a severe deficiency of peripheral B cells and immunoglobulins (Igs),[16] due to an arrest of B cell development around the large pre-B cell stage.[15] The CBA/N mouse strain harbors a loss-of-function mutation in the *Btk* gene.[30-32] These BTK-mutant mice are known as *Xid* (X-linked immunodeficiency) mice, and do not appear to have a detectable defect in the bone marrow B cell development. Rather, these mice show an impaired differentiation of mature peripheral B cells characterized by a ~50% reduction in total splenic B cells.[32] In particular, there is a severe decrease in long-lived $IgM^{low}IgD^{high}$ mature B cells, illustrating that BTK signaling is crucial for maturation and maintenance of mature peripheral B cells. The transitional block from $IgM^{+}IgD^{-}$ immature B cells to $IgM^{low}IgD^{high}$ mature B cells in BTK-deficient mice is in parallel with findings in XLA patients in which residual B cells are also $IgM^{high}IgD^{low}$.[16] In addition, *Xid* mice lack the population of B-1 cells in the peritoneal and pleural cavities.[32] Because this cell population is important for the production of natural antibodies and is involved in T-cell independent B cell responses, *Xid* mice have severely reduced IgM and IgG3 levels in the serum. In line with the finding that B cells from *Xid* mice still have the capacity to form germinal centers, T-cell dependent immune responses to model antigens appear normal and serum levels of Ig isotypes other than IgM and IgG3 are unaffected.

Next to natural-occurring mutations in BTK as seen in *Xid* mice, several groups created C57BL/6 mice with a targeted deletion of the *Btk* gene.[20, 21] These BTK-deficient mice show impaired differentiation from large pre-B to small pre-B cells in the bone marrow and have a reduced capacity to open the *Ig*λ light chain locus for recombination and therefore show reduced λ light chain usage.[33-35] However, the effect on B cell development is less severe than seen in XLA patients, because BTK-deficient mice present with only a partial block at the pre-B cell stage. Like *Xid* CBA/N mice, also BTK-deficient C57BL/6 mice have peripheral B cells that show severe defects in their differentiation, survival and activating capacity.[20, 21, 32, 35, 36] In addition, both *Xid* and BTK-deficient B cells are not able to induce cyclin D2 expression upon BCR engagement, and as a result they do not reach the S-phase of the cell cycle and do not proliferate.[20, 21, 32, 35-37]

As mentioned earlier, BTK-deficient mice show only a partial block in B cell development at the large to small pre-B cell transition. However, when BTK is knocked-out in combination with either TEC kinase or the SH2 domain leukocyte protein of 65 kD (SLP-65, also known as BLNK), this results in an almost complete arrest at the pre-B cell stage.[38, 39] In addition, BTK-deficient B-lineage cells are outcompeted by BTK-sufficient B cells in heterozygous $BTK^{+/-}$ female mice, as a significant disadvantage was observed for BTK-deficient cells at the transition from pre-B to the $IgM^{hi}IgD^{-}$ immature B cell stage in the bone marrow.[21] This disadvantage was virtually complete in mature splenic or blood B cells in mice,[21] consistent with findings in the peripheral blood B cell compartment in carrier mothers of XLA patients.[40, 41]

In summary, these findings in human and mouse demonstrate that the presence of BTK in the context of BCR-mediated survival signals is crucial for normal B cell development and maturation.

B. BTK activation and the IgM-BCR signaling cascade

BTK is located in the cytoplasm in resting B cells, but can be recruited to the cell membrane after BCR stimulation. Upon triggering of the surface IgM BCR, the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR co-receptors CD79a/b are phosphorylated by Lyn, resulting in the recruitment and activation of Syk (**Figure 1A**). Simultaneously to BCR activation, Lyn will phosphorylate the cytoplasmic tail of the co-receptor CD19, which results in recruitment and activation of phosphoinositide 3-kinase (PI3K). PI3K can also be activated by B cell adaptor for PI3K (BCAP), illustrating that BCR signaling can target PI3K activity independent of CD19.[42] PI3K generates PIP_3, which is essential in recruiting BTK to the cell membrane by interacting with the BTK PH domain.[43, 44] Upon recruitment, activated Syk and Lyn can fully activate SLP-65. This results in phosphorylation of BTK in the kinase domain at Y551, which subsequently leads to full activation of BTK and auto-phosphorylation at Y223 in the SH2 domain.[45-47] Upon full activation and with SLP-65 as a linker protein, BTK can activate Phospholipase Cγ2 (PLCγ2),[48] leading to the activation of several downstream signaling pathways (**Figure 1A**).

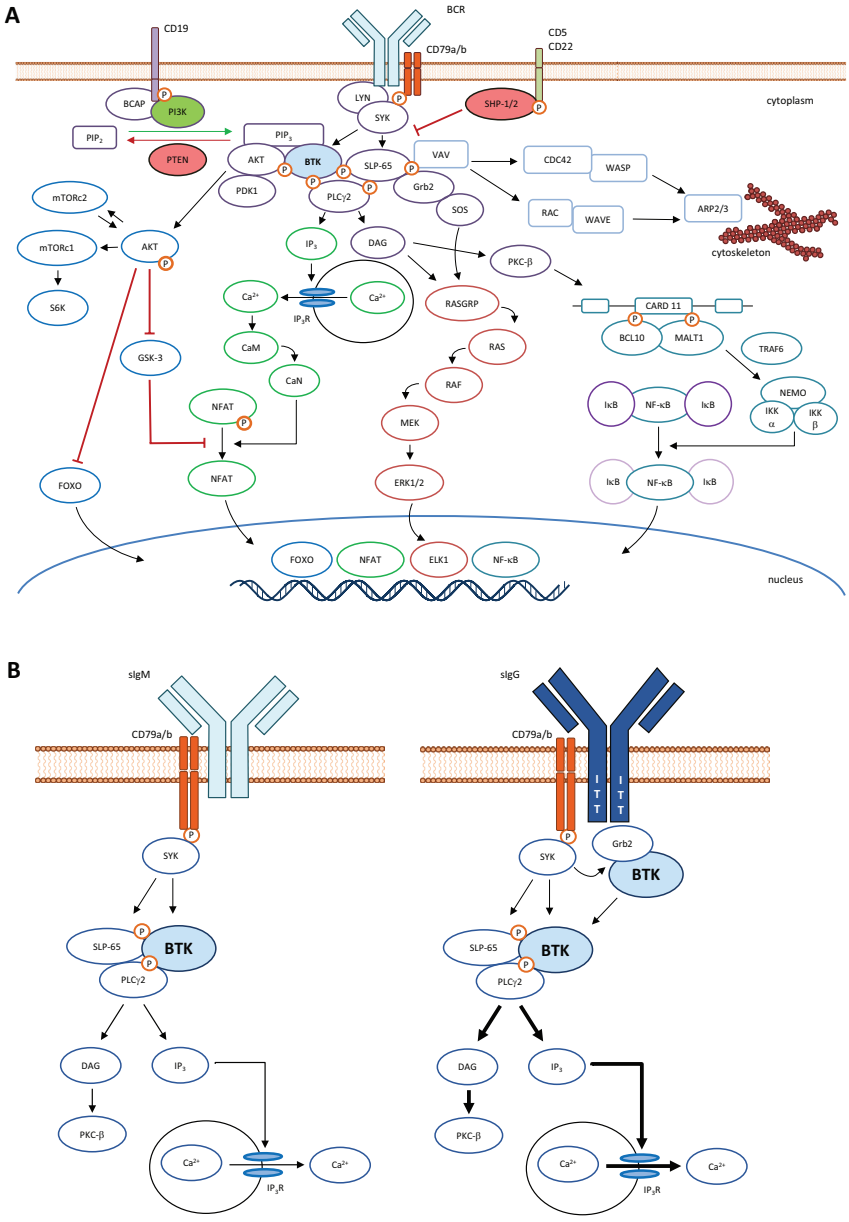

Figure 1. An overview of signaling downstream of the B cell receptor (BCR)

A) BCR signaling cascade showing several important downstream signaling routes. Triggering of the BCR results in the formation and activation of a BTK-PLCγ2-SLP-65 micro-signalosome. This leads to activation of several downstream signaling cascade, such calcium mobilization (green), cytoskeletal rearrangements (grey), AKT-mediated signaling (blue), mitogen-activated protein kinase (MAPK) activation (red), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (turquoise). B) BCR signaling downstream of surface IgM (sIgM; left panel) and surface IgG (sIgG; right panel) BCRs. Bold arrows are indicative of increased signaling in IgG BCR signaling. Immunoglobulin tail tyrosine (ITT) motifs are shown specifically in sIgG BCRs.

C. Class-switched BCR signaling

In contrast to IgM-BCR signaling, the kinetics and signaling cascade of class-switched BCRs were shown to be slightly different. In IgG and IgE signaling, it was shown that the heavy chain domains contain longer cytoplasmic tails compared to IgM and IgD BCRs.[49] These cytoplasmic tails contain phosphorylation motifs called immunoglobulin tail tyrosine (ITT) motifs, which can recruit adaptor protein Growth factor receptor-bound protein 2 (Grb2) via their SH2 domains post ITT phosphorylation by Syk (**Figure 1B**).[49] ITT-bound Grb2 binds BTK, thereby amplifying the response triggered by IgG engagement.[50] Using ITT mutant IgG1 B cells it was shown that the ITT domain is crucial for a competitive advantage of IgG switched B cells *in vivo*,[51] providing an explanation for the requirement of enhanced signaling specifically in switched-memory B cells (**Figure 1B**). In contrast to the signaling domains of IgG and IgE, no studies have been reported on IgA-mediated BCR signaling, so the intracellular signaling mechanism remains to be elucidated.

D. Calcium mobilization

Upon BCR-mediated activation of PLCγ2, PIP_2 can be cleaved into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 can bind to the IP_3 receptor (IP_3R), facilitating the influx of calcium into the cytoplasm (**Figure 1A**). This will activate signaling via Calmodulin (CaM), subsequently binding and activating the phosphatase Calcineurin (CaN). Activated CaN dephosphorylates Nuclear Factor of Activated T cells (NFAT), leading to a conformational change, revealing NFAT's nuclear localization sequence.[52] This allows NFAT to translocate to the nucleus, while in resting conditions it is retained within the cytoplasm due to it constitutive phosphorylated nature. Importantly, BTK-deficient cells showed impaired activation of NFAT upon BCR-mediated signaling.[53] NFAT has been shown to be a crucial transcription factor for controlling B cell activation status, as B-cell restricted NFATc1 deficiency causes dysregulation in B cell cytokine production and class-switching.[54, 55]

E. MAPK and NF-κB signaling

DAG and calcium influx can together lead to the activation of PKC-β, a crucial signaling molecule in the activation of many signaling cascades. PKC-β can phosphorylate caspase-recruitment domain (CARD)11, which in turn can bind B-cell lymphoma 10 (BCL10) and Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1).[56] This complex will recruit Tumor necrosis factor receptor-associated factor 6 (TRAF6), which activates the IκB kinase (IKK) complex by non-degradative ubiquitination.[57, 58] The activated IKK complex, consisting of an α, β , and γ (NEMO) subunit, can subsequently mediate in the phosphorylation and ubiquitination-mediated degradation of IκB.[59] The degradation of IκB allows NF-κB to translocate to the nucleus, resulting in signals associated with B cell survival (**Figure 1A**). When mice are deficient for BTK, BCR-mediated NF-κB activation does not occur, demonstrating the key role of BTK in NF-κB signaling.[60, 61]

Activation of mitogen-activated protein kinase (MAPK) signaling has been shown to depend to protein kinases in the BCR pathway, especially Syk, Lyn and BTK.[62] The MAPK signaling cascade is initiated by DAG-activated RASGRP (RAS guanyl-releasing protein), which functions as a guanine nucleotide exchange factor (GEF) specifically activating RAS. Alternatively, Grb2 can be phosphorylated by SLP-65.[63] This subsequently activates the GEF Son of Sevenless (SOS), which acts on Ras-GTPases leading to activation of Ras.[64] Upon activation of Ras, Raf and MEK will get activated, eventually leading to the phosphorylation and translocation of extracellular signal-regulated protein kinase 1/2 (Erk1/2) to the nucleus. Erk has been shown to be essential for pre-B cell survival by contributing to phosphorylation of transcription factor Elk1 and resulting in transcription of Myc,[65] contributing to stimulation of B cell proliferation.

F. Cytoskeleton organization and rearrangement

The activation of cytoskeleton remodeling is crucial for antigen processing and receptor re-localization, as super resolution microscopy revealed that nanoscale reorganization of receptor complexes facilitates downstream signaling upon activation via the BCR.[66, 67] SLP-65 and CD19 activation are essential for activation and phosphorylation of VAV. Activated VAV associates with WAS protein and CDC42 or RAC and WAVE,[68] resulting in activation of the cytoskeleton machinery by targeting ARP2 and ARP3 (**Figure 1A**). Severe problems in B cell activation can occur when cytoskeletal reorganization is hampered, as illustrated by the phenotype of the primary immunodeficiency Wiskott-Aldrich syndrome (WAS).[69] B cells from WAS patients and total WAS protein-deficient mice showed reduced migrating capacity.[70] Moreover, B cell-specific WAS protein knock-out mice showed a markedly reduced marginal zone B cell population, increased splenic germinal center B cells and plasma cells and other features that are indicative of autoimmune disease.[71] B cell-specific WAS protein knock-out mice showed altered repertoire selection in transitional B cells compared to wild-type mice,[72] illustrating how crucial control of cytoskeletal mobilization is to normal B cell function.

G. AKT signaling

Next to recruiting BTK, PIP_3 also binds the PH domain of protein kinase B (AKT) (**Figure 1A**), which can be fully activated by PI3K, PDK1 and mammalian target of rapamycin complex 2 (mTORc2).[73, 74] There is evidence that BTK may directly interact with AKT and that BCR-mediated AKT activation partially relies on BTK and Syk.[75, 76] In addition, we have recently shown that AKT signaling is increased in TEC-deficient mice after BCR stimulation, and that phosphorylation of the S6 kinase protein, which is downstream AKT, was dependent on BTK-kinase activity.[77] This may implicate competition between BTK, TEC and AKT for PIP_3 recruitment sites, and that BTK-mediated AKT phosphorylation occurs more efficiently in the absence of TEC. However, the exact mechanism of interaction and activating capacity of AKT by BTK remains to be defined. The AKT signaling pathway has many downstream effects, promoting survival and proliferation. AKT signaling enhances cellular survival

by inhibiting nuclear localization of the pro-apoptotic fork head box O (FOXO) protein, which induces a pro-apoptotic transcriptional signature (**Figure 1A**). In addition, AKT can inhibit the dephosphorylation of NFAT by activating the kinase GSK-3 and promote protein synthesis via mTOR complex 1 (mTORc1) and S6 kinase (**Figure 1A**). AKT signaling is controlled by phosphatase activity of phosphatase and tensin homolog (PTEN), which may act as a negative regulator of AKT signaling by dephosphorylating PIP_3. PTEN-deficient B cells show highly active AKT signaling, leading B-cell hyperproliferation, enhanced resistance to apoptosis and to *in vivo* production of auto-antibodies.[78] In addition, PTEN was shown to be more highly expressed in immature B cells, resulting in lower AKT levels and increased sensitivity to apoptosis.[79] This suggests that negative selection of peripheral B cells is associated with downregulation of PIP_3 levels and AKT-mediated survival.

I. BTK IN OTHER SIGNALING PATHWAYS

As extensively discussed above, BTK is a crucial signaling molecule downstream of the BCR. However, BTK is also expressed downstream of many other receptors, including pattern-recognition receptors (PRRs), chemokine receptors and Fc receptors.

A. Toll-like receptors

PPRs are germline-encoded receptors, recognizing specific molecular signatures derived from microbial origin or self-derived molecules from damaged cells. One of the most well-known and best defined group of PRRs is the family of Toll-like receptors (TLRs). TLRs are composed of a horseshoe-like ectodomain with leucine-rich repeats that recognize specific structures. The ectodomain is attached to a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates the TLR signaling cascade. To date, 10 human TLRs and 11 functional murine TLRs have been defined (**Table 1**). TLRs expressed on the cell surface mainly recognize bacterial structures, e.g. TLR4 and TLR5 recognize lipopolysaccharide (LPS) and flagellin, respectively. In contrast, TLR3, TLR7/8 and TLR9 are expressed within endosomes and are sensors of nuclear material (**Table 1**).

Table 1. Overview of Toll-like receptors expressed in mice and humans

TLR	Species	Assembly	Location	Adaptor protein	Ligand
TLR1	Mouse Human	Heterodimer with TLR2	Plasma membrane	MyD88, TIRAP	Triacyl lipopeptides
TLR2	Mouse Human	Homodimer, also heterodimerizes with TLR1 and TLR6	Plasma membrane	MyD88	Peptidoglycan
TLR3	Mouse Human	Homodimer	Endosomal	TRIF	dsRNA
TLR4	Mouse Human	Homodimer	Plasma membrane	MyD88, TIRAP TRIF, TRAM	LPS
TLR5	Mouse Human	Homodimer	Plasma membrane	MyD88	Flagellin
TLR6	Mouse Human	Heterodimer with TLR2	Plasma membrane	MyD88, TIRAP	Diacyl lipopeptides, lipoteichoic acid
TLR7	Mouse Human	Homodimer	Endosomal	MyD88	ssRNA
TLR8	Human	Homodimer	Endosomal	MyD88	ssRNA
TLR9	Mouse Human	Homodimer	Endosomal	MyD88	dsDNA, CpG-methylated DNA
TLR10	Human	Homodimer, also heterodimerizes with TLR1 and TLR2	Plasma membrane	MyD88	Unknown
TLR11	Mouse	Homodimer, possible heterodimer with TLR12	Endosomal	MyD88	Profilin
TLR12	Mouse	Homodimer, possible heterodimer with TLR11	Endosomal	MyD88	Profilin
TLR13	Mouse	Homodimer	Endosomal	MyD88	Bacterial 23S Ribosomal RNA, vesicular stomatitis virus (VSV)

Upon triggering of TLRs, adaptor proteins Myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF) are recruited to the TIR domains (**Table 1**; **Figure 2A**).[80, 81] These adaptor molecules activate the transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1) complex via interleukin-1 receptor-associated kinase (IRAK) and TRAF6 for MyD88.[82] Alternatively, the TRIF-dependent pathway does not use TRAF6, but receptor-interacting protein 1 (RIP1) to activate the TAK1 complex.[83, 84]

Subsequently, the TAK1 complex, consisting of TAK1 and the TAK1 and MAP3K7-binding proteins 1 and 2 (TAB)1/2, and TRAF6 can activate the NEMO/IKKα/IKKβ complex, leading to the degradation of IκB and the release of activated NF-κB to the nucleus.[85] Alternatively, TRIF can activate Interferon regulatory factor 3 (IRF3) via a cascade involving TRAF3 and TANK binding kinase 1 (TBK1). NF-κB and IRF3 are crucial transcription factors for cellular activation, leading to production of cytokines, type I Interferon (IFN type I) and cellular survival and differentiation.

The role of BTK in TLR signaling has been widely studied, identifying its role within the signaling cascade on several levels. BTK was shown to interact with the TIR domains of TLR4, TLR6, TLR8 and TLR9, MyD88, MyD88 adaptor-like protein (MAL) and IRAK, but not with TRAF6 (**Figure 2A**).[86, 87] BTK is also involved in TRIF-dependent signaling, as BTK-deficient macrophages were unable to respond to virus-induced TLR3 ligation.[88]

Crosstalk and synergistic signaling between BCR and TLR signaling has been described in the context of autoimmune disease.[89] More importantly, another study showed that BTK is the crucial mediator in this BCR and TLR synergistic signaling by modulation of calcium mobilization.[90] This could be the rationale for the role of BTK in BCR/TLR synergy required for autoreactive B cell survival and proliferation (**Figure 2A**). Likewise, it has been shown that intracellular expression of MHC class II molecules promote TLR signaling in antigen-presenting cells by maintaining activation of BTK.[91] Together, this illustrates the important role of BTK in TLR and TLR/BCR synergistic signaling in the context of autoimmunity.

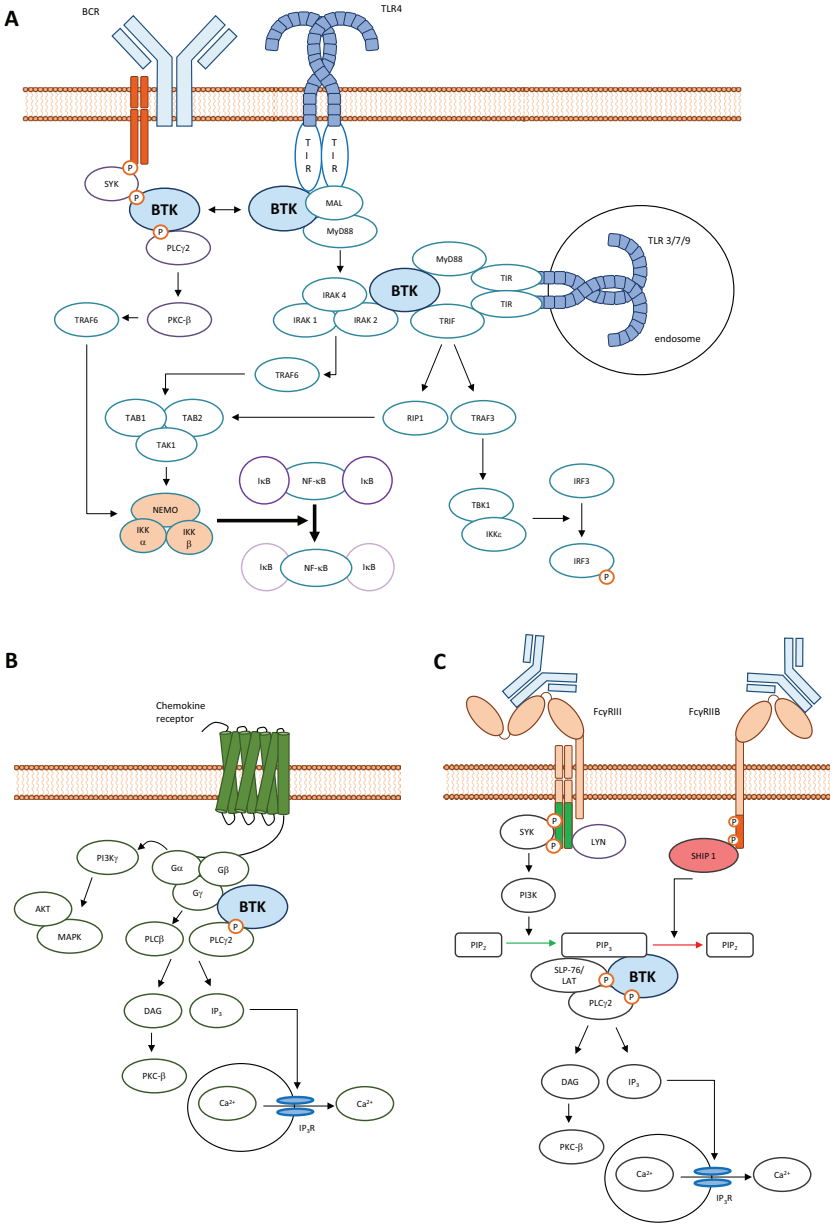

Figure 2. Btk signaling in various receptor signaling pathways

Signaling pathways downstream of A) Toll-like receptors, either signaling via TRIF or MyD88. Btk binds to several places proteins in TLR signaling and can potentially synergize with BCR signaling. Bold arrows are indicative of increased synergistic signaling by TLRs and BCRs. B) Chemokine receptor use Gα and Gβ/γ signaling post stimulation. Btk can bind to these subunits and thereby induce signaling of PLCγ2. C) Activating Fcγ receptors (FcγRIII) and inhibitory Fcγ receptors (FcγRIIB) are able to modulate the availability of PIP_3, as activating FcγR promote generation of PIP_3 by Syk-PI3K and inhibitory FcγRs promote SHIP-1 activation leading to hydrolyzes of PIP_3 into PIP_2.

B. NOD-like receptor and STING signaling

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are a class of cytosolic PRRs. The family of NLRs includes numerous receptors, such as the NODs and NACHT, LRR and Pyrin domain containing proteins (NLRPs). NOD1 and NOD2 signaling has been well-characterized, as they activate RIP kinases to induce activation of NF-κB and MAPK activation.[92, 93] NLRPs are well known for their inflammasome activity. Inflammasomes are large cytosolic protein complexes, consisting of sensory receptors such as NLRP1 and NLRP3, which are able to activate caspase-1 signaling, resulting in processing of pro-inflammatory cytokines.[94] For example, caspase-1 mediates cleavage of pro-IL-1β and pro-IL18 into their active forms IL-1β and IL-18, respectively. Although all inflammasomes recognize PAMPs or DAMPs, NLRP3 might be the most clinically relevant receptor, as it is responsive to a great range of stimuli. It was recently shown that BTK is a crucial component in NLRP3 inflammasome activation of macrophages by direct physical interaction with NLRP3.[95, 96] Also in a model of cerebral ischemia, infiltrating macrophages showed decreased caspase-1 and subsequent IL-1β activation after treatment with ibrutinib, a small molecule BTK inhibitor.[95] Interestingly, NLR signaling is partially complemented by other PRR signaling cascades, as TLR signaling provides transcription of the IL-1β precursor. This suggests that BTK could mediate in TLR-NLR complementary signaling, also potentially contributing to mechanisms of autoimmunity. Moreover, inflammasome activity was impaired in peripheral blood mononuclear cells from XLA patients, suggesting that inflammasome deficiency may contribute to the XLA phenotype.[96]

Another class of sensory receptors for self and non-self nucleic acids involves the cGAS-STING signaling pathway. Cytosolic DNA can bind to cyclic GMP-AMP synthase (cGAS) that produces cGAMP. In turn, cGAMP will bind Stimulator of Interferon genes (STING), subsequently causing TBK-1-mediated activation of IRF3-regulated transcription of IFN type I. cGAS is the most well-defined sensory unit to activate STING. However, there are several more cytosolic sensors such as IFI16 and DDX41. Although not much is known how DDX41 senses nucleic acids, it was recently reported that BTK binds DDX41 and STING via interactions with the BTK kinase and SH3/SH2 domains in murine embryonic fibroblasts and macrophages.[97] BTK can phosphorylate DDX41, thereby enabling DDX41 to bind DNA and regulate the recruitment of STING and downstream signaling.[97] This implies that activated BTK can assist DDX41-mediated IFN production, showing a mechanism in which BTK is an important amplifier in cross-talk between cellular signaling pathways.

C. Chemokine receptors

Attracting cells to instruct their migration pattern is crucial for proper localization in steady state and inflammation. As a class of chemotactic cytokines, chemokine gradients are essential for regulating the migratory pathways of cells that are already present in tissues or recirculating. To date, 50 chemokine receptor ligands and 20 signaling chemokine receptors have been identified,[98] associated with recruitment to different anatomical locations.

Chemokine receptors are expressed on all leukocytes and signal via a G-coupled protein domain. Lymphocytes are fully dependent on chemokine-mediated migration within a lymph node. In the follicles of the lymph node, follicular dendritic cells (FDCs) attract $CXCR5^+$ B cells by producing CXCL13. Also T cell localization around the follicle is controlled by chemokine migration: CCR7-positive T cells are recruited by CCL19 and CCL21-producing fibroblastic reticular cells. CXCR4 is also used within a germinal center (GC) response by B cells, as upregulation is required for trafficking into the GC dark zone.[99] In addition, CXCR4 is an important receptor for bone marrow homing, which is a crucial process for the maintenance of long-lived plasma cells.[100]

As mentioned above, chemokine receptors are G-protein coupled receptors that consist of seven transmembrane spanning domains and an intracellular domain composed of α, β and γ G-protein subunits. Upon binding of chemokines to the extracellular domain, a conformational change is induced resulting in dissociation of the Gα and Gβγ subunits of the intracellular domain. All G-protein subunits are able to induce PI3K-mediated signaling, leading to the activation of AKT/MAPK activation and PIP_3 conversion (**Figure 2B**, *left panel*). G-proteins can also bind to the TEC and PH domain of BTK and are reported to actively modulate BTK activity in chemokine receptor signaling (**Figure 2B**, *left panel*).[101-103] This could partially be mediated by Syk and is suggested to activate PLCγ2 signaling,[104, 105] linking chemokine and BCR signaling cascades. In addition, multiple studies have shown that BTK is crucial for the maintenance of chemokine receptor expression on B cells, as BTK-deficient and ibrutinib-exposed cells show impaired homing and migration.[104, 105] The observed effect of BTK inhibition on homing and migration could (partially) be due to the role of BTK in cellular adhesion, as BTK is involved in integrin-upregulation upon BCR stimulation.[106]

D. Fcγ Receptors

Antibody production by B cells is crucial for opsonization, neutralization and exterminating pathogens. This will lead to activation of the complement system and thereby supports the activity of innate immune cells. Antibodies can stimulate these innate immune cells via Fc receptors (FcR) expressed on their cell surface, which are specific for either IgG, IgM, IgA or IgE. FcR signaling is important in assisting innate cell function to clear targeted epitopes, either by modulating signaling or assisting in phagocytic uptake of immune complexes. Also in the context of autoimmunity, excessive antibody production or differential FcR signaling itself can lead to exaggerated responses via FcR signaling. As a consequence, harmful inflammatory mediators may be produced, which will be discussed below.

BTK is crucial in FcR signaling in various granulocytes and mast cells, which will be discussed in the section on BTK function in myeloid cells below. Although not much is known about BTK in IgA and IgM FcR signaling, BTK was shown to be important for IgG-specific Fc receptor (FcγRs). Fcγ receptors are divided based on their inhibitory or activating capacity. Activating FcγRs signal upon activation via their own intracellular ITAM domains (FcγRII2A) or associate with the ITAM-containing signaling subunit FcR common γ chain

(FcγRI). This results in the activation of Syk, BTK and PI3K-mediated PIP_3 generation and subsequent cellular activation (**Figure 2B,** *right panel*). BTK function in activating FcγR signaling was shown by BTK inhibition experiments in macrophages, resulting in decreased production of IL-1β, IL-6 and TNF-α upon activation of FcγRIII.[107]

In contrast to activating FcRs, FcγRIIB contains an intracellular domain with ITIMs that upon receptor engagement recruit SH2 domain-containing inositol 5′ phosphatase 1 (SHIP1).[108] SHIP1 hydrolyzes PIP_3 into PIP_2, thereby inhibiting activation and membrane-recruitment of BTK and PLCγ2-induced calcium signaling. FcγRIIB signaling was shown to affect and modulate BTK activity through phosphate activity of SHIP1, as SHIP1 deletion increased PIP_3 levels and BTK activity.[44] Conversely, the expression of a membrane-associated BTK chimeric molecule suppressed the inhibitory effects of FcγRIIB-signaling.

E. Other receptor signaling pathways

Next to the receptor signaling pathways discussed above, BTK has also been implicated in various other signaling cascades. For example, BTK is involved in several interleukin receptor (IL-R) signaling pathways. Upon *in vitro* stimulation of Y16 B cells with IL-5, BTK activity was enhanced.[109] In addition, IL-10 mediated MHC class II upregulation is mediated by BTK, as *Xid* mice did not upregulate their MHC class II levels upon IL-10 stimulation *in vitro*.[110] Although these B cell-stimulatory properties of IL-10 are equally mediated by IL-4, the two cytokines appear to act differently, because *Xid* mice responded normally to IL-4-mediated stimulation.[110] Finally, gp130 stimulation by IL-6 was shown to activate both BTK and TEC in a pro-B cell line.[111] Taken together, these findings show that BTK is involved in signaling of several cytokine receptors and may be crucial in the regulation of cytokine-mediated cellular proliferation.

Next to cytokine-mediated signaling, direct cell-cell contact is critical for cellular survival and differentiation. A crucial signaling pathway for B cell maturation and differentiation is CD40-mediated signaling by interacting with the CD40 ligand (CD40L) expressed on activated T cells. In *Xid* mice, it was reported that B cells did not proliferate upon ligation of CD40.[112] In addition, we have shown that naive B cells upregulate BTK protein levels upon *in vitro* CD40 stimulation.[25]

As mentioned above, BTK is involved in the regulation of integrins on the cell surface, thereby regulating cellular adhesion.[106] Next to regulating integrins in B cells, BTK has also been implicated in integrin upregulation in other cell lineages. Signaling downstream of Fas (CD95) in neutrophils was shown to be crucial for integrin upregulation and to be mediated by BTK and PLCγ2.[113] A function for BTK downstream of Fas is intriguing with respect to autoimmune disease, because $Fas^{LPR/LPR}$ mutant mice spontaneously develop systemic autoimmune disease.[114] In addition, we previously showed that BTK-overexpressing mice are more resistant to Fas-mediated apoptosis,[25] suggesting that BTK may be important in B cell survival by controlling Fas signaling.

Also erythroid cells and platelets express both TEC and BTK. Because BTK was found to be phosphorylated by stimulation of the receptors for erythropoetin and the stem cell factor in primary liver-derived erythroid cells,[115] BTK signaling is important in erythroid renewal and protection from apoptosis. BTK is also downstream of collagen receptor glycoproteins, and thereby important for calcium mobilization in megakaryocytes and platelets.[116, 117] The clinical relevance of BTK function in platelets is unclear, since XLA patients do not exhibit overt increased bleeding,[118] although in the context of treatment of CLL patients with ibrutinib an increased risk of bleeding-related adverse events was reported.[119, 120]

II. BTK FUNCTION IN THE MYELOID LINEAGE

BTK is not only expressed in cells from the lymphoid, but also in the myeloid lineage. Expression of BTK has been observed in all myeloid cell types, contributing to signaling pathways such as Toll-like receptors (TLRs), cytokine receptors and G-protein-coupled receptors. However, in contrast to the detailed knowledge of BTK in BCR signaling in B cells, the role of BTK in individual myeloid cell populations is not that well defined. In this section, we will highlight several subsets of cells derived from the common myeloid progenitor and the evidence for BTK-mediated signaling in these subsets.

A. Monocyte/macrophage lineage cells

Macrophages, which have an important function in both innate immunity and activation of the adaptive immune system, derive from erythro-myeloid progenitors from the yolk-sac, as well as from definitive hematopoietic stem cells in fetal liver and bone marrow after birth.[121] Yolk-sac and fetal-derived macrophages give rise to tissue-specific populations such as Kupffer cells, microglia and Langerhans cells.[122] Recirculating monocytes, generated in the bone marrow, can also differentiate into macrophages during inflammatory conditions.[123] Upon encountering pathogens, macrophages are activated and engulf and process these pathogens. Activated macrophages are able to present antigen to T cells, produce various cytokines including IL-12 and TNF-α and reactive oxygen species (ROS), thereby contributing to inflammation and activation of more specific antigenic responses. Activation can be classically by LPS or cytokines, resulting in Th1-promoting environmental signals. Alternatively, macrophages can be primed by more Th2-skewing signals leading to immune regulation. Th1 promoting macrophages are called M1, whereas immune regulatory macrophages belong to the M2 subtype.[124]

BTK and TEC have been described to regulate macrophage stimulating factor receptor (M-CSFR) signaling, a pathway that is required for macrophage differentiation, survival and proliferation.[125] Macrophages express FcγRs on their cell surface, which are crucial for the induction of their phagocytic capacity and cytokine production. FcγR-mediated phagocytosis by monocytes or macrophages is not affected by inhibition of BTK or by the *Xid* mutation, but FcγR-mediated cytokine production is suppressed when cells are treated with specific BTK inhibitors.[107, 126]

BTK is critical for macrophage function through TLR signaling, whereby signaling via TLR4 is crucial for their TNFα and IL-1β-producing capacity.[127] BTK is important in the production of TNFα, as it stabilizes TNF-α transcripts via their 3' untranslated region, probably via MAPK activation.[128] *Xid* mice showed decreased nitric oxide generation and increased IL-12 levels,[129, 130] indicating the importance of BTK in the regulation of macrophage cytokine and ROS production. This would also imply a role for BTK in macrophage polarization to either M1 or M2. It was reported that BTK-deficient macrophages have an impaired ability to polarize into M1 macrophages, as illustrated by reduced expression of the *Tnfα*, *Il12p40* and *Cxcl10 genes*.[131] Instead an enhanced induction of immunosuppressive M2-associated genes, including *Ccl2*, *Ccl17* and *Ccl22,* was observed in response to M1-polarizing stimuli.[131] The importance of BTK for M1 identity was in line with another study, showing impaired phagocytosis and M1 to M2 skewing of macrophages, as demonstrated by increased expression of the M2-associated markers CD206 and CD11b, in CLL after treatment with BTK-inhibitor ibrutinib.[132] In contrast, the increase in IL-12 levels in *Xid* mice and M1-skewing of macrophages in a pancreatic cancer model after BTK inhibition show that the exact role of BTK in macrophage polarization is highly debatable.[127, 133] In this context, it is of note that the interpretation of data obtained with ibrutinib may be complicated by known off-target effects of this first-generation BTK-inhibitor, e.g. on EGFR, ITK, and TEC family kinases.

Finally, BTK is involved in differentiation and maturation of osteoclasts, which are bone resorbing cells and are derived from the monocyte cell lineage. RANK and ITAM-mediated signaling are crucial for the differentiation of these cells, which was shown to depend on expression of TEC and BTK.[134, 135]

B. Dendritic cells

Dendritic cells (DCs) are another subset of antigen-presenting cells, next to macrophages and B cells. Both in human and in mice, there are four main subsets of DCs: plasmacytoid DCs, two subsets of conventional DCs (cDC1 and cDC2) and inflammatory monocyte-derived DCs.[136] Plasmacytoid DCs are the main producers of type I IFNs and have the capacity to present antigens upon stimulation of their TLRs by viral antigen. cDCs are derived from pre-cDC precursors, which seed peripheral tissues with cDCs upon migration from the bone marrow. cDC1s are specialized in antigen cross-presentation on MHC class I to $CD8^+$ T cells, production of IFNγ in response to TLR3 ligation and the polarization of Th1 cells. On the other hand, cDC2s tend to induce Th2 and Th17 polarization. Finally, monocyte-derived DCs originate from circulating monocytes, which are classified into classical and non-classical monocyte subpopulations. Tissue inflammation is often accompanied by a large influx of monocytes, illustrating their important role in the innate immune response. Whereas non-classical monocytes mainly patrol the vascular wall, classical monocytes migrate into tissues and give rise to mononuclear phagocytes. These cells can acquire both macrophage and DC characteristics, but are nowadays considered as a separate lineage.[136]

As DC subsets have only been recently defined, the role of BTK is not clearly defined in these DC subsets, but mainly in monocyte-derived cells. Monocytes from XLA patients normally differentiated into DCs *in vitro* and matured upon stimulation with LPS and were equally able to prime naive T cells compared to monocytes derived from healthy donors.[137] *Xid* mice were shown to have a Th2-skewed immune response and increased IgE production upon parasitic challenge.[138] This was attributed to a DC-mediated effect, given that adoptive transfer of *in vitro* activated BTK-deficient DCs resulted in higher serum IgE levels than transfer of BTK-sufficient DCs.[139] This is likely due to an increased maturation status of BTK-deficient DCs upon LPS stimulation and their increased capacity to stimulate T cells.[139] It is conceivable that reduced autocrine secretion of IL-10 by BTK-deficient DCs contributes to this DC phenotype, since IL-10 has the capacity to impair DC maturation.[139] A recent study, investigating the role of BTK and TLR signaling in cDCs from the bone marrow, showed that *in vitro* stimulation of cDCs via TLR7 and TLR9 signaling depends on the presence of BTK.[140]

Taken together, these findings illustrate that BTK has various functions in DCs, but remains poorly defined due to DC plasticity and heterogeneity.

C. Granulocyte populations

Polymorphonuclear cells, generated in the bone marrow, comprise several distinct lineages of granulocytes: neutrophils, eosinophils, basophils and mast cells. Granulocytes contain granules containing anti-microbial peptides that assist in the clearance of pathogens and the induction of inflammation.

Neutrophils are the most abundant granulocytes and are crucial in the first line of defense by phagocytosis and neutrophil extracellular trap formation. Moreover, they have been implicated in adaptive immune responses because of their capacity to interact with lymphocytes. Neutrophils have been reported to be recruited by Th17 cells and to interact with these cells.[141, 142] Splenic neutrophils assist in marginal zone B cell function and have the capacity to induce Ig class-switch recombination and somatic hypermutation by providing signals such as B cell activating factor (BAFF), a proliferation-inducing ligand (APRIL) and IL-21.[143] Although XLA patients and BTK-deficient mice have normal granulocyte numbers in the circulation, BTK is involved in the neutrophil development, attraction and function.[144-147] In addition, BTK is important for regulation of ROS production by human neutrophils via TLR-dependent mechanisms.[148]

Mast cells, which play a role in the pathology of several autoimmune diseases,[149-151] highly express BTK, as well as three other members of the TEC kinase family, Itk, TEC and Rlk.[152] Within mast cells, BTK mainly signals downstream of the high-affinity IgE receptor (FCεRI), as well as c-kit and the IL-3 receptor.[153-155] In addition, BTK was shown to be dispensable for TLR-mediated mast cell activation.[156] FCεRI has no intrinsic enzyme activity, therefore FCεRI-mediated cell activation solely relies on activation of non-receptor tyrosine kinases.[157] The role of BTK in the downstream signaling of the FCεRI parallels its role in BCR signaling: upon crosslinking of FCεRI, the ITAMs of the FCεRI are phosphorylated,

resulting in recruitment of Lyn and activation of Syk.[157-159] Syk then phosphorylates the linker for the activation of T cells (LAT), which in conjunction with the SLP-76 linker, functions as a scaffold for signaling complexes, such as BTK and PLCγ.[160, 161] Syk and BTK both activate PLCγ1 and PLCγ2, leading to calcium demobilization and activation of PKC isoforms. Even though mast cell development is not altered in absence of BTK, upon FCεRI cross-linking mast cells of BTK-deficient mice showed impaired degranulation and histamine release.[153, 162] In addition, BTK positively regulates production of several cytokines by mast cells, such as IL-2, IL-4, TNF-α and GM-CSF.[163] Besides the role of BTK in degranulation and histamine release in mast cells, BTK also regulates apoptosis via the c-Jun NH_2-terminal kinase (JNK) and p38 pathway. Upon IL-3 deprivation *Xid* or BTK-deficient mast cells died by apoptosis more slowly than wild-type mast cells.[155] BTK-deficient bone marrow-derived mast cells were unable to activate JNK, causing a defect in apoptosis.[155] Additionally, treatment of mast cells with the BTK-inhibitor ibrutinib efficiently blocked mast cell degranulation,[164] as well as release of TNF-α, IL-8 and MCP-1.[165]

Eosinophils and basophils are crucial in allergic responses and fighting parasitic infections by their anti-microbial granules. In addition, eosinophils and basophils modulate B cell functions: eosinophils were shown to be important in IgA class-switching of B cells and plasma cell maintenance in the lamina propria.[166] IL-6 and IL-4 derived from basophils support plasma cell survival and Ig production.[167, 168] Similarly to mast cells, human basophils show hampered FcεR responsiveness after treatment with ibrutinib.[169-171] Other than FcεR signaling, the role of BTK in eosinophils and basophils remains unclear.

All in all, these findings illustrate that - despite the limited myeloid defects initially reported in XLA patients and *Xid* mice - BTK functions in nearly all cells of the myeloid lineage. Nevertheless, its exact role in various myeloid subpopulations remains to be defined and warrants further investigation.

III. BTK IN DIFFERENTIATION AND SURVIVAL OF B CELL POPULATIONS

A. BTK function in differentiation of mature B cells

BTK is of great importance for overall B cell survival, illustrated by the almost complete lack of peripheral B cells in XLA patients and reduced numbers of mature $IgD^{high}IgM^{low}$ B cells in *Xid* and BTK-deficient mice.[16, 20, 21, 32, 36] Furthermore, the residual $IgM^{+}IgD^{-}$ B cells in XLA patients are differentially selected and express a unique antibody repertoire compared to healthy B cells, suggesting that BTK-deficient B cells might be selected to express autoreactive antibodies.[172]

Furthermore, even though marginal zone B cells are present in BTK-deficient mice, the survival capacity of the marginal zone B cells is severely impaired.[173, 174] The role of BTK in B cell survival in BTK-deficient mice seems to be masked by the presence of several other compensatory survival signals, including T cell-mediated BTK-independent signaling via CD40 on B cells. This is evident from the introduction of CD40-deficiency, T-cell receptor (TCR) β-deficiency or the *nude (nu/nu)* mutation, characterized by the lack of T cells, in BTK-deficient or *Xid* mice, which resulted in an almost complete block of B cell differentiation within the transitional B cells compartment at the T1 to T2 transition.[175-177]

Notably, in BTK-deficient mice, $CD5^{+}$ B cells (B1a cells) are completely absent, indicating the particular importance of BTK for development of B1 cells.[20, 21, 32, 36, 178] B1 cells constitute a distinct population of B cells that is long-lived and self-renewing and mainly reside in the peritoneal and pleural cavities. B1 cells play an important role in the first line of defense against microbial infection by spontaneous production of non-specific antibodies in a T cell-independent manner, so-called circulating 'natural antibodies'.[179, 180] In contrast to regular B2 cells, which are deleted from the repertoire when their BCR is autoreactive, B1 cells in mice are positively selected for self-reactivity, e.g. recognizing phosphatidylcholine, which apparently selects for protective effector functions of these cells.[181]

Transgenic expression of human BTK restored the phenotype of the $BTK^{-/-}$ mice.[182, 183] Hereby, $CD5^{+}$ B1 cell numbers and serum IgM and IgG3 levels were increased to wild-type levels and the immune response to T cell-independent type II antigens was in normal range. Although BTK deficiency leads to a complete absence of B1 cells in *Xid* mice, a 14-day treatment with BTK inhibitors PF-303 or ibrutinib did not diminish presence of B1 cells in the peritoneal cavity, indicating that BTK might only be required for the development, but not for persistence or function of B1 cells.[25, 184]

For decades, it has been debated whether B1 and B2 cells derive from a single progenitor, according to a selection or induced model, or from distinct progenitors and thus forming two individual cell lineages, i.e. the lineage model.[185] Montecino-Rodriguez et al. provided the first evidence for the existence of a distinct B1 progenitor by identification of $lin^{-}AA4.1^{+}CD19^{+}B220^{lo\text{-}neg}$ B1-specific progenitor cells (B1P) in the fetal liver.[186]

However, Montecino-Rodriguez et al. did not study the developmental heritage of the B1P cells, which was studied first by Esplin et al.,[187] who investigated the developmental relationship of B1P cells to conventional lymphoid progenitors. They found that B1P cells slowly arise from early lymphoid progenitors, and more rapidly from common lymphoid progenitors, suggesting a common ancestry between B1 and B2 cells. In 2011, Yoshimoto et al. demonstrated that B1P cells can also derive from hemogenic endothelium of the yolk sack and para-aortic splanchnopleura (P-Sp) at embryonic day (E) 9-9.5.[180] The B1P cells then migrate to the fetal liver where they mature and differentiate into B1 and marginal zone B cells. Taken together, this suggests that B1P cells in the fetal liver are developmentally heterogeneous, derived from at least two distinct sources, namely from the HSC-generated common lymphoid progenitor cells, opposed by Esplin et al.,[187] and from the HCS-independently derived B1Ps originated from the yolk sack and P-Sp.[180, 188] Remarkably, even though BTK-deficient mice completely lack peripheral B1 cells, Esplin et al. found increased numbers of B1Ps in BTK$^{-/-}$ bone marrow, indicating negative feedback regulation of BTK on the development of B1P cells.[187] Unfortunately, the molecular mechanism of this link between increased B1P cell numbers but absence of B1 cells in BTK-deficient mice has not yet been elucidated, but might be of considerable interest.

B. Effects of BTK on B cell survival

BTK links BCR signaling to NF-κB activation,[60, 61] which is responsible for expression of anti-apopotitic genes such as Bcl-x_L in B cells, showing that BTK is essential for cell survival.[189, 190] Bcl-2 proteins regulate cell survival via mitochrondial integrity by controlling the activity of pro-apoptotic proteins Bax and Bak.[191] A1, a family member of the Bcl-2 family, is a critical mediator of BCR-dependent B cell survival; overall knockdown of A1 leads to loss of mature peripheral B cells and a lack of BCR signaling leads to a loss of A1 expression, inducing apoptosis.[192] Interestingly, A1 expression is controlled by Syk and BTK and inhibition of either one reduces A1 expression and induces apoptosis.

Constitutive BTK and/or NF-κB activation in B cells have been implicated in several B cell malignancies, such as mantle cell lymphoma (MCL), activated B cell-like diffuse large B cell lymphoma (ABC-DLBCL) and CLL,[24, 193, 194] as recently reviewed in.[195] Inhibition of BTK significantly reduces survival and proliferation of primary tumor cells and tumor cell lines *in vitro* and improves progression-free survival and overall survival of MCL and CLL patients, indicating that BTK is an effective therapeutic target in B cell malignancies.[23, 24, 119, 196-200]

Even though BTK-mediated NF-κB activation and B cell survival has mainly been linked to BCR signaling, Shinners *et al.,* proposed that BTK also contributes to BAFF receptor signaling.[201] BAFF is a member of the TNF family and functions as a survival factor for immature and mature B cells by activating the NF-κB pathway.[202, 203] BAFF-overexpressing mice develop a lupus-like autoimmune phenotype and in serum of SLE patients increased levels of BAFF were found, demonstrating the importance of BAFF signaling in autoimmunity.[204-206] Shinners *et al.,* showed that upon stimulation with BAFF,

BTK$^{-/-}$ B cells are unable to activate both the classical and the alternative NF-κB pathway, indicating that BTK is involved in both NF-κB pathways.[201] Another indication for BTK being involved in BAFFR-signaling is that B cell survival via BAFF receptor-mediated NF-κB activation is reduced in PLCγ2-deficient mice.[207] However, these findings in BTK$^{-/-}$ B cells are not uniform, as another group reported that *Xid* B cells have no defective activation of the alternative NF-κB signaling pathway, but the expression of the APRIL and BAFF receptor transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) was severely reduced.[208]

Another role of BTK in B cell survival is through its involvement in the JNK1 signaling pathway. In DT40 chicken B cells, activation of JNK1 via the BCR induced apoptosis and required both Syk and BTK, but not Lyn.[62] Whereas healthy human B cells showed JNK1 activation upon BCR stimulation, B cells from XLA patients were not capable of inducing the JNK1 response.[209] A BCR-induced JNK1 response is dependent on PLCγ2 and Rac1, both of which are activated by BTK.[210-212] XLA B cells fail to activate Rac1 and, likewise, ibrutinib treatment significantly reduces Rac1 activation.[209, 213] BTK regulates Rac1 activation independently of PLCγ2 since Rac1 activation seems normal in PLCγ2 deficient DT40 B cells.[209]

C. BTK and GC responses

GCs are lymphoid structures - consisting of B cells, T cells and FDCs - that are formed via stimulation of the BCR and CD40 on naive B cells by a T cell-dependent antigen. Activation of naive B cells leads to their migration to the border of the T and B cell zone or the intra-follicular region, were B cells start to proliferate and form GCs through long-lived interactions with antigen-specific T cells.[214-217] To increase BCR affinity, GC B cells introduce non-random single-base pair changes in the Ig V genes by somatic hypermutation, in particular in the complementarity-determining *regions* (*CDRs*), which are the parts of the Ig V region variable chains where they specifically bind to antigen. In addition, BCRs undergo class-switch recombination, whereby the BCR isotype changes from IgM to IgG, IgA or IgE.[217, 218] Both somatic hypermutation and class-switch recombination are induced by the activation-induced cytidine deaminase (AID) enzyme.[219, 220] Competition for a limited amount of antigen ensures that B cells will go into apoptosis, if the new BCR is less affine for the antigen or autoreactive. Hereby, the capacity of B cells to proliferate is controlled by follicular T helper cells, which provide selective help on the basis of the amount of antigen captured and presented by the GC B cell.[221] Tight regulation of these processes in the GC response is of great importance, since incorrect regulation can give rise to persistence of autoreactive B cells and long-lived autoantibody responses, causing autoimmune diseases.[222]

The precise role of BTK in GC responses is not studied in large detail. BTK-deficient mice do develop GCs, but primary responses to T cell-dependent model antigens, such as trinitrophenol keyhole limpet hemocyanin (TNP-KLH), are diminished.[223] Nevertheless, secondary immune responses of *Xid* or BTK-deficient mice to T cell-dependent model antigens appear unaffected.[20, 32, 36] By contrast, we recently found that influenza virus-infected

BTK-deficient mice display more severe weight loss compared to wild-type littermates.[174] Even though the mice were able to form GCs, the total numbers of GC B cells in the lung draining lymph nodes were approximately 6-fold decreased.[174] Moreover, influenza virus-specific antibody levels in serum were significantly reduced, indicating that BTK does play a major role in the GC function. This would also be supported by the finding that GC formation requires the NF-κB pathway downstream of the BCR, since B cells with inactive NF-κB signaling failed to develop into GC B cells.[224]

D. Regulation of BTK protein expression

Precise regulation of BTK levels may be critical for BCR signaling, as it was shown that BTK levels are upregulated after BCR stimulation.[225] In addition, sub-physiological expression of transgenic BTK was not sufficient to rescue the BTK-deficient B cells, whereas physiological levels of BTK provide full rescue of B cell differentiation, survival and responsiveness.[183, 226]

BTK regulates itself on a transcriptional level, as the BTK promotor contains an NF-κB binding site.[227] In addition, BTK was shown to be extensively regulated by posttranscriptional mechanisms. MicroRNAs (miRNAs) are important regulators of mRNA and several specific miRNAs have been reported to target BTK mRNA. miR-185 was shown to be critical for regulation of BCR signaling in follicular B cells by targeting BTK, as B-cell-specific deletion led to decreased miR-185 expression and consequently a skewed BCR repertoire and autoimmune disease.[228] More recently, it was shown that six miRNAs target BTK with high specificity and that miR-210 and miR-425 are significantly reduced in patients with CLL.[229] Interestingly, treatment with HDAC inhibitors on primary CLL samples resulted in upregulation of these miRNAs and consequently downregulation of BTK mRNA and protein levels.[229]

E. Transgenic overexpression of BTK in B cells

We illustrated a possible role of BTK in autoimmunity by B-cell specific overexpression of human BTK in transgenic mice driven by the CD19 promoter region (CD19-hBTK mice).[25] These BTK-overexpressing mice spontaneously develop systemic autoimmunity resembling SLE and SjS phenotypes, from the age of 20 weeks. Peripheral B cells in CD19-hBTK mice express approximately 3-fold higher BTK protein levels compared to wild-type B cells. This leads to enhanced BCR-mediated B cell activation displayed by a stronger Ca^{2+} influx and higher expression of activation markers *in vivo*, as well as *in vitro* upon BCR stimulation. Interestingly, particularly the T-cell co-stimulatory surface molecules CD80, CD86 and MHCII were increased. Furthermore, B cells of BTK-overexpressing mice were more resistant to apoptosis and had enhanced NF-κB activation. Inhibition of BTK kinase activity by ibrutinib reduced the BCR hyper-responsiveness, anti-IgM-induced Ca^{2+} influx and expression of activation markers of BTK-overexpressing B cells.

From ~14 weeks onwards, BTK-overexpressing mice spontaneously developed GCs and had

increased plasma cell formation in the spleen. In the serum of aging BTK-overexpressing mice high anti-nuclear autoantibody levels were present. Additionally, they started to develop infiltrations of lymphocytes in several organs, including the lungs, kidney and salivary glands and show signs of kidney damage. As CD19-hBTK mice show high BTK expression throughout all B cell subsets, it remained conceivable that B cell repertoire selection in the bone marrow was altered in these mice. However, the autoimmune phenotype of BTK-overexpressing mice appeared independent of changes in the BCR repertoire, because MHC-II-driven BTK overexpression, which is restricted specifically to mature peripheral B cells (and myeloid cells), resulted in a similar phenotype.[25]

The autoimmune phenotype of aging CD19-hBTK mice was also dependent on BTK kinase activity, since treatment of BTK overexpressing mice with ibrutinib resulted in loss of spontaneous GC and plasma cell formation in the spleen. Additionally, mice with 15- to 20-fold increased expression of the kinase-inactive BTK^{K430R} mutant in B cells did not produce anti-nuclear autoantibodies or develop signs of autoimmune pathology.[25] These findings support the requirement of BTK kinase activity for the development of the autoimmune phenotype seen in BTK-overexpressing mice. Furthermore, the autoimmune phenotype is also dependent on B-T cell interactions: when crossed onto a CD40L–deficient background, BTK-overexpressing mice did not develop autoimmune pathology and B cells no longer showed enhanced IL-6 production, although increased IL-10 and IgM autoantibody production were not abolished.[230]

BTK overexpression also affected CLL development, since crosses of the IgH.ETμ CLL mouse model with CD19-hBTK mice demonstrated accelerated CLL onset and increased mortality,[231] consistent with a correlation between autoimmune disease and development of B cell malignancies.[232]

IV. BTK IN B CELL CYTOKINE PRODUCTION

As discussed above, BTK is a crucial signaling molecule downstream of many receptors and is involved in cytokine signaling and production in several cell-types. In this section, we will discuss the role of BTK in B cell cytokine production in health, infection and autoimmune diseases.

B cells do not only secrete antibodies, they are also very potent producers of cytokines in health and disease. An important cytokine that is produced by B cells is IL-10, which is a well-known regulatory cytokine. However, autocrine IL-10 could also act as a human B cell proliferation factor in steady state.[233] All B cell subsets are able to produce IL-10, but the main producers of IL-10 are B1 cells and immature B cells.[234, 235] IL-10-producing B cells have been reported to assist in alleviating active disease in various autoimmune models. B cell-derived IL-10 was shown to be important in controlling the disease course in a collagen-induced arthritis (CIA) model.[236] In a model of experimental autoimmune arthritis (EAE), Fillatreau and colleagues showed that autoantigen-specific B cells produced IL-10 and that IL-10 production by B cells was crucial to recover from this Th1 driven autoimmune

disease.[237] Within the B cell population, plasma cells are large contributors to B cell lineage IL-10 production in both models of *Salmonella typhimurium* infection and EAE.[238] In contrast to regulatory effects, IL-10 was shown to be important in anti-nuclear autoantibody production in context of murine SLE models.[239] BTK is important for the regulation of B cell IL-10 production, because BTK-deficient B cells show strongly impaired IL-10 production upon activation with TLR9 ligand,[240, 241] even though these BTK-deficient B cells showed increased TLR-9 pro-inflammatory responsiveness.[241] In addition, we previously showed that splenic B cells from transgenic BTK-overexpressing mice have increased IL-10 production when compared with wild-type littermates, independent of the expression of CD40L.[230] Taken together, these findings illustrate that BTK is a crucial signaling molecule involved in production of B-cell-derived IL-10.

As previously discussed, BTK is known to signal downstream of the IL-6R family. How IL-6 production by B cells is regulated and if this depends on BTK remains to be elucidated. We recently reported that IL-6 is increased in splenic B cells in mice with B-cell-specific BTK overexpression.[230] However, this increase is largely lost on a CD40L-deficient background and thus partially due B-T cell interaction *in vivo*, suggesting BTK plays a role in regulating B cell derived IL-6 via T cell-dependent mechanisms.

Secretion of cytokines is often specific to certain subsets of B cells and modes of activation. However, B cells can also produce cytokines to maintain self-homeostasis and homeostasis of lymphoid tissue. B cell cytokine production affects the induction and maturation of lymphoid tissue, as is extensively reviewed in Shen et al.[242] Splenic development is crucially dependent on B cell cytokine production, as lymphotoxin $\alpha 1\beta 2$ ($LT\alpha 1\beta 2$) produced by B cells is required for establishment of the splenic T cell zone stroma and marginal zone formation.[243-245] Even the splenic follicles are dependent on $LT\alpha 1\beta 2$ expression, as this together with TNF-α induces development of FDCs. Interestingly, FDCs produce CXCL13, which activates $LT\alpha 1\beta 2$ production by B cells, creating a feed-forward loop.[246] Next to splenic organization, B cells have been shown to be crucial in the induction of other lymphoid tissues. In the absence of lymphoid tissue inducer cells in an induced colitis model, B cells were shown to be crucial for induction of tertiary lymphoid tissue by $LT\alpha 1\beta 2$.[247] To date, there is no direct evidence that BTK interferes with B cell-mediated $LT\alpha 1\beta 2$ production in lymphoid tissue induction. However, we have previously shown that BTK-mediated autoimmune disease leads to formation of ectopic GCs in salivary glands and lungs.[25] A non-obese diabetic mouse model for Sjögren's disease showed that immune cell infiltrates and local BTK expression levels are decreased in salivary glands upon inhibition of the $LT\beta$ receptor.[248] In addition, BTK expression levels were shown to correlate with the number of infiltrating T cells in the parotid gland of SjS patients.[26] This would suggest that overexpression of BTK could have implications in ectopic lymphoid tissue induction.

V. BTK AND B-T CELL INTERACTION IN AUTOIMMUNITY

A. CD40-CD40L interaction and B cell survival

B-T cell interaction through CD40-CD40L has previously been suggested to facilitate the survival of autoreactive B cells in the periphery.[249] In the absence of CD40 or $CD4^+$ T cells, peripheral B cell numbers in BTK-deficient mice were dramatically decreased, compared to BTK-deficient or CD40 deficient mice, and the cells retained an immature B cell phenotype.[176, 177, 250] They failed to respond to T cell-dependent and independent antigens, and GCs are not formed. This important effect of CD40 signaling is restricted to the periphery, as B cell development in the bone marrow was not affected.[176] The transition of immature to mature naïve B cells, via the transitional B cell stage, is accompanied by a ~50% reduction in the number of autoreactive BCRs present in the B cell pool (~40% to ~20%).[251] This maturation step, and subsequent homeostatic proliferation of mature naïve B cells requires CD40L expression on $CD4^+$ T cells.[249] In the absence of CD40L, B cells are dependent on BCR signaling, and the number of autoreactive BCRs decreases, indicating that CD40-CD40L interaction promotes the survival of autoreactive B cells. Signaling through CD40 induces upregulation of BAFFR and TACI expression, which are important survival factors for B cells.[249] It has been suggested that naïve $CD4^+$ T cells are responsible for these signals, indicated by their constitutive expression of CD40L mRNA, but downregulation of extracellular protein expression through interaction with CD40.[252] This signaling can be cell extrinsic, as BM chimeras in which CD40L-deficient and sufficient autoreactive cells were compared showed no difference in maturation between these cells.[252]

Conversely, overexpression of BTK in mouse B cells enhances the survival of autoreactive B cells.[25] These B cells show enhanced cytokine production, and increased activation marker expression already on naïve B cells.[230] Overactivation of B cells in these mice is sufficient to disrupt T cell homeostasis and promote an autoimmune phenotype. This requires CD40L expression by T cells, not only to promote T cell differentiation to follicular helper T cells and pro-inflammatory cytokine production, but also for full activation of B cells. In the absence of CD40L, enhanced expression of activation markers by naïve B cells and B cell cytokine production is abrogated, suggesting that T cells are required to allow full pathogenic capacity of BTK-overexpressing B cells.[230] On the other hand, regulatory T cells are essential for the inhibition of autoreactive B cells: mutations in the FoxP3 gene cause the severe autoimmune disease IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked). In IPEX patients, central tolerance is unaffected, but peripheral naïve B cells show increased autoreactivity.[253] Treg cell function is lost in these patients, and instead T cells express higher levels of CD40L, PD-1 and ICOS.

Together, these data show that T cells play an important role in the transition from immature to mature naïve B cells and are crucial for maintaining peripheral B cell tolerance **(Figure 3)**.

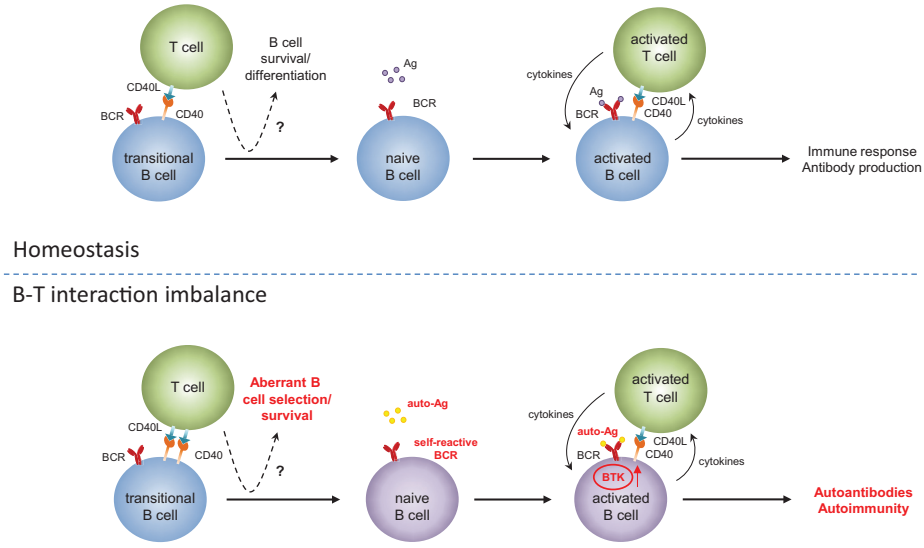

Figure 3. B-T cell interaction in homeostasis and autoimmunity

Top: Transitional B cells receive signals through the BCR and through CD40-CD40L interaction with T cells. This regulates peripheral selection of non-autoimmune B cells, leading to their survival and differentiation into naïve mature B cells. Upon sensing an antigen, B cells can interact with activated T cells to form memory B cells or antibody producing plasma cells.

Bottom: An imbalance in B-T cell interaction may aberrantly select for autoreactive B cells and support their activation, which may subsequently accumulate in the periphery and sense auto-antigens. Pathogenic B cells may have higher BTK expression and activation, produce more pathogenic cytokines and drive autoimmunity by activating T cells leading to autoantibody production.

B. BTK and the FcμR

T cells may also regulate B cell survival through Fas-L expression by induction of Fas-mediated apoptosis. B cells from BTK overexpressing mice are less susceptible to this form of apoptosis *in vitro*.[25] The IgM-Fc receptor (FcμR, also known as Toso or Faim3), was shown to inhibit apoptosis through Fas in B cells, although these data are controversial.[254, 255] Nevertheless, mice deficient for the FcμR display increased B1 cell numbers and higher serum levels of natural autoantibodies.[256] A link between the FcμR and BTK may be found in CLL patients, in which the FcμR was overexpressed and associated with progressive disease.[257, 258] Although CLL B cells display an anergic phenotype, BTK is often constitutively active in these cells, and expression of the FcμR can be induced through BCR stimulation.[257] It is therefore possible that increased BTK expression in CLL and autoimmune patients can enhance the expression of the FcμR, thereby limiting B cell sensitivity to Fas-mediated apoptosis and enhancing survival of malignant or autoreactive B cells.

VI. BTK IN ANIMAL MODELS OF AUTOIMMUNITY

Systemic autoimmunity can arise through the aberrant activation or survival of autoreactive B or T cells. Indications that tight regulation of BCR signaling is essential to maintain tolerance come from studies with mice deficient for important inhibitors of signaling. Mice deficient for CD72, Siglec-G, FcγRIIB, or its downstream phosphatase SHIP-1, spontaneously develop a lupus-like autoimmune phenotype.[259-262] In addition, Siglec-G or CD22-deficient mice show earlier or more severe development of disease in lupus or arthritis mouse models.[263, 264] On the other hand, SLP-65-deficient mice are more susceptible to EAE, due to a decreased immune inhibitory capacity of B cells through loss of IL-10 production.[265] Interestingly, *Xid* mice show decreased susceptibility to EAE.[130] Differences between these studies may be due to the different modes of EAE induction (different peptides and different adjuvants), which could trigger different pathways in different cells.

The importance of BTK activity in BCR signaling-driven B cell survival has prompted many studies into the role of BTK in autoimmune disease. Apart from enhanced B cell survival, BTK may promote several other key features of autoimmunity. B1a cells require BTK for survival, and absence of BTK led to a total loss of B1a cells, whereas increased BTK protein or activation increased B1a cell numbers.[20, 25, 36, 266, 267] B1a cells may contribute to autoimmunity through production of autoantibodies or cytokines, and BTK is required for IL-10 production by these cells.[266, 268-270] Similarly, BTK is crucial for marginal zone B cell survival, and these cells may become autoimmune through TLR9 activation of marginal zone precursor cells.[173, 271] TLR9 is often associated with autoimmunity, as it senses double stranded DNA (dsDNA) and BTK is involved in downstream TLR9 signaling.[90]

Enhanced BTK protein levels in B cells in mice also promote spontaneous GC formation in the spleen, and constitutive activation of BTK or increased protein expression in B cells induce the formation of autoantibodies, even when T cell help is abrogated.[25, 230, 267] Furthermore,

increased BTK expression in B cells drives pro-inflammatory cytokine production by B and T cells, and induces immune cell infiltration in lungs and salivary glands of aging mice.[230] Using *Xid* or BTK-deficient mice, or BTK inhibitors in a broad range of autoimmune models (**Tables 2 and 3**), the role of BTK in autoimmunity has been further studied.

A. Arthritis models

BTK-deficiency shows protective effects in a number of autoimmune arthritis mouse models. *Xid* mice are completely protected from developing collagen induced arthritis (CIA), an autoimmune mouse model for RA, despite the mutation being crossed onto the highly susceptible DBA/1 strain.[272] These mice failed to generate an auto-antibody response against collagen type II, with very low titers present in the serum compared to non-*Xid* controls.

Similarly, in the spontaneous K/BxN mouse model of arthritis, BTK-deficiency protects mice against full-blown disease through decreased autoantibody levels.[273] This model depends both on the innate and the adaptive immune system.[274] In $BTK^{-/-}$ K/BxN mice, splenic B cell numbers are decreased and auto-antibodies are almost undetectable in serum, while total IgG levels are only mildly decreased compared to controls. This is remarkable, as GC B cells and follicular helper T cells are decreased both in the spleen and draining popliteal lymph nodes in these mice. Total numbers of splenic macrophages and neutrophils are not decreased in BTK-deficient K/BxN mice, but macrophage recruitment to the joints was significantly decreased. As a result, although there were signs of mild inflammation in the joints, there was no cartilage damage or bone destruction in the absence of BTK.[273] Both the innate and the adaptive immune system appear to be affected in the $BTK^{-/-}$ K/BxN mice, but transfer of serum from K/BxN mice to BTK-deficient non-obese diabetic mice (collagen antibody induced arthritis model (CAIA)), which bypasses the adaptive immune response, did induce arthritis with a similar severity to BTK-sufficient non-obese diabetic mice. Macrophage activation in the joints was comparable between BTK-deficient and sufficient mice, indicating that protection in $BTK^{-/-}$ K/BxN mice was not mediated through macrophage intrinsic BTK-signaling.[273] Therefore it was concluded that in this model BTK contributes to autoimmune arthritis primarily through its role in B cell signaling and not through innate immune components.

Although these data suggest that the role of BTK in arthritic mouse models is primarily in B cells, the development and function of other cells may also be dependent on BTK. In the context of arthritis and joint destruction, BTK may play an important role in the regulation of osteoclasts. Both BTK and TEC kinase are highly expressed in osteoclasts, and show redundant roles in osteoclast differentiation similar to B cell development: while the reduction in osteoclast differentiation was limited in TEC-deficient mice, it was severe in BTK-deficient and almost completely absent in double-deficient mice.[134] Consequently, BTK/TEC double-deficient mice developed severe osteopetrosis. Here, BTK plays a role in RANKL-induced calcium influx and NFATc1 activation, which drives osteoclastogenesis.[134, 135] However, in the human-TNF transgenic (hTNFtg) mouse model of arthritis, BTK deficiency cannot

prevent severe inflammation, cartilage damage and bone destruction in the joints.[275] Normal osteoclast formation was observed in hTNFtg.BTK$^{-/-}$ mice, whereas osteoblast differentiation and function appeared to be impaired. These data suggest that residual TEC activity in osteoclasts is sufficient for normal osteoclast development in arthritic joints.

In addition to direct effects on humoral and innate immunity, BTK deficiency may also indirectly prevent the development of autoimmunity by altering the immune response against pathogens. In a *Staphylococcus aureus*-induced model of septic arthritis, *Xid* mice did develop arthritis, but both incidence and severity of disease were greatly reduced.[276] Mice had lower levels of autoantibodies in the serum and an altered cytokine profile in the spleen, where IFNγ was increased compared to controls. Interestingly, despite lower levels of bacteria-specific antibodies, these mice showed a decrease in bacterial load. This protection was dependent on the increase in IFNγ, as IFNγ neutralizing antibodies led to high mortality in these mice.[276] Although the source of IFNγ was not identified in this model, it is likely that B cells are responsible for the protective effect.

B. Lupus models

Early studies with *Xid* mice indicated that BTK deficiency is protective in animal models of lupus nephritis. NZBxNZW F_1 mice harboring the *Xid* construct showed delayed and decreased development of anti-DNA autoantibodies, did not develop proteinuria, and showed prolonged survival compared to controls.[277] Even after immunization with dsDNA with the addition of Freund's complete adjuvant, NZBxNZW *Xid* mice fail to produce autoantibodies. Similar results were obtained when the *Xid* construct was crossed into BXSB mice, which develop a lupus-like phenotype and coronary artery disease.[278] In this model, anti-DNA and anti-erythrocyte antibodies were significantly decreased in *Xid* BXSB mice, and they were completely protected from renal and cardiac pathology. Protective effects of BTK deficiency were also found in *Lyn*-deficient mice, which develop an autoimmune phenotype characterized by autoantibodies to nuclear antigens or dsDNA, increased B cell sensitivity to BCR stimulation and myelo-erythroid hyperplasia causing splenomegaly.[279] In the pristane-induced lupus mouse model, *Xid* mice were also completely protected from disease development.[280]

Crossed onto the MRL.lpr/lpr lupus susceptible mouse strain, which develops lymphoproliferative disease, *Xid* mice showed delayed mortality and a decrease in autoantibodies and glomerulonephritis.[281, 282] However, the protective effect on the autoimmune phenotype was less pronounced than in NZBxNZW or BXSB mice. This is also true for *Xid* mice with the *me* ("motheaten") mutation, which affects the *Ptpn6* gene encoding SHP1, also known as hematopoietic cell phosphatase (HCP).[283] *Me* mice develop a severe autoimmune phenotype characterized by lung pathology and skin lesions caused by accumulation of neutrophils, which often leads to auto-amputations of limbs and early mortality at 6-8 weeks of age.[284] Although autoantibodies are almost undetectable in serum of *me.Xid* mice, skin pathology, including auto-amputations, and mortality are unchanged

compared to control *me* mice.[285] The importance of SHP-1 in a broad range of hematopoietic cells, causing both autoimmune and other defects in these mice, may underlie this difference with other models. A similar effect was seen in C3H.gld/gld mice, which develop a B and T cell-mediated lupus-like autoimmune phenotype. These mice develop lymphadenopathy through expansion of T cells, which is not affected by the *Xid* mutation, while *Xid* C3H.gld/gld mice do show a significant reduction in serum auto-antibodies, without affecting total antibody levels.[286] These studies show that BTK can be an important player in lupus mouse models, but may not be involved in all aspects of the autoimmune phenotype.

C. Other autoimmune models

BTK-deficient non-obese diabetic mice show a severely reduced susceptibility to development of autoimmune diabetes.[287] Despite having no effect on T cell proliferation, activation and numbers of B and T cells infiltrating the pancreas, BTK deficiency reduced the incidence of diabetes from ~69% to ~17%. This partial protection can be primarily attributed to a significant reduction of anti-insulin antibodies through altered BCR repertoire selection and secondarily to the strongly decreased production of IL-10 by splenocytes, which may normally contribute to β-cell damage. Introduction of an insulin-specific Ig heavy chain transgene restored the autoimmune phenotype.[287]

In another diabetes model in which mice carry an Ig heavy chain transgene which has insulin-binding capacity after pairing with certain Ig light chains (125Tg mice), BTK-deficiency specifically impaired survival of mature, but not transitional B cells.[288] Furthermore, targeted insertion of an anti-insulin Ig light chain gene into the Ig κ locus, thereby allowing recombination of ~50% of B cells away from autoreactivity, showed that BTK deficiency mostly reduced autoreactive rather than non-autoreactive B cells in these mice. However, the few remaining autoreactive B cells were sufficient to maintain the autoimmune phenotype.[288]

BTK was also investigated in other BCR transgenic autoimmune models. In mice harboring a BCR specific for red blood cells, crosses with *Xid* mice protect against the development of hemolytic anemia.[289] Similarly, *Xid* mice carrying the 56R anti-DNA Ig transgene did not produce anti-DNA antibodies, although DNA specific B cells were present in these mice.[290]

Taken together, these studies show that BTK plays an important role in murine models of many different autoimmune diseases, in particular when autoantibodies are required for the disease.

D. BTK inhibitors in arthritis animal models

The studies described above focus almost exclusively on the effects of BTK deficiency on B cells and autoantibody production. Although BTK is mostly required for normal B cell development and function, effects on other cell types may also play a role in protection from autoimmunity. This is illustrated by many studies of BTK inhibition in autoimmune animal models.

The first small molecule BTK inhibitor approved for clinical use in the treatment of B cell malignancies is ibrutinib.[119, 195, 199, 291] This inhibitor binds covalently to the cysteine residue Cys-481 and irreversibly inhibits BTK kinase activity.[292] The efficacy of this inhibitor was also tested in several arthritis models (**Table 2**). When mice were treated orally with ibrutinib for 11 or 18 days, starting shortly after onset of CIA, they showed a decrease in clinical arthritis score in a dose-dependent manner. The decrease with the highest dose of ibrutinib was comparable or superior to that observed with dexamethasone treatment, while vehicle-treated mice showed an increase in clinical score over time.[22, 165] This was accompanied by a ~50% decrease in collagen type II-specific antibodies in the serum.[22] However, ibrutinib did not only affect B cells in these mice. In addition to decreased lymphocyte numbers in the synovial fluid, macrophage and granulocyte numbers were also significantly decreased.[165] This was reflected by the synovial levels of cytokines produced by these cells, and decreased inflammation, pannus formation and cartilage and bone damage in the joints. *In vitro* experiments showed that ibrutinib inhibits the production of cytokines by murine macrophages upon FcγR stimulation.[165] Additionally, ibrutinib may affect osteoclasts, as *in vitro* inhibition experiments showed decreased osteoclast differentiation and bone resorbing activity in bone marrow derived monocytes.[293]

Successful inhibition of the non-humoral compartment by ibrutinib *in vivo* is even more evident from the CAIA model, where the humoral immune response is bypassed by injection of collagen specific antibodies in mice. When animals were orally treated starting at the moment of antibody transfer, high doses of ibrutinib completely protected against arthritis development, while a lower dose delayed and decreased the symptoms.[165] Immune cell infiltration of the synovium was nearly undetectable and pannus formation and bone loss were reduced in ibrutinib treated mice. These data are in contrast with the results from K/BxN.*Xid* mice, which did not appear to be protected from arthritis upon antibody transfer.[273] A possible explanation could be that ibrutinib does not only inhibit BTK, but also inhibits other kinases that may be important for myeloid cells, albeit with a lower binding capacity.[294]

Table 2. BTK inhibition in mouse models of rheumatoid arthritis

inhibitor	model	effect	ref
Ibrutinib (PCI-32765)	CIA (mice)	After onset: reduces joint inflammation, pannus formation, bone destruction, cytokines in synovium and auto-antibodies in serum of DBA/1 mice	22, 165
	CAIA (mice)	Before onset: reduces inflammation of the synovium, and reduced pannus formation and bone loss in DBA/1 mice	165
	AIA (rats)	After onset: reduces joint inflammation, affected joint count and bone destruction, increases OPG and reduces RANK-L in serum	300
	in vitro	Reduces cytokine production by murine macrophages upon FcγR stimulation	165
		Reduces osteoclast differentiation and bone resorbing activity	293
CGI1746	CIA (mice)	Before onset: prevents arthritis and auto-antibodies in serum;	107
		After onset: significantly reduces joint inflammation, pannus formation and cartilage and joint destruction in B10.RIII mice	
	CIA (rats)	After onset: significantly reduces joint inflammation in Lewis rats	107
	CAIA (mice)	Before onset: inhibits arthritis, decreases cytokine levels in the joints but does not affect normal macrophage mediated antibody clearance in SCID mice; reduces arthritis scores in Balb/c mice injected with K/BxN serum	107
	in vitro	Inhibits BCR signaling and proliferation of murine B cells, inhibits FcγRIII signaling mediated pro-inflammatory cytokine production in murine macrophages	107
RN486	CIA (mice)	Before onset: inhibits arthritis development dose dependently, no arthritis at highest dose, decreases autoantibody levels in serum, synovial inflammation, pannus formation cartilage damage and bone loss in DBA/1 mice;	295
		After onset: blocks progression of arthritis in DBA/1 mice	
	CAIA (mice)	Before onset: completely prevents arthritis in Balb/c mice	295
	AIA (rats)	Before onset: inhibits arthritis development dose dependently, completely protects against disease in combination with methotrexate, limiting joint and systemic inflammation in Lewis rats	295
	in vitro	Inhibition of anti-IgD stimulated, but not LPS stimulated CD69 expression on murine splenic and blood B cells	295
BMS-986142	CIA (mice)	Before onset: reduces arthritis scores, lower autoantibody levels in serum, decreased joint inflammation and bone loss in DBA/1 mice; synergistic therapeutic effect with methotrexate, etanercept or CTLA-4-Ig at suboptimal doses in DBA/1 mice	296
	CAIA (mice)	Before onset: reduces arthritis scores with low dose and almost no arthritis with high dose treatment in Balb/c mice	296
CC-292	CIA (mice)	After onset: dose dependently inhibits weight loss, synovial inflammation, pannus formation, cartilage and bone damage in DBA/1 mice; correlation between BTK occupancy and treatment efficacy	298
HM71224	CIA (mice)	After onset: dose dependently inhibits weight loss, arthritis scores, bone loss, reduces autoantibodies and IL-6 in serum in DBA/1 mice; correlation between BTK occupancy and treatment efficacy	297
CHMFL-BTK-11	AIA (rats)	After onset: reduces joint inflammation, affected joint count and bone destruction, increases OPG and reduces RANK-L in serum	300
GDC-0834	CIA (rats)	Before onset: dose dependently reduces joint inflammation, reduces phosphorylation of BTK at Y223 in Lewis rats	299

Similar results were obtained with CGI1746, a highly selective BTK inhibitor that stabilizes the non-phosphorylated enzyme conformation upon binding BTK, thereby inhibiting BTK kinase function.[107] *In vitro*, CGI1746 inhibits BCR signaling and B cell proliferation in a dose-dependent manner. *In vivo* in CIA, twice daily treatment of mice with CGI1746 initiated before onset of arthritis could almost completely prevented the development of disease, whereas dexamethasone control treatment only decreased disease severity. Collagen-specific autoantibodies were decreased in serum of CGI1746 treated mice. In addition, treatment in the effector phase or in established disease, when B cells and autoantibodies are no longer essential, significantly reduced joint inflammation, pannus formation and joint destruction in both mice and rats. Again these protective effects may be in part mediated through monocyte and macrophage inhibition, as CGI1746 could inhibit FcγRIII-mediated, but not TLR2 or TLR4 mediated production of pro-inflammatory cytokines by murine macrophages *in vitro*.[107] *In vivo*, CGI1746 was effective in two CAIA models. Treatment before disease onset in severe combined immunodeficiency (SCID) mice injected with anti-collagen type-II antibodies completely prevented arthritis. Pro-inflammatory cytokines in the joints were significantly decreased compared to non-treated controls, but macrophage-mediated antibody clearance was not compromised. In Balb/c mice injected with K/BxN serum, arthritis did develop but disease scores were markedly decreased.[107] These data are more in line with results in K/BxN.*Xid* mice discussed above,[273] and suggest that macrophages may be only partly inhibited by CGI1746, or that other cells that also produce cytokines in the joints may be more effectively inhibited than macrophages.

RN486 and BMS-986142 are both reversible small molecule BTK inhibitors. Interestingly, both these inhibitors have been studied as monotherapy, as well as in combination with other standard of care treatments in animal arthritis models.[295, 296] RN486 treatment before onset of CIA inhibited arthritis development in a dose-dependent manner, with complete protection reached at the highest dose given, exceeding therapeutic effects of dexamethasone.[295] Autoantibody levels, synovial inflammation, pannus formation, cartilage damage and bone loss were significantly decreased. When treatment was started after onset of disease, progression of arthritis was blocked, despite the limited effects on autoantibody levels. Also with this inhibitor, the effects at this stage of disease were probably mediated through inhibition of the myeloid compartment, as treatment of mice in the CAIA model completely abrogated disease development.[295] In adjuvant-induced arthritis (AIA) in rats, RN846 treatment inhibited both arthritis and systemic inflammation in a dose-dependent manner. Interestingly, when both RN486 and methotrexate were given at suboptimal doses that alone would only reduce arthritis to ~50% of controls, this combination completely abrogated disease development. This finding shows that BTK inhibition may be an interesting therapeutic for combination strategies in the treatment of RA. Importantly, combination treatment significantly reduced inflammatory markers in serum, including factors that attract or stimulate monocytes and macrophages, indicating that combination therapy not only alleviates arthritis, but also affects markers of systemic disease, which contribute significantly to disease burden.[295]

Treatment with BMS-986142 started before onset of disease similarly reduced CIA in mice in a dose-dependent manner.[296] Splenic plasma cell numbers, autoantibody levels, joint inflammation and bone loss were all decreased upon treatment. This effect was not only mediated through B cell inhibition, but also through inhibition of the non-humoral compartment, because treatment in CAIA reduced arthritis scores, joint inflammation and bone resorption. Interestingly, combination of a suboptimal dose of BMS-986142 with methotrexate had a synergistic therapeutic effect on CIA clinical scores, despite limited effects on plasma cell numbers in the spleen and autoantibody levels.[296] Similar synergistic effects were observed when BMS-986142 treatment was combined with etanercept, a TNF-α inhibitor, or CTLA-4-Ig, which inhibits T cell activation through co-stimulation.[296] These studies show a clear therapeutic potential for BTK inhibition in combination treatment of RA, although in both studies treatment efficacy was only assessed when it was started before onset of clinical symptoms.

A number of other BTK inhibitors have also shown therapeutic efficacy in arthritis models. HM71224 and CC-292 were both tested in mouse CIA.[297, 298] Treatment after onset of disease reduced weight loss, arthritis scores and bone erosion in mice in a dose-dependent manner. Serum cytokine and autoantibody levels were also decreased. Importantly, in both studies the efficacy of BTK inhibition was correlated with BTK occupancy by the inhibitor, where a low dose of the inhibitor showed lower occupancy and also limited effects on arthritis.[297, 298] The inhibitor GDC-0834 was shown to inhibit phosphorylation of BTK at Y223 in blood of naïve Balb/c mice, and in Lewis rats subjected to CIA, in which the inhibitor dose dependently reduced disease symptoms.[299] Another inhibitor, CHMFL-BTK-11, was effective in the rat AIA model, where it had similar beneficial effects as ibrutinib, reducing joint inflammation and bone destruction, increasing serum OPG levels and decreasing serum RANK-L levels.[300]

Finally, the highly selective irreversible BTK inhibitor acalabrutinib (ACP-196), which showed promising safety and efficacy profiles in patients with B cell malignancies and recently received accelerated FDA approval,[23, 301] is currently in a clinical trial in RA patients on methotrexate background therapy (www.clinicaltrials.gov), but results of this study have not been reported to date.

E. BTK inhibitors in lupus mouse models

Several BTK inhibitors were also tested in mostly spontaneous lupus mouse models (**Table 3**). MRL.lpr/lpr mice treated orally with ibrutinib for 12 weeks from 8 weeks of age, when the disease phenotype is still very mild, showed a significant reduction in proteinuria, serum blood urea nitrogen (BUN) levels, serum anti-dsDNA antibodies and histological kidney damage.[22] Similarly, treatment of MRL.lpr/lpr mice with HM71224 from 8 weeks of age effectively reduced disease symptoms.[302] B cell activation, splenomegaly and lymphadenopathy were reduced in these mice. Skin lesions were completely absent, BUN levels, proteinuria and renal inflammation were significantly decreased and survival improved in treated compared

to non-treated animals. Treatment efficacy was even more pronounced in the NZBxNZW model, where mice treated from 18 weeks of age showed a 100% survival with the highest dose used, compared to ~67% in non-treated controls. Serum BUN and creatinine levels, proteinuria and kidney damage were also significantly improved in this model, indicating that BTK inhibition may be effective in a broad range of lupus-like etiologies.[302]

Table 3. BTK inhibition in mouse models of SLE

inhibitor	model	effect	ref
Ibrutinib (PCI-32765)	MRL.lpr/lpr	Start at 8 weeks old: reduces proteinuria, BUN, serum anti-dsDNA, interstitial nephritis and perivascular inflammation	22
	Sle1	Start at 4 months old: reduces splenomegaly by reducing B and T cells, reduces IgG, but not IgM autoantibodies in serum	303
	Sle1Sle3	Start at 4 months old: reduces splenomegaly (B cell and myeloid cells reduced, T cells more naïve), IgM and IgG autoantibodies in serum and glomerulonephritis	303
RN486	NZBxNZW	Start at mild onset of proteinuria: reduced glomerulosclerosis, Ig and C3 depositions and macrophage infiltration in kidneys and IgG autoantibodies (but not IgM) in serum, reduces plasma cells IFN-inducible gene expression in the spleen	307
PF-06250112	NZBxNZW	Start at 26 weeks: reduces glomerulonephritis, prevents severe proteinuria, reduces splenic plasma cells and autoantibodies in serum, inhibits B cell activation and reduces splenic memory T cells	305
	Anti-GBM Ab	Before onset: dose dependently reduces IC-mediated proteinuria	305
G-744	NZBxNZW (+ IFNα-expressing adenovirus)	3 weeks after adenovirus treatment: increases survival, decreases proteinuria, reduces kidney damage, splenic B and T cell activation, GC formation and plasma cell numbers	309
HM71224	MRL.lpr/lpr	Start at 8 weeks old: inhibits B cell activation, splenomegaly, lymphadenopathy, skin lesions, proteinuria, kidney damage	302
	NZBxNZW	Start at 18 weeks old: reduces B cell activation, splenomegaly, lymphadenopathy, serum BUN and creatinine, proteinuria and kidney damage and improves survival	302
BI-BTK-1	NTS	Before onset: dose dependently reduces proteinuria, serum BUN, creatinine levels and kidney damage in 129sv/J mice	306
		After onset: reduces proteinuria and kidney damage	
M7583	BXSB-*Yaa*	Before onset: decreases proteinuria, kidney damage and serum autoantibody levels (except anti-RNA antibodies) and improves survival. Reduces activated B and T cell numbers and expression of genes associated with kidney infiltration	310
	Pristane-induced	2 months after pristane injection, but before onset of disease: reduces arthritis and autoantibodies in serum, except anti-RNA, but does not affect IFN type I induced gene expression in DBA/1 mice	310

Mice harboring the *sle1* locus, which develop a mild lupus-like phenotype, were protected from splenomegaly when treated with ibrutinib through drinking water.[303] This was due to a significant decrease in the number of splenic B and T cells, although monocyte and macrophage populations were not affected compared to untreated controls. Interestingly, ibrutinib prevented the formation of IgG, but not IgM autoantibodies in these mice, indicating the involvement of BTK signaling in the induction of Ig class-switching. Mice harboring both the *sle1* and *sle3* loci develop a much more severe autoimmune phenotype featuring spontaneous glomerulonephritis.[304] When these mice were treated with ibrutinib, starting before onset of disease, both IgM and IgG anti-nucleosome, anti-histone and anti-dsDNA levels were decreased.[303] Like in *sle1*-only harboring mice, treatment in *sle1.sle3* mice led to reduced splenomegaly. Splenic B cell populations and memory, but not naïve, T cells were decreased. Moreover, in these mice also myeloid cells were affected and decreased compared to untreated controls. The shift from memory to a more naive phenotype of T cells may be an indirect effect of B and myeloid cell inhibition, but might also be a consequence of TEC or Itk inhibition by ibrutinib. Glomerulonephritis was decreased in these mice, although intra-renal cellularity was not affected save for a drop in B cell numbers.[303] These data indicate that also in mouse lupus, myeloid cells may be inhibited by BTK inhibition.

The NZBxNZW lupus mouse model has been used to assess the efficacy of several BTK inhibitors. The effects of BTK inhibition in monocytes or effector cells in lupus was investigated in two studies with PF-06250112 and BI-BTK-1. NZBxNZW were treated with PF-06250112 orally for 12 weeks from 26 weeks of age, when anti-dsDNA antibodies can be detected in the serum, and severe proteinuria is developing.[305] Kidney pathology was inhibited in a dose dependent manner, and severe proteinuria was completely prevented by treatment. A reduction in plasma cells in the spleen was also dose-dependent, and B cell numbers and activation were reduced upon treatment. Both IgG and IgM-producing plasma cells were reduced in the spleen, resulting in significantly lower levels of anti-dsDNA antibodies. PF-06250112 affected memory, but not naïve T cells. Similar to the CAIA model, lupus can be induced in an FcγR-dependent way by passive transfer of autoantibodies, in this case anti-glomerular basal membrane antibodies. Treatment of mice, starting shortly before transfer of auto-antibodies, significantly decreased proteinuria in a dose-dependent manner, even when antibody and C3 depositions in the glomeruli remained, indicating that immune complex mediated disease could be inhibited with PF-06250112.[305] In another model of passive antibody transfer, by injecting nephrotoxic serum into 129sv/J mice, the BTK inhibitor BI-BTK-1 likewise inhibited proteinuria, and reduced serum BUN and creatinine levels in a dose-dependent manner.[306] BTK inhibition reduced kidney damage, without affecting the levels of autoantibodies present in the serum, indicating that downstream effectors are the target of this inhibition. Importantly, when BI-BTK-1 treatment was started after disease onset, it also effectively reduced proteinuria and kidney damage, showing that BTK inhibition is also effective in later effector stages of disease in lupus mouse models.[306]

Treatment of NZBxNZW mice with RN486 in chow after onset of mild proteinuria led to a reduction of proteinuria at the end of the study compared with the start of treatment, whereas in untreated controls mice proteinuria was increased.[307] Glomerulosclerosis and

IgM, IgG and C3 depositions in the kidneys were reduced in treated mice. B cell activation was suppressed by treatment, leading to decreased serum anti-dsDNA IgG levels. Like in *sle1* mice, IgM autoantibodies were not affected. In line with this, the number of autoreactive IgG-producing but not IgM-producing plasma cells was decreased in the spleen, although total splenic IgG-secreting plasma cells were not altered, nor were IgM or IgG plasma cells in the bone marrow. Apart from B cells, monocytes are also affected by treatment. Significantly fewer macrophages infiltrated the kidneys, and expression of monocyte related genes associated with lupus in the kidneys was reduced. In addition, in the spleen the expression of IFN-inducible genes was decreased. Importanlty, these genes are increased in 2/3 of SLE patients and make up the IFN type I signature.[308]

The effect of BTK inhibition in IFNα-mediated disease was more specifically addressed in a study testing the BTK inhibitor G-744.[309] To this end, an adenovirus expressing murine IFNα was administered to NZBxNZW mice at 14-15 weeks of age, and oral treatment with G-744 was started 3 weeks later. At the end of the study, all G-744 treated mice survived, while only 40% of vehicle-treated controls were still alive. Autoantibody levels, proteinuria and kidney damage were significantly decreased. GC B cell and splenic IgM and IgG plasma cells numbers were decreased, as was the expression of T cell activation markers. These results were comparable to treatment of NZBxNZW mice that were not injected with adenovirus, and shows that BTK inhibition may be effective in inhibiting the type I IFN signature.[309]

This finding was confirmed in a study with the BTK inhibitor M7583 in the pristine-induced SLE mouse model.[310] Upon pristane injection, DBA/1 mice develop an autoimmune phenotype characterized by the presence of autoantibodies often associated with SLE.[311] In addition to nephritis mice can develop arthritis, which is similar to arthritis in SLE patients and depends on TLR7 and type I IFN.[311] Treatment of mice before onset of disease significantly reduced arthritis and most autoantibody levels, with the exception of anti-RNA antibodies. This was related to a reduction in activated B and T cells and plasma cells in the spleen.[310] Interestingly, the expression of type I IFN-induced genes was not affected in these animals, suggesting that the effects of BTK inhibition are more downstream. Similarly, in the BXSB-Yaa lupus mouse model, which is also TLR7 dependent, treatment of mice with M7583 significantly reduced proteinuria and kidney damage, and improved survival.[310] Like in the pristane model, autoantibodies except those recognizing RNA were decreased. In the kidneys of treated mice expression of several genes associated with infiltrating immune cells was also reduced.

Together, these studies show that different BTK inhibitors, with different modes of inhibition and both reversible and irreversible, are very effective in multiple models of autoimmunity (**Tables 2 and 3**). Not only B cell-driven models show improvement upon treatment, also models that are driven by myeloid cells or other effector cells can be treated with these inhibitors. Most often the benefits of BTK-inhibition observed in murine models are comparable to or superior to the standard treatment of care used in patients. These promising results make BTK inhibition an interesting new therapeutic option for autoimmune patients, particularly in combination therapy strategies.

VII. BTK IN HUMAN AUTOIMMUNE DISEASE

A. Autoimmunity in XLA

In animal models of autoimmunity, loss of BTK mostly protects against disease development, and pharmacological inhibition of BTK can decrease disease symptoms even after onset of the disease. However, in human XLA patients, the few B cells that do leave the bone marrow and enter the periphery have a highly selective BCR repertoire. These BCRs show increased secondary Igκ and Igλ recombination, are polyreactive, and recognize self-antigens.[172] Although residual peripheral B cells in XLA patients have a recent immigrant phenotype, these cells have intact CD40L signaling, can undergo class-switching and can differentiate into antibody producing plasma cells.[16, 312] These data show that BTK is not only required for normal B cell development, it is also essential for normal BCR selection and B cell tolerance. A fraction of XLA patients show clinical symptoms of autoimmunity,[313] and there are indeed several case reports describing XLA patients that developed an autoimmune disease.

Case reports describe XLA patients who developed polyarthritis strongly resembling RA, Kawasaki disease or type I diabetes mellitus, but B cells or autoantibodies were largely undetectable, suggesting that in these patients the disease was T cell mediated.[314-316] It is conceivable that B cells, or the near absence of B cells may indirectly support the development of autoimmune disease, as XLA patients show lower numbers of Treg cells.[317] However, there are also reports of autoimmunity in XLA patients that suggest that residual B cells may be directly involved in the disease. In a patient who developed membranoproliferative glomerulonephritis, low levels of antibodies were found before the onset of intravenous Ig (IVIG) treatment.[318] This disease is immune complex mediated, so the IVIG treatment the patient received could be a causal factor. However, modulation of the IVIG regimen did not reduce the symptoms. Instead, the successful treatment of this patient with methylprednisolone pulse therapy suggests that endogenous B cells may be responsible for immune complex formation. Another report describes a patient that developed limited cutaneous scleroderma.[319] XLA was not formally proven by identification of a mutation in BTK, but the patients had very low B cell numbers and a history of recurrent infections. In this patient, autoantibodies were detected, indicating that residual B cells may play a role. Finally, two related XLA patients were shown to have developed gastrointestinal symptoms resembling IBD.[320] These patients both had a mutation in BTK that greatly decreased but not totally ablated BTK expression, leading to a partial or hypomorphic phenotype with low levels of IgM detectible in the serum. A survey revealed that patients with a hypomorphic phenotype have a higher chance of developing IBD, implying residual B cells may be involved.

B. BTK in autoimmune disease

Increasing evidence suggests that altered BTK activity and rewiring of BCR signaling may play a role in the loss of B cell tolerance in human autoimmune disease. In active RA patients receiving various treatments, BCR signaling in unstimulated $CD27^{-}IgD^{+}IgM^{low/-}$ B cells is increased.[321] These B cells normally have an anergic phenotype, but harbor BCRs that can recognize self-antigens.[322] In RA patients, phosphorylation of BLNK, JNK and SHP2 were significantly increased in these unstimulated 'anergic' B cells, although maximum phosphorylation upon BCR stimulation was comparable to cells from healthy controls.[321] This shows that not only activating signals are increased, but also cell intrinsic BCR inhibitory signals are increased in RA patients, possibly due to chronic stimulation with self-antigens. In another study, phosphorylation of Syk was also found to be increased in RA patients, particularly in patients with high anti-citrullinated protein antibody (ACPA) levels.[323] However, pSyk did not correlate with other clinical parameters or disease severity. In line with this, several clinical trials with the Syk inhibitor fostamatinib showed a moderate therapeutic effect in RA.[324]

BTK protein expression is differentially regulated between different human peripheral B cell subsets, with increased protein levels in non-switched memory B cells compared to naive and switched memory B cells.[26] In addition, BTK protein levels are upregulated upon stimulation through the BCR and TLRs. The functional role of this differential regulation is yet unclear, but changes in BTK protein correlate with changes in BTK phosphorylation. In a majority of untreated SjS and RA patients, BTK protein levels were increased compared to healthy controls in all these B cell subsets, including naïve B cells.[26] Increased BTK protein was strongly correlated with autoantibodies in these patients, as in RA, only $ACPA^{+}$ patients had high BTK levels, and in SjS, rheumatoid factor levels correlated with BTK protein levels. In addition, BTK levels in B cells correlated with Th17 cell numbers and follicular helper T cell activity in peripheral blood of RA patients, and with numbers of infiltrating T cells in the salivary glands of SjS patients. This associates increased BTK expression with autoantibodies and T cell activity, two important markers of disease activity. When SjS patients were treated for 24 weeks with abatacept, a CTLA-4-Ig aimed at inhibiting T cell activation/co-stimulation, increased expression of BTK protein in naïve B cells of these patients decreased to levels found in healthy controls.[26] This is in line with our data from BTK overexpressing mice showing a pro-inflammatory B-T cell interaction loop.[230] Similar results were obtained for phosphorylation of Syk in RA patients treated with abatacept, indicating an important role for T cells in the aberrant activation of B cells in autoimmunity.[323]

Mouse studies suggest that altered BTK expression or activity may change B cell survival and selection in favor of auto-reactivity.[25, 267] However, recent data from SLE patients suggests that BTK may also be involved in autoimmunity through autoantibody independent B cell function. BTK interacts with the transcription factor Bright/ARID3a to enable transcription of the immunoglobulin heavy chain.[325, 326] This requires intact BTK kinase activity, as the BTK phosphorylation target general transcription factor IIi (GTF 2I or TFII-I) is a crucial third molecule in this transcription factor complex.[327] It is conceivable that the phenotype of BTK

deficient or *Xid* mice can partly be explained by the loss of Bright function, as mice expressing dominant negative Bright mimic several characteristics of BTK deficient mice, including reduced mature B cell numbers and decreased serum IgM.[328] Bright also contributes to the BCR signaling threshold by blocking calcium influx and BTK and TFII-I phosphorylation, independent of its role in immunoglobulin heavy chain expression.[329] In mice overexpressing Bright, splenic transitional B cells were increased and differentiation was skewed towards the marginal zone subset.[330, 331] Furthermore, these mice produced autoantibodies already at a very young age.[330, 331] In human SLE patients, Bright was expressed on naïve B cells, which is not the case in healthy controls, and expression was correlated with disease severity.[332] However, Bright expression did not correlate with autoantibody production by B cells, nor skewing towards certain BCR specificities, suggesting that the connection to disease severity is through antibody independent mechanisms. Whether BTK also plays a role in these processes remains to be investigated, but BTK protein expression was also found to be upregulated in B cells of SLE patients and correlated with disease severity.[333]

CONCLUDING REMARKS

A growing body of literature supports the concept that BTK is a crucial signaling molecule in selection, survival and activation of pathogenic B cells in human autoimmunity. It plays a role not only in autoantibody production, but also in pathogenic cytokine production, IFN type I signaling and aberrant activation of other immune cells by B cells. Use of BTK inhibitors ibrutinib and acalabrutinib in the clinic in the treatment of several B cell lymphoma have shown that BTK is not only involved in BCR signaling, but also in TLR and chemokine receptor signaling, and in signaling pathways in cells of the tumor microenvironment in for example the bone marrow.[105, 334-336] In addition, its role in other immune cells, the availability of a wide range of BTK small molecule inhibitors and the fact that ibrutinib and acalabrutinib are generally well tolerated by patients with B cell malignancies, make BTK an interesting new therapeutic target in autoimmunity. Together with the promising results from autoimmune mouse models with BTK inhibition, in particular in combination strategies, this has prompted several phase I and phase II clinical trials with BTK inhibitors in RA (HM71224, CC-292, M2951, Evobrutinib and GS-4059) and SLE (BIIB068 and MSC2364447C) patients (www.clinicaltrials.gov). These and future studies will soon show the potential of BTK inhibition in these patients with respect to long-term treatment.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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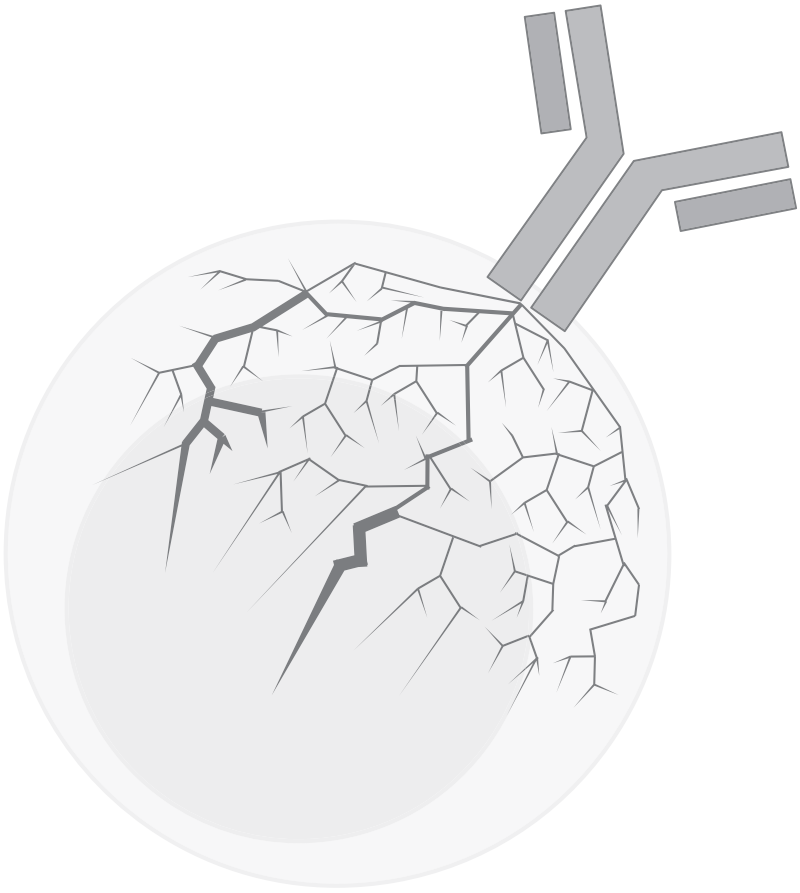

CHAPTER 3.1

Phosphoflow protocol for signaling studies in human and murine B cell subpopulations

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ABSTRACT

B cell receptor (BCR) signaling, involving phosphorylation of various downstream molecules, including kinases, lipases and linkers, is crucial for B cell selection, survival, proliferation and differentiation. Phospho-flow cytometry (phosphoflow) is a single-cell-based technique to measure phosphorylated intracellular proteins, providing a more quantitative read-out than western blotting. Recent advances in phosphoflow basically allow simultaneous analysis of protein phosphorylation in B cell (sub)populations, without prior cell sorting. However, fixation and permeabilization procedures required for phosphoflow often affect cell surface epitopes or monoclonal antibody conjugates, precluding the evaluation of the phosphorylation status of signaling proteins across different B cell subpopulations present in a single sample. Here, we report a versatile phosphoflow protocol allowing extensive staining of B cell subpopulations in human peripheral blood or various anatomical compartments in the mouse, starting from freshly isolated or frozen cell suspensions. Both human and mouse B cell subpopulations showed different basal and BCR stimulation-induced phosphorylation levels of downstream signaling proteins. For example, peritoneal B-1 cells and splenic marginal zone (MZ) B cells exhibited significantly increased basal (*ex vivo*) signaling and increased responsiveness to *in vitro* BCR stimulation compared to peritoneal B-2 cells and splenic follicular B cells, respectively. In addition, whereas stimulation with anti-IgM or anti-Igκ light chain antibodies resulted in strong pCD79a and pPLCγ2 signals, IgD stimulation only induced CD79a – but not pPLCγ2 phosphorylation. In summary, the protocol is user-friendly and quantifies BCR-mediated phosphorylation with high sensitivity at the single-cell level, in combination with extensive staining to identify individual B cell development and differentiation stages.

INTRODUCTION

Intracellular signal transduction is an integral part of cell biology and crucial for transfer of activation, survival, proliferation and differentiation signals received by immune cells from their environment. B cells are crucial in the protection against pathogens, as they can sense antigen and - upon activation - can differentiate into plasma cells that produce highly specific antibodies. Signaling is crucial for selection of B cells based on the functionality and specificity of their antigen receptor (1, 2). B cell receptor (BCR) repertoire selection is a process that is largely determined by BCR signaling strength, as B cells should not respond too strongly or too weakly to encountering antigen. When signaling molecules downstream of the BCR are aberrantly expressed or activated, this can result in altered B cell selection and defective clonal deletion of autoreactive B cells (3, 4). Moreover, several BCR signaling molecules function as important regulators of cell proliferation and survival in various B cell malignancies. Small molecule inhibitors of BCR-associated kinases, such as idelalisib and ibrutinib/acalabrutinib, which target phosphoinositide 3-kinase (PI3K) and Bruton's tyrosine kinase (BTK), respectively, have shown impressive anti-leukemia activity in clinical studies (5). Therefore, expression and activity of signaling molecules downstream of the BCR play a crucial role in B cell proliferation and survival as well as in fine-tuning of the BCR repertoire.

The BCR is non-covalently associated with the transmembrane proteins CD79a (Ig-α, MB1) and CD79b (Ig-β, MB2), which form heterodimers. Following BCR engagement, biochemical alterations in this BCR complex lead to the recruitment and activation of Src kinases such as Lyn. Upon activation, phosphorylated Lyn activates the cytoplasmic tail of CD19 with help of B cell adaptor for PI3K (BCAP) (6). This will recruit and activate PI3K, which is responsible for the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3), a crucial process that attracts cytoplasmic kinases with a plekstrin homology (PH) domain to the cell membrane (7). Lyn also phosphorylates the intracellular tyrosine-based activation motifs (ITAMs) of CD79a and CD79b, thereby creating docking sites for spleen tyrosine kinase (SYK). Subsequently, phospho-SYK (pSYK) activates and recruits SH2 domain leukocyte protein of 65 kD (SLP-65), also known as SLP-65. SLP-65 is present in a complex with the Cbl-interacting protein of 85 kDa (CIN85) (8, 9) and its phosphorylation results in activation and stimulation of the recruited PH domain-harboring kinase BTK. Upon SLP-65-mediated recruitment, BTK will phosphorylate phospholipase Cγ2 (PLCγ2)(10). This complex of SLP-65, BTK and PLCγ2 is crucial for mobilization of intracellular calcium and for the activation of further downstream signaling routes, including mitogen-activated protein (MAP) kinase-mediated signaling of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and the NF-κB signaling cascade. Furthermore, both PI3K-derived signals and BTK have the capacity to activate the serine/threonine-specific protein kinase B (PKB/AKT) signaling pathway (11-13). AKT signaling is critical for the induction of proliferation via the ribosomal protein S6 and for the inhibition of nuclear translocation of pro-apoptotic FOXO proteins.

Traditionally, the method of choice for detection of phosphorylated proteins has been western blotting (14). Although this technique is effective to detect protein phosphorylation, it has several limitations: (i) it requires large numbers of purified cells and thus does not allow for single-cell analyses; (ii) it is not sensitive enough to quantify subtle changes in phosphorylation, (iii) it is time-consuming as it often takes several days to obtain results and (iv) results are hard to quantify. Phosphoflow is a more recently developed technique that is quick, based on single-cell analysis, and potentially allows for the quantification of the phosphorylation status of proteins in different cell populations present in a single sample (15, 16). However, the latter poses a challenge, as fixation and permeabilization procedures required for phosphoflow often affect cell surface epitopes or conjugates of the monoclonal antibodies that recognize these epitopes. Although some fixation and permeabilization procedures are less destructive, they may not be suitable to measure the phosphorylated protein of interest with sufficient sensitivity.

The expression and activity of signaling molecules in B cells, together with the balance between activating and inhibitory pathways, is crucial for the outcome of BCR signaling. This balance may be different across B cell subpopulations. For example, differences in mTOR signaling were found between marginal zone (MZ) B cells or follicular (Fol) B cells (17) and play a role in the development of these two subsets (18). Over the past years, several groups have focused on signaling differences between B cell subpopulations in various organs from mouse and human-derived material, by different protocols for phosphoflow cytometry or phospho-kinase arrays (17, 19-21). Here, we present a phosphoflow method that combines extensive B cell subpopulation identification using cell surface markers combined with sensitive detection of phosphorylation of several crucial BCR-associated protein kinases in human and mouse B cells, starting from freshly isolated or frozen cell suspensions. Using the protocol, we were able to identify subtle differences between B cell subpopulations, e.g. demonstrating increased signaling and responsiveness to BCR stimulation of MZ B cells and peritoneal B-1 cells, when compared with Fol and B-2 cells, respectively. The described protocol additionally should allow for simultaneous staining of nuclear proteins, thus staining of phosphoproteins in combination with transcription factors, such as T-bet and IRF4.

MATERIAL AND METHODS

Patient material and mice

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors were obtained from buffy coats via Sanquin (location Rotterdam Centrum). Experimental procedures were approved by the ethics committee of the Erasmus MC.

C57BL/6J mice were obtained from Charles River. All animal experiments complied with national laws and institutional regulations. Prior to performing experiments, all experimental protocols were reviewed and approved by our institutional Erasmus MC Committee for animal experiments (DEC) and nationally by the central committee for animal experiments (CCD).

Isolation and processing of PBMCs from whole blood

Fifteen mL of Ficoll-Paque™ Plus (GE Healthcare) was added to the 50mL Leucosep tube (Greiner Bio-one) and spun down at 1000*g* for 1 min at room temperature (RT). Next, a maximum of 25mL of whole blood was gently loaded in each Leucosep tube, ensuring the Ficoll remains beneath the filter in the tube and that blood does not mix with the Ficoll. The Leucosep tube containing whole blood and Ficoll was centrifuged on very low deceleration at 1000*g* for 10 min at RT. The whole solution above the filter (containing PBMCs, Ficoll and plasma) was transferred to a regular 50mL tube containing 15mL RPMI 1640 Glutamax™ (Lonza) – 5% FCS (Gibco) (RPMI-5% FCS). The respective 50mL tube was centrifuged at 400*g* for 7 min at 4°C. Afterwards, the supernatant was removed and cells were resuspended in 1-5mL RPMI-5% FCS to directly proceed to phosphoflow experiments. Freezing and thawing of viable cells was performed according to standard laboratory procedures. After thawing, pellets were resuspended in 1mL of RPMI-5% FCS and the yield of viable cells was checked by cell counting. Cells were kept at 4°C for <2 h before proceeding with the *in vitro* stimulation.

Isolation and acquisition of single-cell suspensions of murine cells

C57BL/6 mice were sacrificed by cervical dislocation and subsequently fixed on a porous surface using needles. A midline incision over the abdomen was performed to retract the skin while keeping the peritoneum intact. The peritoneum was flushed using a 10mL syringe, containing 5mL of ice-cold PBS and 5mL of air, attached to the 25-gauge x ⅝ inch needle. After gentle flushing and shaking for about 1 min, the peritoneal lavage was collected by aspirating the peritoneal cavity using a 10mL syringe and subsequently placed in a 15mL tube on ice. Immediately after collecting the peritoneal lavage, the peritoneal cavity was opened and the spleen and Peyer's Patches were carefully extracted and placed in ice-cold PBS. Also, one femur was extracted and placed in ice-cold PBS.

Next, single-cell suspensions of both tissues were acquired. Peritoneal lavage cells were centrifuged at 400*g* for 7 min at 4°C and the supernatant was discarded. Peritoneal lavage cell pellets were resuspended in 1mL of RPMI-2% FCS (Capricorn) and kept on ice. Spleen tissue was mechanically disrupted using a 100μm cell strainer (Corning) on a 50mL tube, using a plunger of a 2mL syringe. During and following processing of the spleen, strainers were flushed with 5mL of cold RPMI-2% FCS to acquire all cells. Single-cell suspensions of Peyer's Patches were acquired using similar procedures as for the spleen, except that the patches were mechanically disrupted using a plunger of a 1mL syringe and that cells were acquired in a total volume of 300μl. Femurs were crushed using a mortar, followed by pipetting the suspension over an 100μm cell strainer (Corning) on a 50mL tube. During and following processing of the femurs, strainers were flushed with a total of 3mL of cold RPMI-2% FCS to acquire bone marrow (BM) cells. After processing, single-cell suspensions were counted and kept at 4°C up to 2 h before proceeding with the *in vitro* stimulation.

Phosphoflow protocol

In vitro Stimulation and Fixation

Stimulation and staining of cells were performed in 96-wells round-bottom plates, using $5*10^5$ per well ($2\text{-}10*10^5$ cells were technically optimal). Per well, we plated $5*10^5$ cells in a volume of 50μL in each well on ice, keeping the cells at 4°C at all times. For stimulation times <1 h, the 96-wells round bottom plate was placed in a water bath at 37°C, ensuring that only the bottoms of the wells were in contact with the water. For stimulation times >1 h, the stimulus was added while the cells were on ice and subsequently incubated at 37°C in a 5% CO_2 humidified incubator. It is important to note that targets such as Akt or components of the NF-κB signaling pathway require 1-3 hours resting prior to stimulation on fresh murine cells, as they are sensitive to mechanical stress of isolation or temperature fluctuation. This resting period also enables the incubation of cells with inhibitor molecules prior to stimulation, because effective inhibition of BCR signaling generally requires pre-incubation with inhibitors for 1-3 hours at 37°C, depending on the inhibitor.

The stimulating agent (**Table 1**), diluted in RPMI 2-5% FCS, was added by pipetting 50μL of stimulus (in a two-fold concentration) to the wells containing 50μL of sample. In addition, 10μL RPMI 2-5% FCS containing fixable live/dead viability stain (Invitrogen) was added 10 min prior to fixation. The eBioscience™ FoxP3 / Transcription Factor Fixation and Permeabilization buffer (Invitrogen) was diluted 1:1 with the supplied Fixation and Permeabilization diluent (Invitrogen). To fix the cells, 100μL of diluted fixation buffer – containing 50μL of fixation and permeabilization concentrate and 50μL of diluent - was added for 10 min at 37°C when stimulation time was due. After fixation, plates were centrifuged at 400*g* for 3 min at 4°C and supernatant was removed. Plates were subsequently washed by adding 200μL eBioscience Wash Buffer (10x diluted in milli-Q; Invitrogen) to all wells, followed by centrifugation at 400*g* for 3 min at 4°C. The washing step was performed twice. Fixed cells were stored in a volume of 200μl of eBioscience Wash Buffer at 4°C (up to 5 d without impacting sample quality).

Table 1. Stimuli used to engage BCR and co-receptor signaling in human and mouse B cells.

Stimulus	Working Concentration	Manufacturer	Cat. No.
Human			
Goat F(ab')2 Anti-Human IgM-UNLB	20µg/mL	Southern Biotech	2022-01
Goat F(ab')2 Anti-Human Ig-UNLB	20µg/mL	Southern Biotech	2012-01
Recombinant Human CD40L	2µg/mL	R&D	6245-CL/CF
Recombinant Human IL-4 protein	250ng/mL	R&D	204-IL-CF
Phorbol 12-myristate 13-acetate (PMA)	50ng/mL	Sigma Aldrich	P8139
Mouse			
AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgM	20-25µg/mL	Jackson ImmunoResearch	115-006-075
Goat anti-mouse IgK-UNLB	25µg/mL	Southern Biotech	1050-01
Goat anti-mouse IgD, antiserum	100x diluted	MD Biosciences	2057001
Purified Rat anti-Mouse CD40 (clone 3/23)	20µg/mL	BD Biosciences	553787
CpG ODN 1668 - DNA sequence (ZOO FZE FOE ZZO OZE FZE OT)	100nM	Invitrogen	n.a.
Lipopolysaccharide (LPS) *E.Coli* 026:B6	400ng/mL	Sigma Aldrich	L8274
Pervanadate (derived from Sodium Orthovanadate)	25µM	New England Biolabs [1]	P0758S [1]

[1] Manufacturer and catalogue number (Cat.No.) correspond to the product Sodium Orthovanadate, which is the essential product required to create the pervanadate solution.

Direct fixation for ex vivo (basal) measurements

Parallel to stimulated cells, $5*10^5$ cells per well were transferred in a 96-wells round-bottom plate at a volume of 100µL per well on ice, keeping the cells at 4°C at all times. The procedure was performed at 4°C at all times, as we noticed that temperature alterations could greatly influence the basal level of phosphorylation. Samples were stained with the fixable live/dead viability stain (Invitrogen) for 10 min and subsequently fixed with pre-diluted eBioscience fixation and permeabilization buffer (Invitrogen) for 10 min at 4°C. After 10 min of fixation, plates were centrifuged at 400*g* for 3 min at 4°C and supernatant was removed. Plates were subsequently washed by adding 200µL eBioscience Wash Buffer to all wells, followed by centrifugation at 400*g* for 3 min at 4°C. The washing step was performed twice. Fixed cells were stored in a volume of 200µl of eBioscience Wash Buffer at 4°C (up to 5 d without impacting sample quality).

Flow cytometry procedures

To proceed with flow cytometry procedures, fixed cell samples were centrifuged at 400*g*, supernatant was removed and cells were resuspended with 40μL antibody staining mix to intracellularly stain for cell surface markers such as CD3, B220/CD19, IgD and IgM (**Table 2**) in eBioscience Wash Buffer, in the presence of an FcR-blocking agent (Human TruStain FcX; Biolegend) to avoid non-specific binding of the antibodies. The samples were incubated in the dark for 30 min at 4°C. Subsequently, plates were washed with eBioscience Wash Buffer and samples were resuspended with 40μL eBioscience Wash Buffer containing the phospho-target antibody of choice and incubated for 30 min at RT in the dark. When the antibody targeting the phospho-protein was an unconjugated rabbit-derived antibody, an additional incubation step was included with a donkey anti-rabbit PE-conjugated antibody (Jackson ImmunoResearch) in eBioscience Wash Buffer for 15 min at RT in the dark. Upon completion of the staining, samples were washed with MACS Buffer (0.5% BSA and 2mM EDTA in PBS) and centrifuged at 400*g* for 3 min at 4°C. The supernatant was removed and cells were resuspended in 50μL MACS buffer. Samples were transferred into insert tubes and measured using a flow cytometer within 1 d.

Prior to measuring, the flow cytometer was calibrated by compensating for all conjugates within the phospho-stainings, using UltraComp eBeads™ (Invitrogen). In order to analyze B cell subpopulations, we acquired 20,000-50,000 B cells. Because the cell size of the fixed cells decreased due the fixation and permeabilization procedure, as compared with non-fixed cells, the voltage of the forward scatter (FSC-A) was increased accordingly. Measurements were performed on an LSRII flow cytometer (BD Biosciences), and results were analyzed using FlowJo software v9 or v10 (TreeStar Inc).

Upon request, we will provide a written out step-by-step protocol supplied with additional information.

Table 2. Antibodies used for Phosphoflow cytometry on human and mouse samples.

Antibody	Conjugate	Clone	Manufacturer	Cat. No.	Dilution
Human					
CD3	Alexa Fluor 700	UCHT1	Invitrogen	56-0038-42	200x
CD3	BV711	UCHT1	BD Biosciences	563725	200x
CD19	FITC	HIB19	BD Biosciences	555412	15x
CD19	AF700	HIB19	BD Biosciences	561031	15x
CD27	BV421	M-T271	BD Biosciences	562513	10x
CD38	BV785	HIT2	Biolegend	303530	15x
IgD	BV605	IA6-2	BD Biosciences	563313	400x
IgM	Biotin	G20-127	BD Biosciences	555781	400x
IgA	FITC	n.a.	Southern Biotech	2050-02	1000x
IgG	BV786	G18-145	BD Biosciences	564230	80x
pSTAT6 (Y641)	PE	A15137E	Biolegend	686004	8x
Mouse					
B220	AF700	RA3-6B2	Invitrogen	56-0452-82	200x
CD21 / CD35	FITC	eBio4E3	Invitrogen	11-0212-85	100x
CD23	Biotin	B3B4	BD Biosciences	553137	400x
CD3	APC-eFluor 780	17A2	Invitrogen	47-0032-82	50x
CD3	BV421	145-2C11	BD Bioscience	562600	400x
CD5	BV421	53-7.3	BD Biosciences	562739	200x
CD5	PE	53-7.3	Invitrogen	12-0051-81	200x
CD43	PE	S7	BD Biosciences	560663	100x
CD93	BV650	AA4.1	BD Biosciences	563807	100x
CD95	FITC	Jo2	BD Biosciences	554257	200x
IgD	BV711	11-26c.2a	BD Biosciences	564275	2000x
IgK	Biotin	187.1	BD Biosciences	559750	4000x
IgL	Biotin	R26-46	BD Biosciences	553433	5000x
IgM	PE-Cy7	II/41	BD Biosciences	25-5790-82	400x
Human and mouse					
Fixable Viability Dye	eFluor 506	n.a.	Invitrogen	65-0866-14	200x
Streptavidin	APC-eFluor 780	n.a.	Invitrogen	47-4317-82	200x
Streptavidin	BV786	n.a.	BD Biosciences	563858	200x
pCD79a (Y182)	Alexa Fluor 647	D1B9	Cell Signaling Technologies	29742S	50x
pSYK (Y348)	PE	I120-722	BD phosflow	558529	8x
pSLP-65 (Y84)	PE	J117-1278	BD phosflow	558442	8x
pBTK / pITK (Y223/ Y180)	PE	N35-86	BD phosflow	562753	400x
pPLCγ2 (Y759)	Alexa Fluor 647	K86-689.37	BD phosflow	558498	8x

Antibody	Conjugate	Clone	Manufacturer	Cat. No.	Dilution
pERK1/2 (T202/pY204)	PE	20A	BD phosflow	561991	8x
pS6 (S240/244)	-	D68F8	Cell Signaling Technologies	5364	200x
pAkt (S473)	-	D9E	Cell Signaling Technologies	4060	100x
Anti-rabbit	PE	-	Jackson ImmunoResearch	711-116-152	400x
IkBα	PE	MFRDTRK	Invitrogen	12-9036-42	8-50x

Additional protocols to study signaling

Alternative phosphoflow protocol

To stain other phosphoflow targets, such as pSTAT6 or other pSTAT proteins, an alternative staining protocol with limited subset-defining markers was used. Upon stimulation and live/dead viability staining, cells were fixed with an equal volume of 100μL BD Cytofix Fixation Buffer (BD Biosciences; cat.no. 554655). After 10 min fixation at 37°C, cells were centrifuged at 400*g* for 3 min followed by two consecutive washes with MACS Buffer. Cells were stained with a staining mix containing monoclonal antibodies to extracellular markers CD19 and CD3, for 30 min at 4°C in the dark. Subsequently, cells were washed with MACS Buffer and, after centrifuging and removal of supernatant, permeabilized with 150μL BD Perm buffer III (BD Biosciences; cat.no. 558050) for 30 min at -20°C in the dark. After permeabilization and washing twice with MACS Buffer, cells were stained in MACS Buffer for the respective phospho-targets for 30 min at RT in the dark. Finally, cells were resuspended in a volume of 50μL of MACS buffer for measurements on an LSR-II flow cytometer (BD Biosciences).

IkB staining protocol

After stimulation, cells that were stimulated to measure IkBα degradation were kept on ice at all times. First, cells were centrifuged at 400*g* for 3 min at 4°C and supernatant was removed. After washing with MACS buffer, a staining mix for extracellular markers in MACS buffer was added and incubated for 30 min at 4°C in the dark. Next, cells were washed with PBS and centrifuged at 400*g* for 3 min at 4°C. After the supernatant was removed, the staining mix containing a live/dead viability marker in PBS was added and incubated for 10 min at 4°C in the dark. After staining for cell viability, cells were fixed using 2% paraformaldehyde (PFA) for 10 min at 4°C in the dark and subsequently permeabilized using 0.5% saponin in MACS buffer for 10 min at 4°C in the dark. Finally, fixed and permeabilized cells were stained in 0.5% saponin in MACS buffer for IkBα protein for 30 min at 4°C in the dark. Cells were resuspended in a volume of 50μL for measurements on an LSR-II.

Western Blotting

Naïve B cells from murine spleens were MACS-purified as previously described (22). Next, we stimulated 1.5*10^6 cells B cells with αIgM (25μg/mL), CpG (1μM) or added RMPI 2% FBS alone for 3 hours at 37°C. After 3 hours we lysed the cells using a Carin lysisbuffer supplemented with PefaBloc SC (Roche), Alpha-Prot Complete (Roche) and sodium orthovanadate. Afterwards, total lysate fractions derived from 1.0*10^6 cells were loaded on a 10% SDS-PAGE gel. After running the gel and blotting, the blot was blocked in 2% non-fat dry milk and Tris-buffered saline, 0.1% Tween 20 (TBST) buffer and subsequently stained overnight at RT for rabbit-derived pAkt-S473, rabbit-derived pS6-S240/244 (both Cell Signaling Technologies) and mouse-derived Actin (Chemicon/Merck) antibodies in 2% BSA. The next day, after washing 4 times with TBST buffer, we stained Actin with anti-mouse IgG IRDye 680RD secondary antibody (LI-COR Biosciences) and pAkt-S473 or pS6-S240/244 with anti-rabbit IgG IRDye 800CW secondary antibody (LI-COR Biosciences) for 2 h at RT in the dark. After washing 4 times, fluorescence intensity of each target was measured using the Odyssey imaging system (LI-COR Biosciences) and Image Studio v5.2 (LI-COR Biosciences).

RESULTS

Validation and titration of phospho-antibodies

Setting-up a phosphoflow protocol can be challenging, as each phospho-target of choice requires simultaneous titration of the phosphoprotein-specific antibody, as well as the determination of the optimal concentration of the stimulus and the optimal stimulation time. Therefore, we first established the concentration at which the antibody specific for the phosphorylated protein worked best. We performed several test stainings using a known strong stimulus and compared the signals obtained to unstimulated or medium-only controls. For example, anti-IgM (αIgM) stimulation was most optimal for testing an antibody staining for phosphorylated-CD79a (pCD79a-Y182; phosphorylated Ig-α). For BCR-restricted targets or BCR-specific stimulation, T cells were very useful as internal negative controls, because T cells should remain unaffected by αIgM stimulation and/or do not express certain BCR-restricted molecules such as CD79a (**Figure S1A**). When investigating more downstream target proteins, such as S6, ERK or NF-κB, it was useful to test strong activation via Toll-like receptors (TLRs) or with phorbol 12-myristate 13-acetate (PMA) in addition to stimulation with αIgM antibodies. Furthermore, we used $F(ab)_2$ fragments instead of complete antibodies. $F(ab)_2$ fragments have the advantage that they are not bound by Fc-receptors present on B cells, which would not only be associated with background staining, but also with interfering (de)phosphorylation events induced by Fc-receptor signaling (23). We validated the antibodies by including isotype and Fluorescence Minus One (FMO) controls in the set-up of the staining procedure, thereby verifying the signal intensities of the phospho-specific antibodies.

The manuscript protocol allows for extensive staining of different cell types, because the fixation and permeabilization conditions used are relatively mild and do not affect the most commonly used markers to identify B cell subpopulations. However, as in this protocol staining of these markers is performed after permeabilization of the cells, a distinction between intra- and extracellular expression cannot be made. We tested several human and mouse monoclonal antibodies that identify different B cell subpopulations, as stated in **Table 2**. The applied dilutions of these antibodies are generally in the range of regular FACS staining, however, additional verifications were performed. Recommended dilutions for these flow cytometry antibodies are stated in **Table 2**. Another important note is that not all phospho-targets of potential interest could be evaluated using this protocol. Alternative protocols showed a better resolution to stain pSTAT6 protein in IL-4-stimulated B cells compared to our phosphoflow protocol (**Figure S1B**). Even though the pSTAT6-Y641 signal is less pronounced using our phosphoflow protocol, this procedure can still be useful when the research question is focused on specific B cell subpopulations, as the alternative protocol does not allow for extensive staining panels.

Validation of optimal stimulation conditions

Media that are used during stimulation of cells should not contain components that may activate B cells or influence protein stability or phosphorylation status of the phospho-targets studied. For B cells, we found that RPMI 1640 supplemented with a low amount fetal calf serum (FCS), preferentially ~2%, did not activate phosphorylation. Lower amounts of FCS may induce apoptotic signals due to nutrient deprivation. Conversely, we observed that higher percentages (>5%) of FCS may induce non-specific stimulation of signaling routes involved in cellular activation (**Figure S1C**).

We performed dose titrations for αIgM stimulation in murine B cells and found that a dose of 20-25μg/mL is optimal for all phospho-targets tested. Sensitive phospho-targets such as pCD79a-Y182 and pS6-S240/244 were also readily detectable upon stimulation with low dose αIgM (e.g. 4-10μg/mL; **Figure S1D-E**).

We aimed to test the maximal capacity of phosphorylation by inhibiting phosphatase activity by stimulation with 25μM pervanadate/H_2O_2 solution, as described by Wienands et al. (24). Although pCD79a-Y182 levels were strongly increased in B cells stimulated with pervanadate compared to αIgM stimulation, in our hands this was also the case for pervanadate-stimulated T cells (**Figure S1F**). As CD79a is not expressed in T cells, we assume that the observed ~5-6-fold increase in pCD79a levels upon stimulation with pervanadate was due to non-specific background signals, as this is also the case in another published phosphoflow protocol (19).

Read-out of optimal stimulation times

To test the functionality and sensitivity of the phosphoprotein-specific antibodies, we simultaneously determined optimal stimulation times. We found that for most targets design of the suitable range of time-points could be guided by published findings in western blotting experiments or in some cases phosphoflow analyses (22, 25-27). In human peripheral blood mononuclear cell (PBMC) fraction-derived B cells, phosphorylation of signaling molecules upon BCR stimulation was detectable at ~2-5 min, at ~15-30 min or even later, depending on the phospho-target of choice (**Figures 1-2**). For all phospho-targets discussed in this manuscript, we indicated the time-point to detect maximal phosphorylation as well as the observed fold-induction upon stimulation with αIgM for both human and mouse B cells (**Figure S2**) .

Sample analysis and quality control

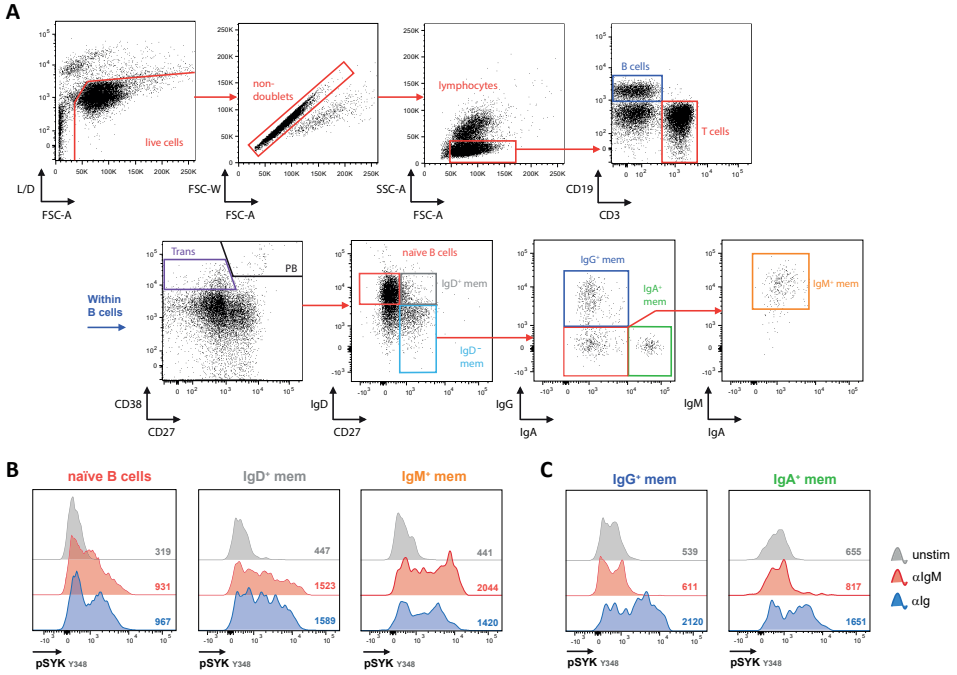
After sample measurement, we used regular methods for flow cytometric procedures for the analysis of the obtained data. We gated B cell subpopulations using specific markers as shown in **Figure 1A** for human peripheral blood B cells and **Figure S3** for subpopulations of B cells from various murine compartments, respectively. Within and across B cell subpopulations, the expression of phosphorylated epitopes was evaluated. We acquired 20,000-50,000 B cells during measuring as this generally resulted in at least a 1,000 cells per B cell subpopulation. The sole exception is for switched memory B cells from PMBCs, due to the low abundance of these cells in healthy volunteers. Upon *in vitro* stimulation, phosphorylation was quantified by comparing fluorescence intensities between unstimulated controls and stimulated samples. As there is no unstimulated control for comparison *of ex vivo* phosphorylation, we used FMO and/or isotype controls, or cell types in which the protein analyzed was not expressed. For example, to analyze the CD79a-Y182 phosphorylation status in B cell subpopulations we used the background signals in T cells, which do not express the CD79a protein.

To quantify protein phosphorylation, we generally used the increase of geometric mean fluorescent intensity (*g*MFI) values when the entire population showed increased phosphoprotein expression upon stimulation (**Figure S1D**). Alternatively, we calculated the increase in the proportion of positive cells, specifically when an increase in phosphoprotein expression was observed in a fraction of the cell population, leading to the appearance of two separate signal peaks (**Figure S1E**).

Optimal detection phosphorylated proteins in individual human peripheral B cell subpopulations

The time points for maximum phosphorylation levels of proteins downstream of the BCR varied from several minutes to hours following stimulation. Therefore, for each phospho-target the optimal time point to detect phosphorylation was determined.

For human B cells, we identified individual B cell subpopulations present in PBMCs based on extracellular markers after gating for single B cells as non-doublet live cells with lymphoid FSC and SSC characteristics that are positive for CD19 (**Figure 1A**). To evaluate if we could reliably quantify phosphorylation in these B cell subpopulations, we stimulated PBMCs either with αIgM or anti-Ig total (αIg). In naïve IgM$^+$ memory and IgD$^+$ memory B cells we observed increased pSYK-Y348 levels upon αIgM and αIg stimulation (**Figure 1B**). As expected, IgG$^+$ and IgA$^+$ memory B cells did not respond to αIgM, but strongly responded to αIg stimulation (**Figure 1C**). These findings show that whereas αIgM antibodies are suitable to study effects of specific IgM-stimulation, without concomitant IgD triggering, αIg can be applied to investigate memory B cell subpopulations.



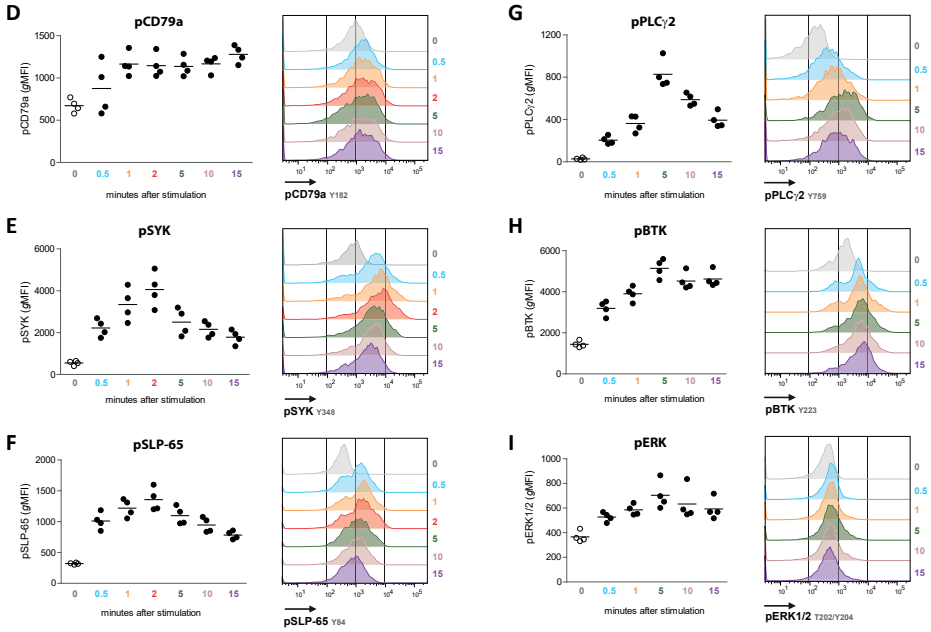


Figure 1. Phosphoflow analysis of human peripheral blood B cells.

(**A**) Gating strategy for human peripheral B cells, gating for live, single lymphocytes. After gating for $CD19^{+}CD3^{-}$ cells (B cells), cells were subsequently gated for plasmablasts (PB, $CD19^{+}CD38^{high}CD27^{+}$) and transitional B cells (Trans, $CD19^{+}CD38^{high}CD27^{-}$). Proceeding with $CD38^{low}$ B cells, we gated for naïve B cells ($CD19^{+}CD38^{low}CD27^{-}IgD^{+}$) and IgD^{+} non-switched memory B cells (IgD^{+} non-sw, $CD19^{+}CD38^{low}CD27^{+}IgD^{+}$). From the IgD-negative memory fraction, we gated for IgA^{+} memory B cells (IgA^{+} mem, $CD19^{+}CD38^{low}CD27^{+}IgD^{-}IgG^{-}IgA^{+}$), IgG^{+} memory B cells (IgG^{+} mem, $CD19^{+}CD38^{low}CD27^{+}IgD^{-}IgG^{+}IgA^{-}$) and IgM^{+} memory B cells (IgM^{+} mem, $CD19^{+}CD38^{low}CD27^{+}IgD^{-}IgA^{-}IgG^{-}IgM^{+}$). (**B-C**) Representative histogram overlays for pSYK-Y348 for naïve B cells, IgD^{+} mem and IgM^{+} mem B cells (**B**) and IgA^{+} mem and IgG^{+} mem (**C**) B cells after 2 min of anti-IgM (αIgM) or anti-Ig total (αIg) stimulation. Geometric mean fluorescence intensity (*g*MFI) values of phosphorylation of CD79a (**D**), pSYK (**E**) and pSLP-65 (**F**), pPLCγ2 (**G**), pBTK (**H**) and pERK (**I**) after stimulation with anti-IgM (αIgM) up until 15 min. Symbols represent individual patients and bars indicate mean values. Histogram overlays of representative examples of the phosphoprotein analyses are shown.

To determine the optimal timing for detection of phosphorylated protein upon BCR stimulation, we analyzed PBMCs from four healthy donors and gated on total $CD19^+$ B cells. Proximal targets of BCR signaling, such as pCD79a-Y182, pSYK-Y348 and pSLP-65-Y84 showed a clear peak in phosphorylation after 2 minutes of stimulation with αIgM $F(ab)_2$ fragments for all four healthy donors (**Figure 1D-F**). After 2 minutes of stimulation, the levels of phosphorylation (quantified by geometric mean fluorescent intensity (*g*MFI)) stabilized or steadily decreased. For pBTK-Y223, pPLCγ2-Y759 and pERK1/2-T202/Y204, 5 min of stimulation with αIgM was optimal for detection of phosphorylation (**Figure 1G-I**). Phosphorylation of ribosomal protein S6 at positions S240/244 took longer than the other targets as it peaked around 30-60 min of stimulation. The timing differed between individual samples and depended on the nature of the stimulus, as e.g. pS6-S240/244 signals after stimulation with anti-CD40 (αCD40) together with IL-4 led to more rapid S6 phosphorylation than BCR stimulation (**Figure 2A-B**). In our hands, optimal S6-S240/244 phosphorylation time points were often slightly more variable across human samples compared to the other BCR targets. We also quantified activation of NF-κB signaling by measuring degradation of IkBα, using 2% PFA for fixation and 0.5% saponin in MACS buffer for permeabilization. We observed that stimulation with αIgM and PMA both resulted in decreased IkBα protein levels upon in B cells (**Figure 2C**).

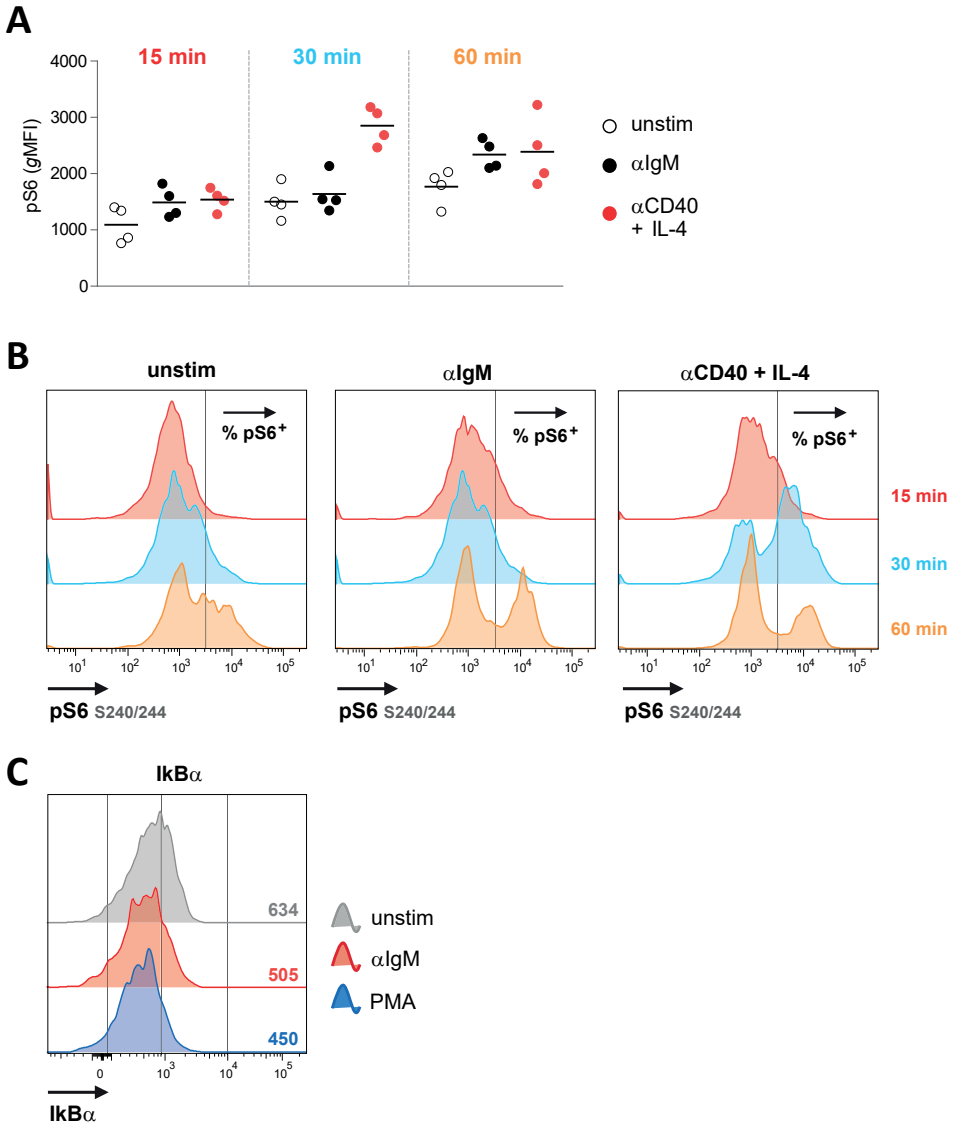


Figure 2. S6 phosphorylation and IkBα degradation in human peripheral blood B cells.
(**A**) Geometric mean fluorescence intensity (*g*MFI) values of phospho-S6 are shown for gated CD19$^+$ B cells present in PBMC fractions that were either unstimulated, or stimulated with anti-IgM (αIgM) and anti-CD40 (αCD40) combined with IL-4 for the indicated times. (**B**) Representative histogram overlays are shown for unstimulated (*left panel*), αIgM stimulated (*middle panel*) and αCD40 + IL-4 stimulated (*right panel*) B cells after culture of 15, 30 or 60 min at 37°C. (**C**) Representative histogram overlay for IkBα protein after stimulation with αIgM or phorbol 12-myristate 13-acetate (PMA) for 30 min at 37°C. Symbols represent individual patients and bars indicate mean values.

Optimal detection of murine B cell signaling by phosphoflow

Our method of fixation and permeabilization allowed for extensive (intracellular) staining of B cell markers to identify individual subpopulations. Phosphorylation of the signaling molecules described above was assessed across different B cell subpopulations in several anatomical compartments of mice. The identification of the main B cell subpopulations present in the spleen, Fol and MZ B cells, is shown in **Figure S3A**. In these splenic B cell subpopulations we investigated the increase in phosphorylation of various BCR targets upon stimulation with αIgM. We observed that pCD79a-Y182, pSyk-Y348, pSLP-65-Y84, pBtk-Y223, pPLCγ2-Y759 and pErk-T202/Y204 all showed optimal peak induction after 5 min of stimulation with αIgM (**Figure 3A**). The optimal detection time of pS6-S240/244 was after 1-3 h of stimulation and phosphorylation was readily detectable up to 24-30 h of stimulation. Phosphorylation of S6-S240/244 was not only induced by αIgM stimulation, but also by αCD40 stimulation or by TLR stimulation via LPS (TLR4) or CpG (TLR9) (**Figure 3B**). Essentially, S6 protein phosphorylation allows for quantitative comparisons of phosphorylation levels across different signaling pathways, because it is induced by various signaling cascades (26, 28, 29). This protocol allowed for sensitive pAkt-S473 measurements upon αIgM and CpG stimulation, as shown in **Figure 5C**. However, pS6-S240/244 could also be used as read-out of Akt signaling, with the advantage that it is a more sensitive measurement (**Figure 3B-C**). When we evaluated IkBα degradation as a measure for NF-κB activation, we observed that IkBα protein expression decreased after 60 min of stimulation with αIgM or CpG (**Figure 3C**). It is crucial to note that IkBα quantification required a different protocol compared to measurements of the BCR/TLR-related phospho-targets tested. Furthermore, it was crucial to rest the murine cells prior to stimulation at 37°C for IkBα**,** as well for pS6-S240/244 and pAkt-S473, because the NF-κB and Akt signaling routes are very receptive to temperature differences or mechanical stress during the processing to acquire single-cell suspensions.

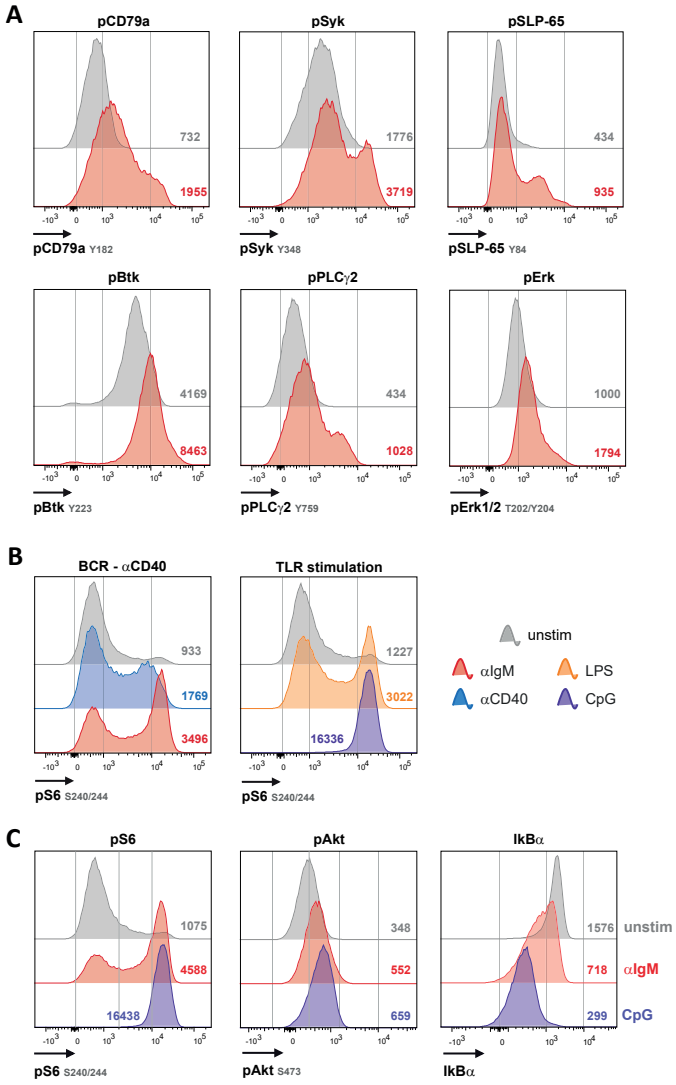


Figure 3. BCR and co-stimulation of mouse splenic B cells.

(**A**) Histogram overlays of representative examples for pCD79a-Y182, pSyk-Y348 and pSLP-65-Y84, pPLCγ2-Y759, pBTK-Y223 and pERK-T202/Y204 in total B cells from splenocyte fractions after 5 min of anti-IgM (αIgM) stimulation. (**B**) Histogram overlays of representative examples for phosphorylation of S6 protein in B cells after 3 h of αIgM or anti-CD40 (αCD40) (*left panel*) and CpG or LPS stimulation (*right panel*). (**C**) Histogram overlays of representative examples for phosphorylation of pS6-S240/244 (*left panel*) and pAkt-S473 (*middle panel*) in splenic B cells after 3 h of pre-incubation followed by 3 h of unstimulated incubation or stimulation with αIgM or TLR9 ligand (CpG). Histogram overlay of representative examples for IkBα degradation (*right panel*) after 3 h of pre-incubation followed by 1 h of unstimulated incubation or stimulation with αIgM or CpG. (**A-C**) Values depicted in histogram overlays represent the corresponding geometric mean fluorescence intensity (*g*MFI) values. Histograms are representative for 4-8 samples from 2 experiments.

Comparison of phosphoflow analysis to western blotting

To validate the obtained phosphorylation signal intensities of our protocol, we compared our protocol to western blotting. MACS-purified naïve B cells from spleen were stimulated with αIgM or CpG for 3 hours and subsequently fixed for phosphoflow or processed for western blotting. To this end, we investigated signaling of pAkt-S473 and pS6-S240/244, because these antibodies are commonly used for both phosphoflow and western blotting. In our hands, phosphorylation of pAkt-S473 and pS6-S240/244 could readily be quantified by western blot (**Figure 4A-B**). However, phosphoflow appeared to be more sensitive and showed less inter-sample variation compared with the western blotting method (**Figure 4C-D**). Major advantages of phosphoflow over western blotting are that it can be performed with only $0.5*10^6$ cells without the need for B cell purification and that it is suitable for comparisons across individual B cell subpopulations within a single sample.

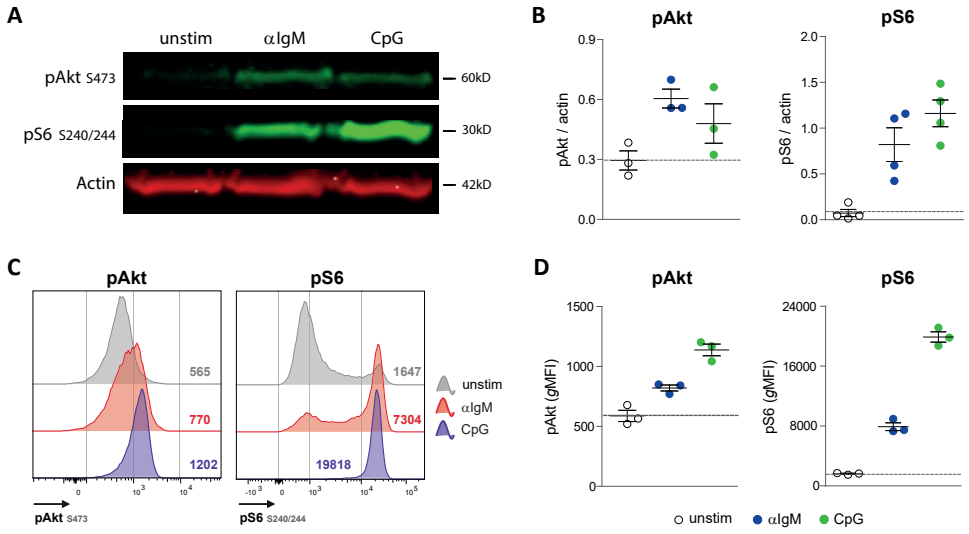


Figure 4. Comparison of phosphoflow analysis to western blotting for pAkt and pS6.
MACS-purified B cells were left unstimulated or stimulated for 3 h with αIgM or CpG. Afterwards, cell lysates were made for western blot (**A-B**) or cell suspensions for phosphoflow (**C-D**). (**A**) Detection of fluorescence on the immunoblot for pAkt-S473, pS6-S240/244 and Actin. (**B**) Ratio of pAkt-S473 and pS6-S240/244 fluorescence intensity relative to Actin for unstimulated or purified B cells stimulated with αIgM or CpG. (**C-D**) Histogram overlays and geometric mean fluorescence intensity (*g*MFI) values for phosphorylation of pS6-S240/244 and pAkt-S473. Symbols represent individual mice and bars indicate mean values. Values depicted in histogram overlays represent the corresponding geometric mean fluorescence intensity (*g*MFI) values. Histograms are representative for 3-5 samples from 2-3 experiments.

Different BCR stimulatory antibodies and analysis of signaling inhibitor efficacy

We also tested whether αIgM showed differential activation of splenic B cells compared to stimulation with other BCR-specific antibodies including anti-κ light chain (αIgK) or anti-IgD (αIgD). We observed that pCD79a-Y182 signals were strongest in mouse splenic B cells stimulated with αIgM or αIgK, whereas stimulation with αIgD resulted in moderate pCD79a-Y182 levels (**Figure 5A**). IgD-induced phosphorylation was not detected for further downstream BCR signaling molecules, such as pPLCγ2-Y759 (**Figure 5B**), implying that αIgD-stimulation only partially activates signaling downstream of the BCR.

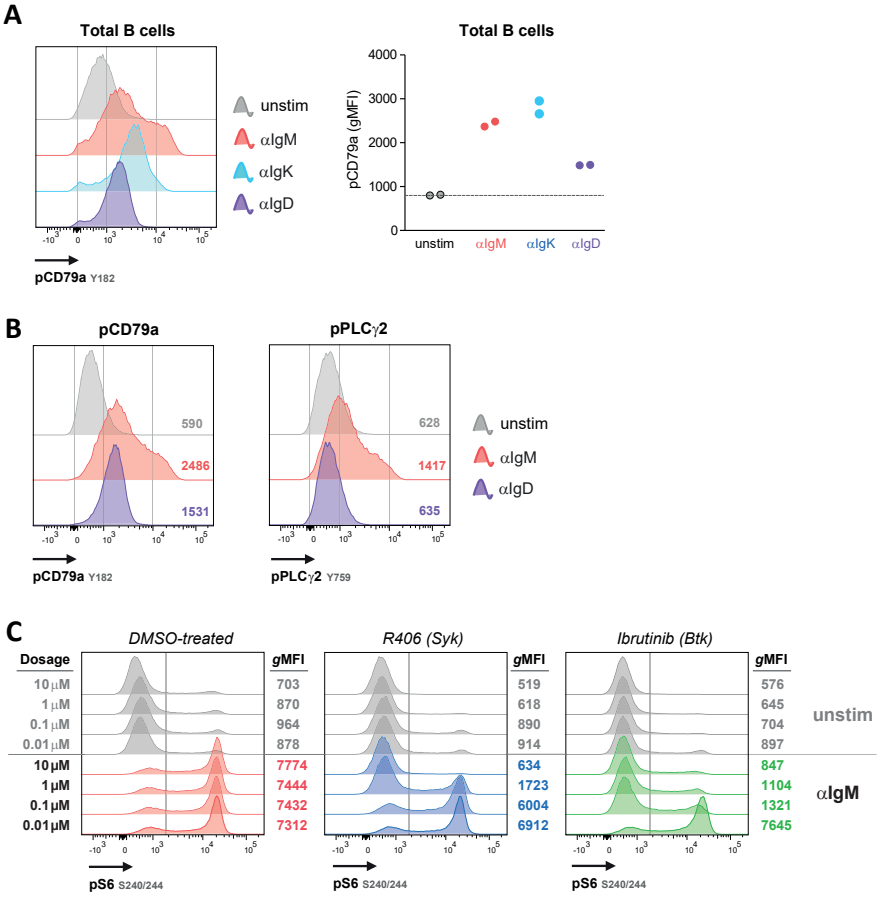
We also tested whether our protocol is suitable for the analysis of effects of *in vitro* treatment with BCR signaling inhibitors. We tested R406, which is an active metabolite of the Syk inhibitor fostamatinib, and the Btk inhibitor ibrutinib, whereby incubations with diluent were used as controls. After 3 h of in vitro treatment with various concentrations of either DMSO diluent, R406 or Ibrutinib, we stimulated B cells with αIgM for 3 h and stained for pS6-S240/244. As shown in **Figure 5C**, S6-S240/244 phosphorylation was not affected by incubations with DMSO diluent. Although both inhibitors showed strong effects on S6 phosphorylation, ibrutinib treatment resulted in complete inhibition of S6-S240/244 phosphorylation at a concentration of 0.1μM, whereas 0.1μM R406 showed a minor effect compared to the DMSO control (**Figure 5C**). This illustrates that pS6-S240/244 analysis provides a sensitive assay to test small molecule inhibitors of proximal BCR signaling.

Analysis of different B cell subpopulations and anatomical locations

As mentioned above, in conjunction with phosphoflow B cells from many organs can be identified using this protocol. We identified the signaling responsiveness to αIgM and/or αIgK of B cell subpopulations in the spleen, bone marrow (BM), lavage from the peritoneal cavity (PerC) and Peyer's Patches (PP). In the spleen we observed that transitional and MZ B cells were very responsive to αIgM stimulation, whereas Fol B cells showed lower levels of PLCγ2-Y759 upon αIgM stimulation (**Figure 5D**). The differences between these subsets were not merely due to IgM expression differences between these subpopulations, as stimulation with αIgK showed similar differences between splenic B cell subpopulations (data not shown). In the BM, we studied whether immature (Imm) B cells showed altered responsiveness compared to recirculating (Recirc) B cells (**Gating strategy in Figure S3B**). Imm B cells showed comparable levels of PLCγ2-Y759 upon αIgM stimulation compared with Recirc B cells (**Figure 5E**). As expected, we observed that pro- and pre-B cells did not respond to αIgM stimulation (**Figure 5E**). B-1a and B-2 cells in the peritoneal cavity were distinguished using surface markers such as CD43, CD5, IgD and IgM (**Gating strategy in Figure S3C**). B-1a cells showed increased basal phosphorylation and increased phosphorylation of pPLCγ2-Y759 upon αIgM stimulation, compared with B-2 cells (**Figure 5F**). In terms of responsiveness, the upregulation of pPLCγ2-Y759 upon αIgM was increased in B-2 cells compared to B-1a B cells. This was even more clear upon stimulation with αIgK,

which resulted in a ~3 fold increase in pPLCγ2-Y759 levels in B-2 B cells, whereas B-1a cells showed a ~2 fold increase upon light chain engagement (**Figure 5F**). Lastly, we studied the responsiveness of germinal center (GC) B cells compared to naïve B cells in the Peyer's patches, as GC B cells are abundant there (**Gating strategy in Figure S3D**). We observed that GC B cells did respond slightly to αIgM stimulation, whereas naïve B cells upregulated pPLCγ2-Y759 more intensely upon IgM engagement (**Figure 5G**). This was expected, as GC B cells are largely IgD-negative and have low expression of IgM. Stimulation with αIgK resulted in a higher upregulation of pPLCγ2-Y759 levels, which was in a comparable range of responsiveness to naïve B cells (**Figure 5G**).

Taken together, these data show that innate-like B cells like MZ and B-1a cells have increased basal phosphorylation and show higher responsiveness upon BCR engagement when compared with local Fol and B-2 B cells. Furthermore, IgD stimulation is able to activate CD79a-Y182 phosphorylation but does not seem to activate the entire signaling cascade that is activated via IgM BCR stimulation.



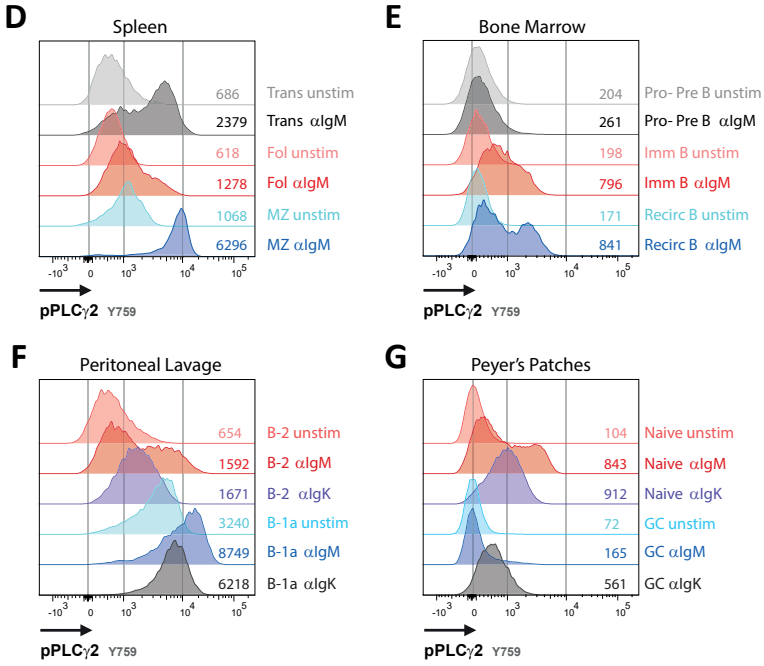


Figure 5. IgD stimulation induces CD79a but not PLCγ2 phosphorylation in mouse splenic B cells.
(**A**) Histogram overlay and geometric mean fluorescence intensity (*g*MFI) values for pCD79a of unstimulated, anti-IgM (αIgM), anti-Igκ light chain (αIgK) and anti-IgD (αIgD) stimulated B cells after 5 min for pCD79a. (**B**) Histogram overlay of pCD79a (*left panel*) and pPLCγ2 (*right panel*) levels in unstimulated, anti-IgM (αIgM), anti-Igκ light chain (αIgK) and anti-IgD (αIgD) stimulated B cells after 5 min of stimulation. Symbols represent individual mice. (**C**) Treatment with R406 (active metabolite of fostamatinib; Syk inhibitor) and ibrutinib (Btk inhibitor) compared to DMSO diluent-treated controls. Signaling of pS6-S240/244 was evaluated after 3 h of pre-treatment and 3 h of stimulation with αIgM or RPMI 2% FCS was added as unstimulated control. Four doses of inhibitor or DMSO control were used: 10μM, 1μM, 0.1μM or 0.01μM. (**D-E**) Histogram overlays of representative examples for phosphorylation of PLCγ2 in splenic B cell subpopulations: transitional (Trans), Follicular (Fol) and Marginal Zone (MZ) B cells (**D**) and bone marrow-derived pro- and pre B cells, immature (Imm) B cells and recirculating (Recirc) B cells (**E**), either unstimulated or upon 5 min anti-IgM (αIgM) stimulation. (**F-G**) Histogram overlays of representative examples for phosphorylation of pPLCγ2 in peritoneal B cell B-1a cells and B-2 cells (**F**) and naïve B cells and germinal center (GC) B cells from Peyer's patches (**G**) either unstimulated or upon 5 min of anti-IgM (αIgM) or anti-Igκ light chain (αIgK) stimulation. Values depicted in histogram overlays represent the corresponding geometric mean fluorescence intensity (*g*MFI) values. Data shown are representative for 2-8 or more samples from 2-3 or more experiments.

DISCUSSION

Here we present a protocol to measure phosphorylation of intracellular signaling molecules by flow cytometry. This method provides several advantages over western blotting, as measurements can be performed on smaller cell numbers, the method is more sensitive, less time consuming and the data is easy to quantify. Furthermore, staining for phospho-targets can be performed in conjunction with antibodies for extracellular markers, allowing the identification of multiple cell types or subpopulations in the same sample, without the need for prior cell purification steps. The protocol allows the evaluation of all B cell-restricted phospho-targets tested in B cell subpopulations, including less abundant cell subsets such as dark- and light zone GC B cells, memory B cells and plasma cells (data not shown).

Phosphoflow also has some drawbacks, as it does not cover the same extent of antibodies that are available for western blotting to detect protein phosphorylation. In addition, not all phospho-targets of choice can be quantified using our protocol. For example, we noted that not all pSTATs are detectable upon stimulation and that our protocol shows a lower sensitivity to detect STAT phosphorylation, compared to alternative protocols. However, we noticed that our protocol is suitable to investigate e.g. pSTAT6-Y641 in specific B cell subpopulations that require multiple markers for their identification. Furthermore, in western blotting analysis background signals associated with non-specific protein binding can be identified by their incorrect apparent molecular weight, but this is not the case in phosphoflow. Therefore, only antibodies with high specificity can be applied in phosphoflow analyses, but the panel of suitable commercially available phosphoflow antibodies is rapidly increasing. Especially BCR-associated NF-κB signaling remains challenging to measure via phosphoflow, as there are very few antibodies that sensitively detect phosphorylation of proteins in this signaling cascade. Although phospho-p65 is detectable with reasonable sensitivity, quantification of IκBα protein degradation seems to be a superior flow cytometry-based alternative.

An important aspect of our phosphoflow protocol is that staining for all markers included occurs after permeabilization, and therefore the expression of cell surface markers such as IgD, B220 and CD3 is detected both extracellularly and intracellularly. Not all traditional B cell markers can be applied in conjunction with phosphoflow, as is illustrated by staining for CD19 in mouse B cells, which does not work in our hands. However, nearly all human peripheral B cell subpopulation markers are efficiently detected using this phosphoflow protocol. Before gating B cell subpopulations, it is crucial to check the compensation matrix and the sample quality. Analysis programs - such as FlowJo - have tools available to check the compensation calculation performed by the instrument and to check the sample quality. If the sample quality is poor in the first or last seconds of measurement, it is advised to exclude these poor-quality regions from analysis. In addition, it is advisable to stain for one phosphoprotein per sample, as we have noticed that staining for multiple phospho-targets per sample may increase the antibody background. Although cell surface markers are very well detectable using this phosphoflow method, the expression of some B cell subpopulation markers may be downregulated, when B cells are analyzed following prolonged activation *in vitro* (> 1 h). This is generally not the case, as stimulation times are typically less than 10

min for most BCR-related protein kinases. Optimal stimulation times are often not conserved between human and mouse and therefore it is advised to test both mouse and human timing separately. Following the determination of the optimal stimulation time, it is recommended to explore which concentration of the stimulus leads to the desired activation window.

Some specific targets including IkBα, pAkt-S473 and pS6-S240/244 - in murine setting - require pre-incubation at 37°C for several hours prior to stimulation to prevent non-specific signals as a result of temperature changes. The pre-incubation for these targets on frozen PBMCs is not recommendable, as the viability of these cells is lower compared to fresh material. This did not seem to be the case for targets directly downstream of the BCR, such as pCD79a-Y182, pSYK-Y348, pPLCγ2-Y759, pSLP-65-Y84 and pBTK-Y223. Importantly, unstimulated controls showed slightly increased S6 phosphorylation over time. This phenomenon suggests that signals other than BCR signaling, such as temperature changes, mechanical stress due to acquisition of single cells or low nutrient availability, may contribute to the activation of this signaling pathway. Downstream targets such as S6 also proved to be very useful for evaluation of effects of various inhibitors of BCR signaling in individual B cell subpopulations.

We observed that innate-like B cells such as MZ and B-1a B cells showed increased basal phosphorylation and responsiveness to BCR engagement when compared to Fol and B-2 B cells. Increased basal phosphorylation of the Akt/mTOR signaling pathway in MZ B cells compared to Fol B cells was previously described (17) and the high responsiveness corresponds with the idea that MZ B cells are pre-activated and should be able act quickly upon BCR ligation (30, 31). Another study did not find differences between MZ and Fol B cells upon BCR stimulation, which could be due to the use of αIgK stimulation (19). In our hands, Igκ engagement showed similar differences to αIgM stimulation, albeit less pronounced. Interestingly, B-1a B cells also showed lowered responsiveness to αIgK stimulation when compared with B-2 B cells from the peritoneal cavity (19). When comparing different BCR stimulating agents in splenic B cells, we found that IgD stimulation results in pCD79a-Y182 activation but does not increased levels of pPLCγ2-Y759. This is in contrast to BCR ligands that activate IgM or Igκ light chain, both resulting in activation of pCD79a-Y182 and pPLCγ2-Y759.

In the BM, immature B cells represent a B cell subpopulation that still needs to undergo peripheral selection, whereas this has already taken place in recirculating B cells. Immature B cells did not seem to differ largely in signaling responsiveness compared to recirculating B cells in the BM. Lastly, we investigated signaling differences between differentiated GC B cells and undifferentiated naïve B cells. Although GC B cells did respond to stimulation with αIgM, GC B cells were much more responsive to αIgK stimulation. This was expected as GC B cells have low surface expression of IgM and lack IgD expression, whereas naive B cells are characterized by high IgD and IgM expression. It was recently shown that signaling in GC B cells and naïve B cells is regulated differently (27, 32). Using our phosphoflow method, staining for GC light and dark zone markers and for specific transcription factors, such as T-bet or IRF4, can be combined for sensitive analysis of phosphorylation.

We also tested whether T cell activation could be measured following cross-linking of CD3 and CD28 of murine T cells or anti-CD3 stimulation alone in human PBMC-derived T cells, and found that this protocol also works as a read-out for many phosphorylation targets, including Zap-70, Slp-76, Itk and Akt (data not shown).

Taken together, our protocol provides a very sensitive and efficient method to measure phosphorylation of various signaling molecules in different B cell subpopulations from several organs using freshly isolated single-cell suspensions or frozen samples without pre-sorting of cells. Following the proposed workflow, the protocol is likely to work for many other phosphorylated targets and cell types, as well as in conjunction with nuclear proteins including transcription factors.

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DISCLOSURE

The authors declare to have no financial conflicts of interest.

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SUPPLEMENTAL MATERIAL

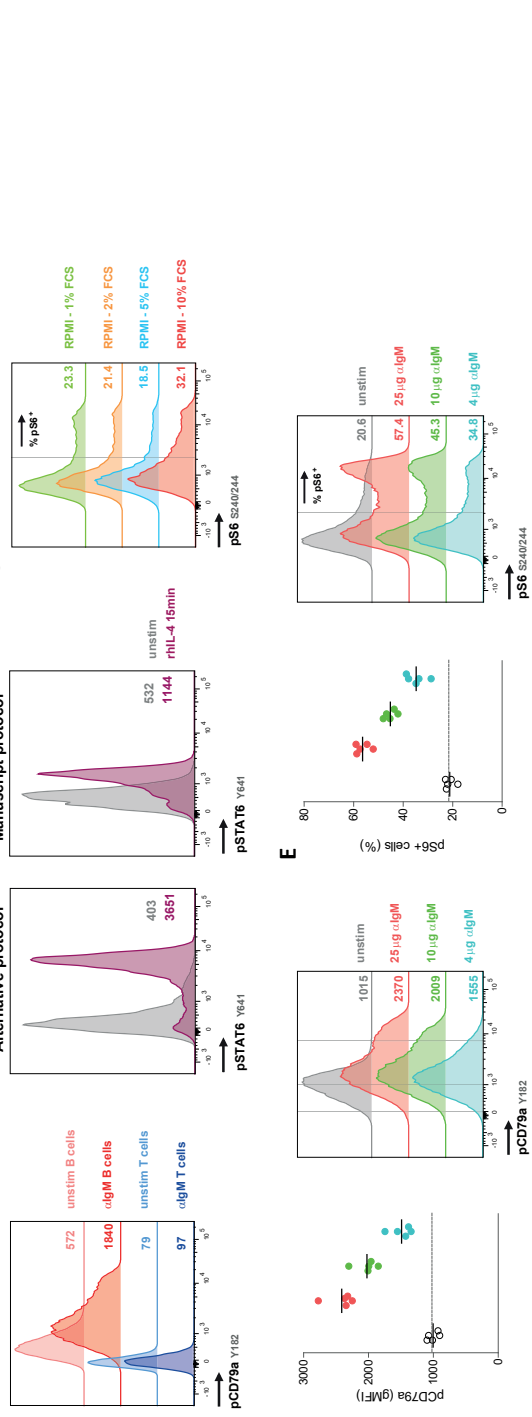


Figure S1. Optimization of the phosphoflow protocol

(**A**) Histogram overlays of representative examples for phosphorylation of CD79a in unstimulated and anti-IgM (αIgM) stimulated B cells and T cells after 5 min of culture at 37°C. (**B**) Representative histogram overlay for phosphorylation of STAT6-Y641 after 15 minutes rh-IL-4 stimulation of PBMCs according to the manuscript or alternative protocol. (**C**) A representative histogram overlay of pS6 levels in unstimulated murine B cells after 3 h of incubation 37°C with 1%, 2%, 5% or 10% of FCS in RPMI. (**D-E**) Quantification and representative histogram overlays for dose titration of αIgM in murine B cells, showing responsiveness after 4, 10 or 25μg/mL αIgM for pCD79a-Y182 (**D**) and pS6-S240/244 (**E**). (**F**) Histogram overlays of representative examples for pCD79a-Y182 after pervanadate stimulation of murine splenic cells. Values depicted in histogram overlays represent the corresponding percentage of pS6-positive cells or geometric mean fluorescent intensity (gMFI) values. Histograms are representative for 2-4 samples from in total 2-3 individual experiments.

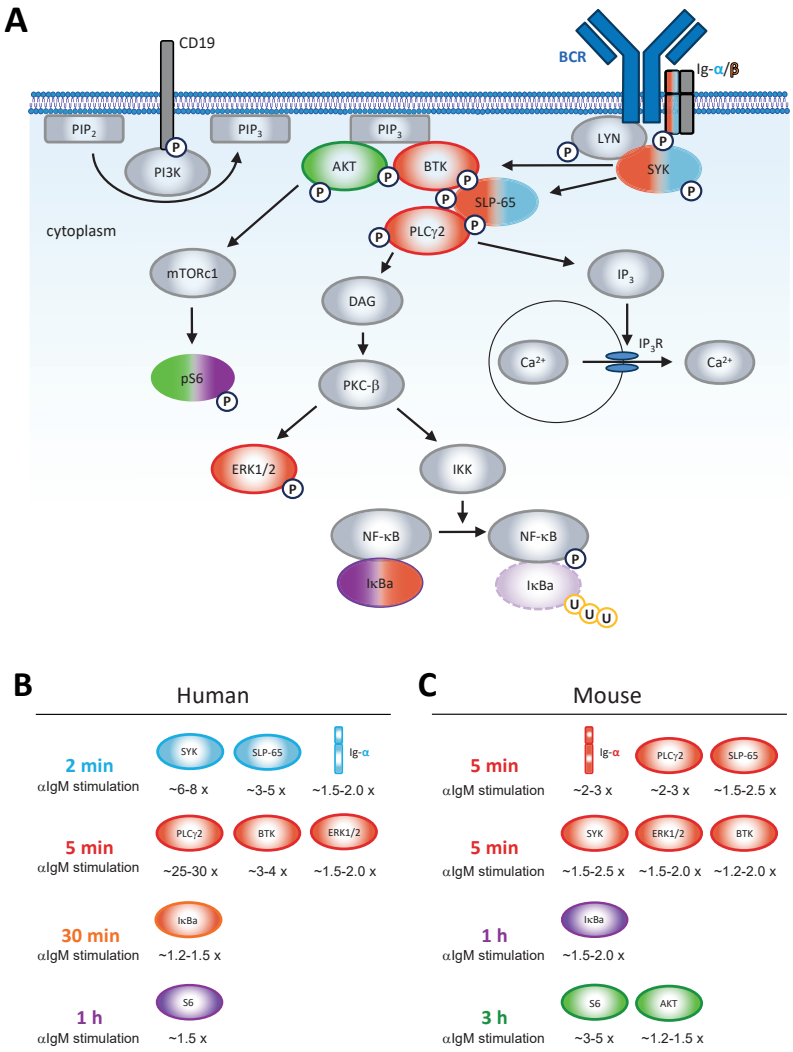


Figure S2. B cell receptor signaling overview and optimal timing of phospho-targets.
(**A**) A) BCR signaling cascade showing several important downstream signaling routes. Triggering of the BCR results in activation of Ig-α and Ig-β by Lyn, that in turn will activate SYK. Activation of SYK together with CD19-mediated activation of PI3K will recruit and activate SLP-65, BTK and PLCγ2. The activation of this complex leads to activation of several downstream signaling cascade, such as calcium mobilization, AKT-mediate signaling and activation of ERK and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Color coding of molecules corresponds with optimal time point to evaluate signaling upon anti-IgM (αIgM). Dual colors indicate differences between human and murine B cells. (**B-C**) Overview of signaling molecules and optimal measuring time as indicate by color coding for human (**B**) or murine (**C**) B cells. Responsiveness upon αIgM stimulation is stated for each target and calculated by dividing the geometric mean fluorescent intensity (*g*MFI) values of αIgM by the *g*MFI value of unstimulated B cells. This is based on 5-10 samples from >2 individual experiments.

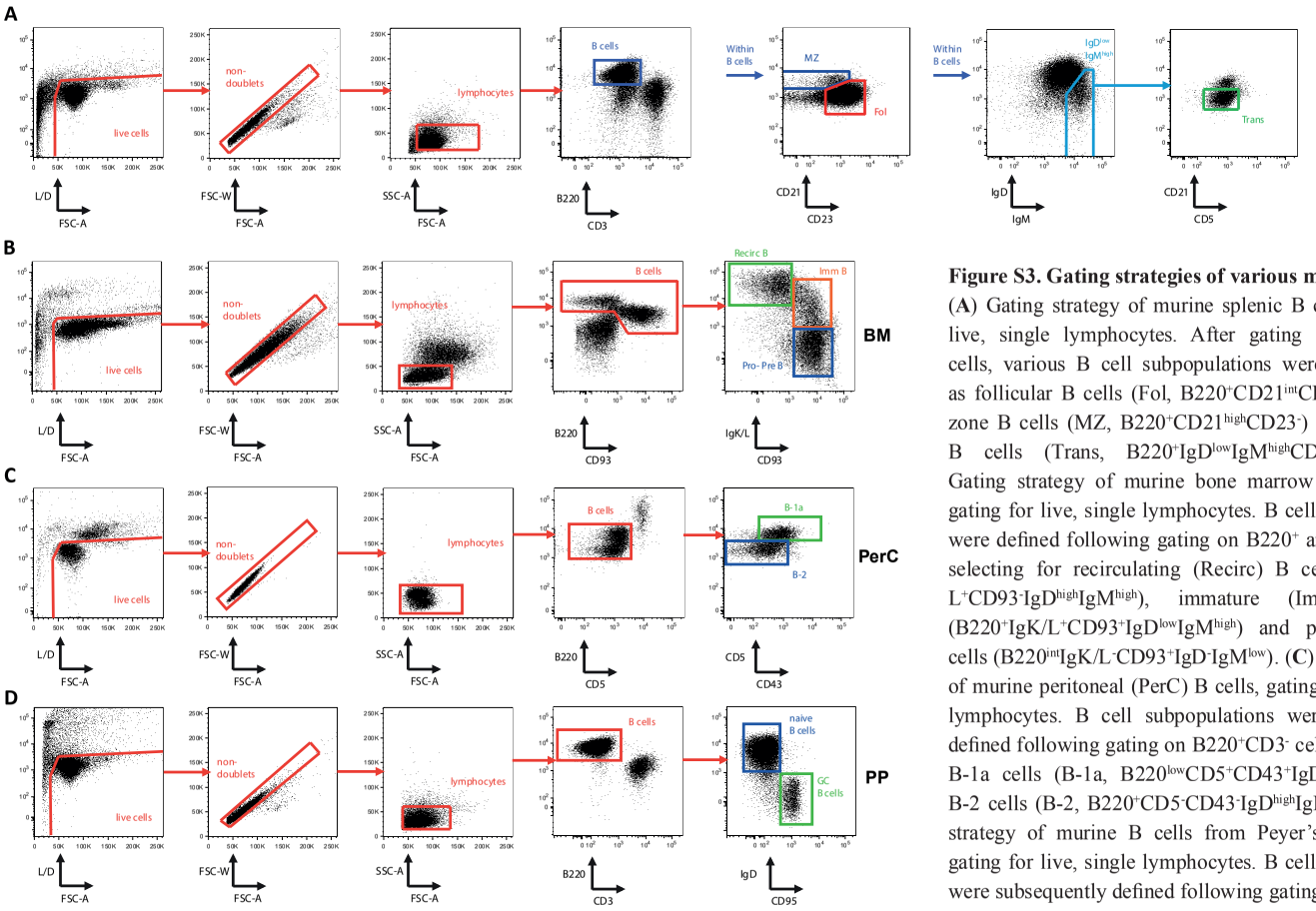
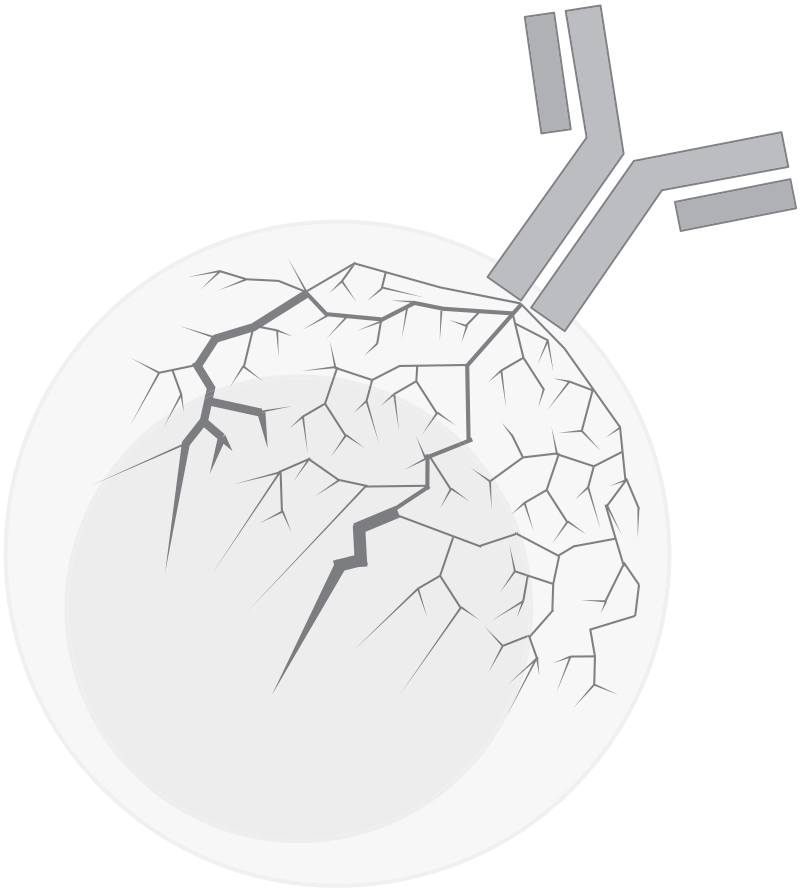


Figure S3. Gating strategies of various murine organs. (**A**) Gating strategy of murine splenic B cells, gating for live, single lymphocytes. After gating for B220^{+}CD3^{-} cells, various B cell subpopulations were defined, such as follicular B cells (Fol, B220^{+}CD21^{int}CD23^{+}), marginal zone B cells (MZ, B220^{+}CD21^{high}CD23^{-}) and transitional B cells (Trans, B220^{+}IgD^{low}IgM^{high}CD21^{-}CD5^{-}). (**B**) Gating strategy of murine bone marrow (BM) B cells, gating for live, single lymphocytes. B cell subpopulations were defined following gating on B220^{+} and B220^{int} cells, selecting for recirculating (Recirc) B cells (B220^{+}IgK/L^{+}CD93^{-}IgD^{high}IgM^{high}), immature (Imm) B cells (B220^{+}IgK/L^{+}CD93^{+}IgD^{low}IgM^{high}) and pro- and pre-B cells (B220^{int}IgK/L^{-}CD93^{+}IgD^{-}IgM^{low}). (**C**) Gating strategy of murine peritoneal (PerC) B cells, gating for live, single lymphocytes. B cell subpopulations were subsequently defined following gating on B220^{+}CD3^{-} cells, selecting for B-1a cells (B-1a, B220^{low}CD5^{+}CD43^{+}IgD^{low}IgM^{high}) and B-2 cells (B-2, B220^{+}CD5^{-}CD43^{-}IgD^{high}IgM^{-}). (**D**) Gating strategy of murine B cells from Peyer's Patches (PP), gating for live, single lymphocytes. B cell subpopulations were subsequently defined following gating on B220^{+}CD3^{-} cells, selecting for naïve B cells (B220^{+}IgD^{+}IgM^{+}CD95^{-}) and GC B cells (B220^{+}IgD^{-}IgM^{low}CD95^{+}).



CHAPTER 3.2

A versatile protocol to quantify BCR-mediated phosphorylation in human and murine B cell subpopulations

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ABSTRACT

Signal transduction is the process by which molecular signals are transmitted from the cell surface to its interior, resulting in functional changes inside the cell. B cell receptor (BCR) signaling is of crucial importance for B cells, as it regulates their differentiation, selection, survival, cellular activation and proliferation. Upon BCR engagement by antigen several protein kinases, lipases and linker molecules become phosphorylated. Phosphoflow cytometry (phosphoflow) is a flow cytometry-based method allowing for analysis of protein phosphorylation in single cells. Due to recent advances in methodology and antibody availability – together with the relatively easy quantification of phosphorylation – phosphoflow is increasingly and more commonly used, compared to classical western blot analysis. It can however be challenging to set-up a method that works for all targets of interest. Here, we present a step-by-step phosphoflow protocol allowing the evaluation of the phosphorylation status of signaling molecules in conjunction with extensive staining to identify various human and murine B cell subpopulations, as was previously published in the original paper by Rip *et al.*, 2020. Next to a description of phosphoflow targets from the original paper, we provide directions on additional targets that play a pivotal role in BCR signaling. The step-by-step phosphoflow protocol is user-friendly and provides sensitive detection of phosphorylation of various BCR signaling molecules in human and murine B cell subpopulations.

BACKGROUND

B cells are a crucial part of the adaptive immune system and play an important role in protection against pathogens. B cells can sense pathogens by innate receptors such as Toll-like receptors (TLRs). The most important receptor for the development and activation of B cells, however, is the B-cell antigen receptor (BCR). The genes encoding the immunoglobulin heavy and light chains that comprise the BCR are assembled during B cell development in the bone marrow through a stepwise process called V(D)J recombination (Tonegawa, 1983). The functionality and autoreactivity of the BCR is checked during three crucial checkpoints in B cell development: (i) productive heavy chain rearrangement is monitored at the pre-B stage by surface deposition of the heavy chain protein together with pre-existing surrogate light chain as the pre-BCR complex; (ii) productive light chain recombination enables surface expression of the BCR, which is checked for auto-reactivity at the immature B cell stage and (iii) upon bone marrow egress when transitional B cell progress to mature, naive B cells in peripheral lymphoid tissues. When expression of (pre-)BCR signaling molecules is altered, B cell development in the bone marrow is severely affected (Martensson *et al.*, 2010; Pieper *et al.*, 2013). Balanced BCR signaling is also crucial for appropriate selection, survival and activation of mature B cells and its distortion is a key factor in the development of autoimmune disease or leukemia (Nishizumi *et al.*, 1995; Kil *et al.*, 2012; Rip *et al.*, 2018).

Upon BCR engagement by antigen, Src family kinases phosphorylate the intracellular tyrosine-based activation motif (ITAM) of CD79a (Ig-α) and CD79b (Ig-β), which are the transmembrane proteins that form a complex with the BCR, as well the cytoplasmic tail of co-receptor CD19. Phosphorylation of the CD79a/b ITAM in turn activates spleen tyrosine kinase (SYK), which subsequently activates the SH2 domain-containing leukocyte protein of 65 kD (SLP-65) linker protein. Simultaneously, phosphorylated CD19 will activate phosphoinositide 3-kinase (PI3K), which in turn phosphorylates and converts phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3). Signaling molecules containing a pleckstrin-homology domain, such as Bruton's tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2), are recruited to the cell membrane by the newly formed PIP_3 (Saito *et al.*, 2001). After BTK recruitment to the cell membrane, SLP-65 mediates SYK-dependent activation of BTK, which in turn will phosphorylate PLCγ2 (Wang *et al.,* 2000). The activation of this signaling complex eventually results in activation of protein kinase B (PKB/AKT) signaling (Craxton *et al.,* 1999), NF-κB translocation to the cell nucleus (Bajpai *et al.*, 2000), calcium mobilization (Fluckiger *et al.,* 1998) and activation of the mitogen-activated protein kinase (MAPK) pathway (*Jiang et al.*, 1998). The complex containing BTK, SLP-65, PLCγ2 together with other PI3K-derived signals are essential for B cell survival, proliferation and differentiation. BCR signal transduction is regulated by the equilibrium of kinase activity and negative regulation of signaling through recruitment of tyrosine phosphatases including SHP-1 (Franks and Cambier, 2018).

Western blotting used to be the method of choice for detection and quantification of phosphorylation of the BCR signaling molecules described above. The main drawbacks of western blotting are the large number of purified cells that are required to perform the analysis and that quantification is not sensitive enough to detect subtle differences between samples. Phosphoflow is a flow-cytometry based technique that allows for quantification of multiple subpopulations within a single sample without prior purification and requires relatively few cells. In addition, phosphoflow cytometry is less time-consuming compared to western blotting. Although for the quantification of protein phosphorylation phosphoflow appeared to be superior to western blotting, the fixation and permeabilization procedure of phosphoflow often affected cellar epitopes or features of the monoclonal antibodies (mAbs) that were required to identify the immune cell population of interest. Due to recent advances in phosphoflow procedures, it is now possible to combine the analysis of phosphorylation of signaling proteins with a comprehensive staining for B cell subpopulations. In addition, more and more mAbs become available for phosphoflow analysis.

Here, we present our step-by-step phosphoflow protocol that is complementary to our original research paper (Rip *et al.*, 2020). The phosphoflow protocol allows for sensitive detection of phosphorylation of numerous BCR signaling molecules in human and murine B cell subpopulations. Next to a description of phosphoflow targets as published in the original paper (Rip *et al.*, 2020), we also present additional BCR signaling targets that can be sensitively quantified using this phosphoflow protocol.

MATERIAL AND REAGENTS

Acquisition of human PBMCs from whole blood

1. Leucosep Tube, 50 ml (Greiner Bio-one, catalog number: 227290)
2. Total peripheral blood (~25 ml) from a healthy donor or patient. In general, 1 ml of blood contains $1*10^6$ PBMCs.
3. Ficoll-Paque™ Plus (GE healthcare, catalog number: 17-1440-03)
4. Fetal bovine serum (FBS, Gibco, catalog number: 10270-106)
   ***CRITICAL**: Heat-inactivate for 1h at 56°C prior to use.*
5. RPMI 1640 Medium containing L-glutamin (Lonza, catalog number: BE12-702F) supplemented with 5% (vol/vol) fetal bovine serum (Gibco, catalog number: 10270-106)
6. 2 ml Cryovial (Starstedt, catalog number: 72.694.006)
7. Freezing medium containing 80% FBS (Gibco, catalog number: 10270-106)
8. 20% dimethyl sulfoxide (DMSO, Sigma Aldrich, catalog number: D2650-100ML)
   ***CAUTION**: DMSO is a toxic product and should be handled with care and discarded.*

Collection of Murine organs

- 10 ml syringes (Braun, catalog number: 4616103V) and 25-gauge x ⅝ inch needle (Braun, catalog number: 4657853)
- C57BL/6J mice (Charles River) that are 8-12 weeks of age, either males and females. All animal experiments should comply with national laws and institutional regulations
- PBS (Gibco, catalog number: 14190169)

Preparation of Murine organs

- 100 µm nylon strainers (Corning, catalog number: 352360)
- 2 ml syringes (BD Biosciences, catalog number: 307727)
- Nunc Cell Culture/Petri dishes (Thermo Scientific, catalog number: 1533066)
- RPMI 1640 Medium, Glutamax™ Supplement (Gibco, catalog number: 61870036) containing 2% (vol/vol) FBS (Capricorn, catalog number: FBS-12A). ***CRITICAL**: Heat-inactivate for 1h at 56°C prior to use.*

In vitro Stimulation of Single-Cell Suspension/Direct fixation for *ex vivo* (basal) measurements

1. 96-wells plate (U-shaped, 310 µl/w, Greiner Bio-one, catalog number: 650201)
2. eBioscience FoxP3/Transcription Factor staining kit (Invitrogen, catalog number: 00-5523-00) containing:
   a. Fixation and permeabilization concentrate
      CAUTION*: This product contains paraformaldehyde, a toxic and mutagenic product. It should be handled with care and discarded as hazardous waste.*
   b. Diluent
   c. 10x Wash Buffer (dilute 10 times in deionized water)
3. Stimuli for BCR signaling and co-receptor engagement, see Table 1.
   CAUTION*: Sodium azide is highly toxic and dangerous to the environment. It should be handled with care and discarded as hazardous waste.*

Table 1. BCR and co-receptor ligands for stimulation of human and murine B cells.

Stimulus	Working Concentration	Manufacturer	Cat. No.
Human			
Goat F(ab')2 Anti-Human IgM-UNLB	20µg/mL	Southern Biotech	2022-01
Recombinant Human CD40L	2µg/mL	R&D	6245-CL/CF
Recombinant Human IL-4 protein	250ng/mL	R&D	204-IL-CF
Mouse			
AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgM	25µg/mL	Jackson ImmunoResearch	115-006-075
Purified Rat anti-Mouse CD40 (clone 3/23)	4 µg/mL	BD Biosciences	553787
Lipopolysaccharide (LPS) *E.Coli* 026:B6	400ng/mL	Sigma Aldrich	L8274

Flow Cytometry Procedures

- Insert tubes (Greiner Bio-one, catalog number: 102280) and 5 ml round bottom polystyrene tubes (Corning, catalog number: 352052).
- UltraComp eBeads™ Compensation Beads (Invitrogen, catalog number: 01-2222-42)
- MACS-buffer containing 0.5% (vol/vol) bovine serum albumin (BSA, Sigma Aldrich, catalog number: A8327) and 2 mM ethylenediaminetetraacetic acid solution (EDTA, Sigma Aldrich, catalog number: 03690) in PBS.
- 10x Wash Buffer (dilute 10 times in deionized water) from the eBioscience FoxP3/ Transcription Factor staining kit (Invitrogen).
- FcR Blocking agent (Human TruStain FcX, Biolegend, catalog number: 422302).
- Antibodies for flow cytometry are listed in Table 2 (phospho-protein-specific antibodies) and Table 3 (antibodies used for an accurate identification of B cell subpopulations). For mouse studies, another 2.4G2 antibody was use from own production.
  CAUTION*: Sodium azide is highly toxic (see above).*

Table 2. Phospho-specific antibodies for the analysis of human and mouse B cells and directions on stimulation timing and antibody dilution.

Antibody	Conjugate	Clone	Manufacturer	Cat. No.	Detection human	mAb Dilution human	Detection mouse	mAb Dilution mouse
pAKT (S473)	-	D9E	Cell Signaling Technologies	4060L	30-60 min	100x	180 min	100x
pAKT (T308)	-	D25E6	Cell Signaling Technologies	13038L	30-60 min	50x	180 min	100x
pBTK/pITK (Y223/Y180)	Alexa Fluor 647	N35-86	BD phosflow	564846	5 min	3000x	5 min	3000x
pCD79a (Y182)	Alexa Fluor 647	D1B9	Cell Signaling Technologies	29742S	1 min	50x	5 min	50x
pERK1/2 (T202/Y204)	PE	20A	BD phosflow	561991	5 min	8x	5 min	8x
pPI3K p85 (Y458)	-	E3UI1H	Cell Signaling Technologies	17366S	1 min	100x	3 min	200x
pPLCγ2 (Y759)	Alexa Fluor 647	K86-689.37	BD phosflow	558498	5 min	8x	5 min	8x
pS6 (S240/244)	-	D68F8	Cell Signaling Technologies	5364	30-60 min	200x	180 min	200x
pSHP-1 (Y564)	-	D11G5	Cell Signaling Technologies	8849S	1 min	100x	3 min	500x
pSLP-65 (Y84)	PE	J117-1278	BD phosflow	558442	2 min	8x	5 min	8x
pSrc (Y418/Y424)	-	EP503Y	Abcam	Ab40660	1-3 min	200x	5 min	500x
pSrc (Y507)	-	5B6	Abwiz Bio	2461S	1-3 min	500x	5 min	1500x
pSYK (348)	PE	I120-722	BD phosflow	558529	2 min	8x	5 min	8x

Equipment

1. Standard laboratory materials or equipment, such as pipettes, tubes, and tips.
2. Tools suitable for sacrificing mice and harvesting organs, including: scissors, tweezers and 70% ethanol
3. Mortar and pestle
4. Stopwatch or timer
5. Water bath incubator and/or CO_2 incubator
6. Multifuge x 3R Refrigerated Centrifuge (Thermo Scientific, catalog number: 75004515) with TX-1000 swinging bucket rotor (Thermo Scientific, catalog number: 75003017).
7. LSR II flow cytometer (BD Biosciences, catalog number: BD LSR-II) equipped with a red, blue and violet laser
8. Flow cabinet
9. Fridge

Software

1. Data acquisition by FACSDiva™ Software (BD Biosciences)
2. FlowJo software (BD Biosciences) to analyze data

Procedure

A. Sample collection

AA. Acquisition of human PBMCs from whole blood (Timing: 60 min)

(Steps 1 to 6 are performed in a sterile environment using a flow cabinet).

1. Add 15 ml of Ficoll to the Leucosep tube and spin down at 1,000 *x g* for 1 min at room temperature (RT).
2. Gently load a maximum of 25 ml whole blood per Leucosep tube.
   CRITICAL*: Make sure the Ficoll is beneath the filter in the tube and that blood does not mix with the Ficoll.*
3. Spin down the Leucosep tube containing whole blood and Ficoll at 1,000 *x g* for 10 min at RT. ***CRITICAL****: Set the deceleration as low as possible to keep the Ficoll gradient separation of the PBMC layer intact.*
4. Transfer the whole solution of peripheral blood mononuclear cells (PBMCs), Ficoll and Plasma to a 50 ml tube containing 15 ml RPMI 1640 supplemented with 5% FBS.
   CRITICAL*: The erythrocyte fraction below the filter should be retained in the Leucosep tube.*

5. Spin down the 50 ml tube at 400 *x g* for 7 min at 4 °C. Afterwards, remove the supernatant and resuspend in 1-5 ml of cold (4 °C) RPMI-2% FBS.
   OPTIONAL*: When performing phosphoflow on fresh PBMCs (non-frozen samples), proceed to Step AA9.*
6. Count cells using standard procedures (manual or automated) and store not more than $50*10^6$ viable cells per vial by adding Freezing medium in 1:1 ratio to the volume in which the cells are held. Add 0.5 ml of Freezing medium to 0.5 ml of medium containing $20\text{-}50*10^6$ cells. Transfer the cells into 2 ml cryovials that are suitable for freezing and immediately freeze the vials at a rate of 1 °C per minute.
   CRITICAL*: As DMSO is harmful to cells, slowly add the freezing medium to the cells in RMPI 5% FBS while gently shaking the tube.*
   PAUSE POINT*: Cells can be kept at -80 °C for several weeks or in liquid nitrogen for years.* ***CAUTION****: DMSO is a toxic product and should be handled with care and discarded.*
7. Vials containing frozen PBMCs are thawed using a 37 °C water bath. Upon reaching the liquid phase, cells are immediately transferred into a 15 ml tube followed by slow addition 10 ml of cold (4 °C) RPMI-5% FBS while gently shaking the tube.
   CRITICAL*: This transfer should happen immediately when the liquid phase is reached during thawing, as DMSO is harmful to the cells.*
8. Spin down at 400 *x g* for 7 min at 4 °C. Afterwards, remove the supernatant and resuspend the pellet in 1 ml of cold (4 °C) RPMI 2% FBS.
9. Count the cells and plate $5*10^5$ cells per well in a 96-wells round bottom plate within a volume of 50 µl RPMI 2% FBS for phosphoflow.
   PAUSE POINT*: Cells can be kept at 4 °C for 1-2 h before proceeding with the phosphoflow protocol at Step B1.*

AB. Collection of murine organs (Timing: 15 min per mouse)

1. Mice are sacrificed by cervical dislocation. The protocol allows for other sacrifice methods that do not affect the anatomical region of the organ of interest, including methods that involve anesthetics.
2. *Peritoneal lavage*. A midline incision over the abdomen is made to retract the skin, keeping the abdomen intact. A 10 ml syringe, containing 5 ml of ice-cold PBS and 5 ml of air, is attached to the 25-gauge x ⅝ inch needle. The peritoneum is subsequently washed by gentle flushing and shaking upon full injection for 1 min. Afterwards, the peritoneal lavage is collected by aspirating the peritoneal cavity with the 10 ml syringe. The lavage is collected in a 15 ml tube and is kept on ice for further processing.
3. *Spleen.* The peritoneal cavity can be opened to carefully extract the spleen. The spleen is located at the left flank in the abdomen and can be extracted using tweezers.

4. *Other organs.* Next, the Peyer's patches can be isolated from the small intestine using curved tip tweezers. Lastly, the femur is extracted by two cuts in the pelvic bone and dislocation of the knee. Harvesting of various other anatomical compartments, including bronchoalveolar lavage (BAL), lungs and lymph nodes is previously described (Li *et al.*, 2017). All organs are placed in ice-cold PBS for further processing.

AC. Preparation of single-cell suspension of various murine organs (Timing: 15 min per mouse)

1. Spleen tissue is mechanically disrupted using a 100 μm cell strainer on a 50 ml tube and the plunger of a 2 ml syringe. During and following processing of the spleen, strainers are flushed with 5 ml of RPMI 1640 GlutamaxTM supplemented with 2% (vol/vol) FBS (RMPI-2% FBS) to acquire all cells. Peyer's patches are processed in a similar manner, using a 100 μm cell strainer and a Petri dish. Strainers are flushed with maximal 200 μl of RMPI-2% FBS to acquire all cells.
   ***CRITICAL**: It is important to immediately put the tubes with single cell suspension back on ice or place them in the fridge at 4 °C after processing.*
2. The femur is processed using a mortar and pestle, crushing the bone and acquiring the bone marrow cells in 3 ml of RMPI-2% FBS. The bone marrow cell suspension is transferred through a 100 μm cell strainer on a 50 ml tube to ensure a single cell solution without parts of bone. The bone marrow cell suspension is kept on ice for upcoming procedures.
3. Peritoneal lavage cells are centrifuged at 400 *x g* for 7 min at 4 °C and the supernatant is discarded. Peritoneal lavage cell pellets are resuspended in 1 ml of RMPI-2% FBS and are kept on ice for upcoming procedures.
4. A cell counting sample is taken from all organs to determine the exact number of cells in each sample.
   ***CRITICAL**: To measure ex vivo basal levels of phosphorylation, cells are kept on ice without any stimulation. Immediately proceed to Step C1.*
   ***PAUSE POINT**: Cells can be kept at 4 °C for 1-2 h before proceeding with the phosphoflow protocol.*

B. In vitro stimulation of single-cell suspension (Timing: 30 min-6 h)

1. Stimulation and staining of cells is performed in 96-wells round bottom plates, using $5*10^5$ per well (200,000–1,000,000 cells is technically most optimal). $5*10^5$ cells per well are transferred in a volume of 50 μl in each well on ice, keeping the cells all time at 4 °C.
2. For stimulation times of less than 1 h, the 96-wells round bottom plate is placed in a water bath at 37 °C. Make sure only the bottoms of the wells are in contact with the water. For stimulation times longer than 1 h, the stimulus is added while the cells are on ice and subsequently incubated at 37 °C and 5% CO_2.

The optimal stimulation time for phosphotargets in human and murine samples are stated in the original paper (Rip *et al.*, 2020) and Table 2.

***CRITICAL**: Some phospho-targets in murine B cells, mainly downstream signaling molecules as NF-κB and AKT/S6, are receptive to temperature changes and mechanical stress due to isolation of single cells. Rest these samples at 37 °C, preferably in an CO_2 incubator, for 3 h prior to stimulation. This is critical for optimal detection and sensitivity of the downstream signaling read-outs. The pre-incubation for these targets on frozen PBMCs is not recommendable, as the viability of these cells is lower compared to fresh material.*

***OPTIONAL**: For experiments including in vitro treatment of samples with BCR signaling inhibitors, incubate cell suspensions 3 h at 37 °C with BCR signaling for effective pre-treatment. If the signaling target requires resting, pre-treatment can be performed during this resting time.*

3. When stimulating cells in a water bath, wait 1-2 min to let the samples reach 37 °C. Add the stimulating agent by pipetting 50 µl of stimulus to the wells containing 50 µl of sample. The unstimulated control samples are supplemented with 50 µl RMPI-2% FBS as stimulation control. In addition, add 10 µl RPMI-2% FCS containing fixable live/dead viability stain 10 min prior to fixation.

   ***CRITICAL**: As the fixable live/dead viability stain is light sensitive and is not optimal in RPMI medium, prepare this solution freshly and add to the cells within 15 min post preparation.*

4. Dilute the eBioscience FoxP3 fixation and permeabilization buffer 1:1 with the supplied diluent. When the stimulation time is over, fix the cells by adding 100 µl of the diluted fixation and permeabilization buffer for 10 min at 37 °C.

   ***CAUTION**: This product contains paraformaldehyde, a toxic and mutagenic product. It should be handled with care and discarded as hazardous waste.*

   ***NOTE**: The supplied fixation buffer is 4x concentrated and is diluted in the supplied diluent by combining both in equal volume. The diluted fixative solution is added in equal volume the cells to reach the recommended working concentration (50 µl of 4x fixation buffer + 50 µl of diluent to the 100 µl of cells in RPMI).*

5. After 10 min of fixation, centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant.

6. Wash by adding 200 µl eBioscience Wash Buffer to all wells and centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant afterwards. Perform this step two times. Proceed to Step D1.

   ***CRITICAL**: Two times washing is critical in order to remove all fixation and permeabilization buffer.*

   ***PAUSE POINT**: Proceed directly to staining for flow cytometry or keep the cells in a volume of 200 µl of eBioscience Wash Buffer at 4 °C. This storage of cells at 4 °C is possible up to 5 days, without impacting the quality of the samples.*

C. Fixation of single-cell suspensions for *ex vivo* measurements (Timing: 30 min)

1. Parallel to stimulated cells, $5*10^5$ cells per well are transferred in a 96-wells round bottom plate at a volume of 100 µl per well on ice, keeping the cells at 4 °C at all times.
   ***CRITICAL**: This procedure must always be performed at 4 °C, as this is a read-out of ex vivo activation and temperature alterations could be of great influence.*
2. Samples are stained with the fixable live/dead viability stain for 10 min at 4 °C in the dark prior to fixation.
   CRITICAL: As the fixable live/dead viability stain is not optimal in RPMI medium, prepare this solution freshly and add to the cells within 15 min post preparation.
3. Fix cells with 100 µl of the diluted fixation and permeabilization buffer for 10 min at 4 °C.
4. After 10 min of fixation, centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant.
5. Wash by adding 200 µl eBioscience Wash Buffer to all wells and centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant afterwards. Perform this step two times.
   ***CRITICAL**: Two times washing is critical in order to remove all fixation and permeabilization buffer.*
   ***PAUSE POINT**: Proceed directly to staining for flow cytometry or keep the cells in a volume of 200 µl of eBioscience Wash Buffer at 4 °C. This storage of cells at 4 °C is possible up to 5 days, without impacting the quality of the samples.*

D. Flow cytometry procedures (Timing: 1-2 h)

1. After the last wash at Step B6 or Step C5, supernatant is removed and the staining mix can be added. Cells are incubated with 40 µl mix to intracellularly stain for cell surface markers such as CD3, B220/CD19, IgD and IgM in eBioscience Wash Buffer. An FcR-blocking agent is added to the staining mix to avoid non-specific binding of the antibodies. Cells are incubated in the dark for 30 min at 4 °C.
   ***CAUTION**: Sodium azide is highly toxic and dangerous to the environment. It should be handled with care and discarded as hazardous waste.*
   ***CRITICAL**: Carefully resuspend the pellet in 40 µl mix, ensuring all cells are in suspension.*
   ***NOTE**: The staining can also be performed for 15 min at room temperature (RT, ~20 °C) in the dark.*
2. Samples are washed by adding 200 µl eBioscience Wash Buffer to all wells and centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant afterwards.
3. Incubate the samples with 40 µl eBioscience Wash Buffer containing the phospho-target antibody of choice for 30 min at RT in the dark.
   OPTIONAL: If the phospho-target antibody is already labeled with a conjugate, proceed to Step D6 upon completion of the incubation time.

4. Samples are washed by adding 200 µl eBioscience Wash Buffer to all wells and centrifuge at 400 *x g* for 3 min at 4 °C. Carefully remove the supernatant afterwards.
5. When the phospho-protein concerns an unconjugated rabbit-derived antibody, incubate with a donkey anti-rabbit PE-conjugated antibody for 15 min at RT in the dark.
6. Samples are washed with MACS Buffer (0.5% BSA and 2 mM EDTA in PBS) and centrifuged at 400 *x g* for 3 min at 4 °C. Remove the supernatant and resuspended in 50 µl MACS buffer. Samples are transferred into insert tubes and measured using a flow cytometer within 1 day.
7. Prior to measuring, calibrate the LSR-II by compensating for all conjugates that are used within the phospho-staining. Preferably use the same antibodies as used in the staining mix and add those to UltraComp eBeads (Invitrogen) according to manufacturer instructions to calibrate the flow cytometer.
8. Acquire a sufficient number of cells to perform B cell subpopulation analysis. This would imply that acquiring between 20,000-50,000 B cells per sample would be sufficient. ***CRITICAL****: Due to the fixation and permeabilization protocol, the cell size will have decreased compared with non-fixed cells. Increase the voltage of the forward-scatter area (FSC-A) accordingly.*
9. Analysis is performed using FlowJo v9 or v10 software.

DATA ANALYSIS

In this protocol manuscript, we include targets described in the original paper (Rip *et al.*, 2020) and provide directions on the analyses of several additional phospho-targets that we recently established.

For each target, it is crucial to first find the best stimulus and identify the most optimal time of stimulation. **Table 2** provides directions on the optimal stimulation time for BCR signaling molecules mentioned in the original paper (Rip *et al.*, 2020) and additional phospho-targets in mice and men. During optimization of the subset and phospho-target staining procedure, all antibodies should be validated by including isotype and fluorescence minus one (FMO) controls, thereby verifying the signal intensities. Following acquisition of the cells by the flow cytometer, compensation can be checked and adjusted when necessary using the FlowJo 10 Software program.

As a data analysis example, **Figure 1** provides an overview of the phosphorylation status analysis for various BCR signaling molecules in B cells from human PBMC fractions, either unstimulated or stimulated by anti-IgM $F(ab')_2$ fragments or recombinant CD40L. Prior to gating B cell subpopulations, we first selected for live, single lymphocytes followed by the identification of B cells with the pan-B cell marker CD19 (**Figure 1A**). We included CD3 in the staining to enable accurate gating of B cells as CD19+CD3- cells. Moreover, $CD19^{-}CD3^{+}$ T

cell fractions can serve as a useful negative control for the analysis of phosphorylation of BCR signaling molecules as T cells are not responsive to anti-IgM stimulation. Within live $CD19^+$ B cells, the populations of transitional B cells and plasmablasts were identified as $CD27^-CD38^+$ and $CD27^+CD38^+$ cells, respectively. B cells with low CD38 expression were subsequently divided into naïve $CD27^-IgD^+$ B cells and $CD27^+IgD^-$ memory B cells (**Figure 1A**).

As a next step, phosphorylation of BCR signaling molecules was analyzed in gated B cell subpopulations. For example, we gated on $CD27^-IgD^+$ naive B cells and analyzed PLCγ2 (Y759) phosphorylation. For this analysis, next to FMO or isotype controls, gated T cells served as a negative staining control, because PLCγ2 is not expressed in T cells. The histogram overlays in **Figure 1B** show that the p-PLCγ2 fluorescence signals of naive B cells were strongly increased upon anti-IgM stimulation, compared with unstimulated naïve B cells. It is of note that unstimulated cells often show low but detectable *ex vivo* phosphorylation of various BCR signaling proteins. Phosphorylation signals were quantified by geometric mean fluorescence intensity (*g*MFI) values, because we observed stimulation-induced intensity shifts of a single peak in the case of p-PLCγ2 (**Figure 1B**). In contrast, phosphorylation is quantified by calculating proportions of positive cells when a bimodal distribution is observed (for example for phosphorylation of ribosomal protein S6 (**Figure 1C**). We usually also include a vertical line for reference that is set either (i) at the peak of the counts of the unstimulated control, when stimulation results in an intensity shift of a single peak; or (ii) to indicate background signals, when stimulation increased the numbers of positive cells in a bimodal staining distribution (as in pS6).

Additional examples include the analysis in total CD19+ B cells fractions of phosphorylation of CD79a and ribosomal protein S6, a downstream target of the AKT signaling pathway (**Figure 1C**). Finally, we show histogram overlays of phosphorylation targets that were not previously described in Rip et al., 2020, including the activating Y418 (in mouse B cells: Y424) and inhibitory Y507 of Src-family kinases, PI3K p85 subunit (Y458) and the Src homology region 2 domain-containing phosphatase-1 (SHP-1; Y564) (**Figure 1D**).

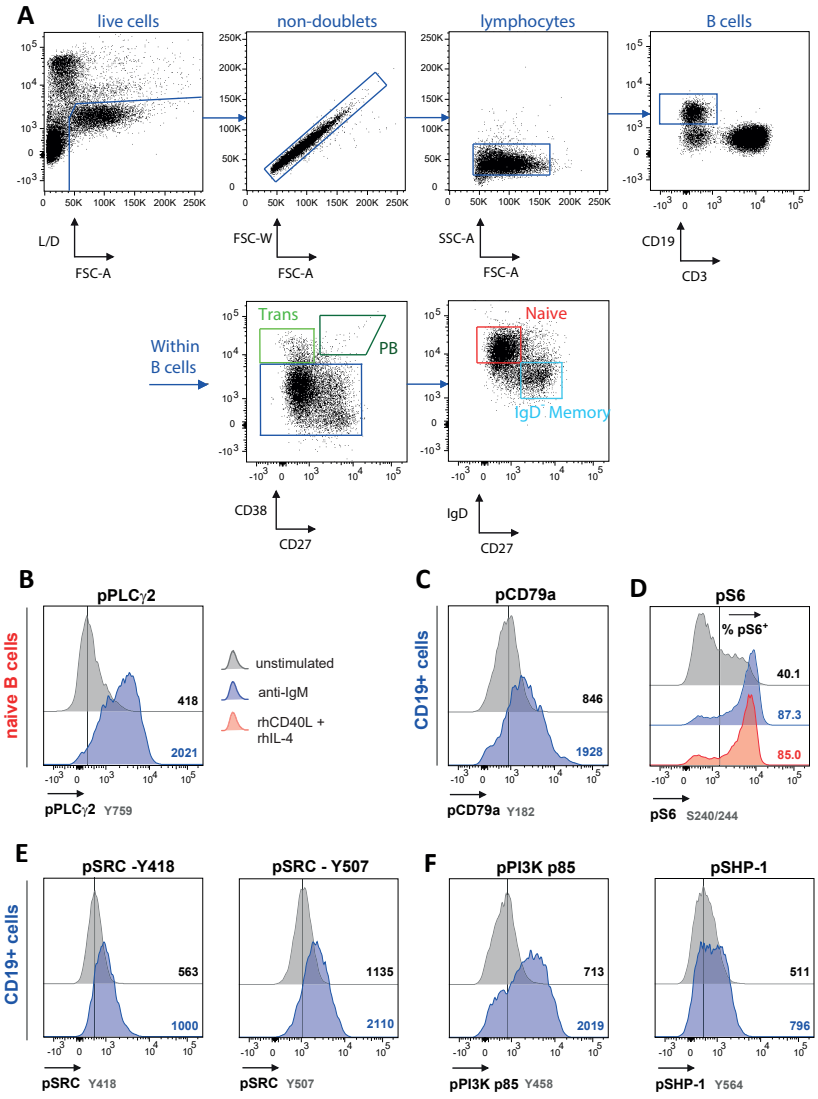
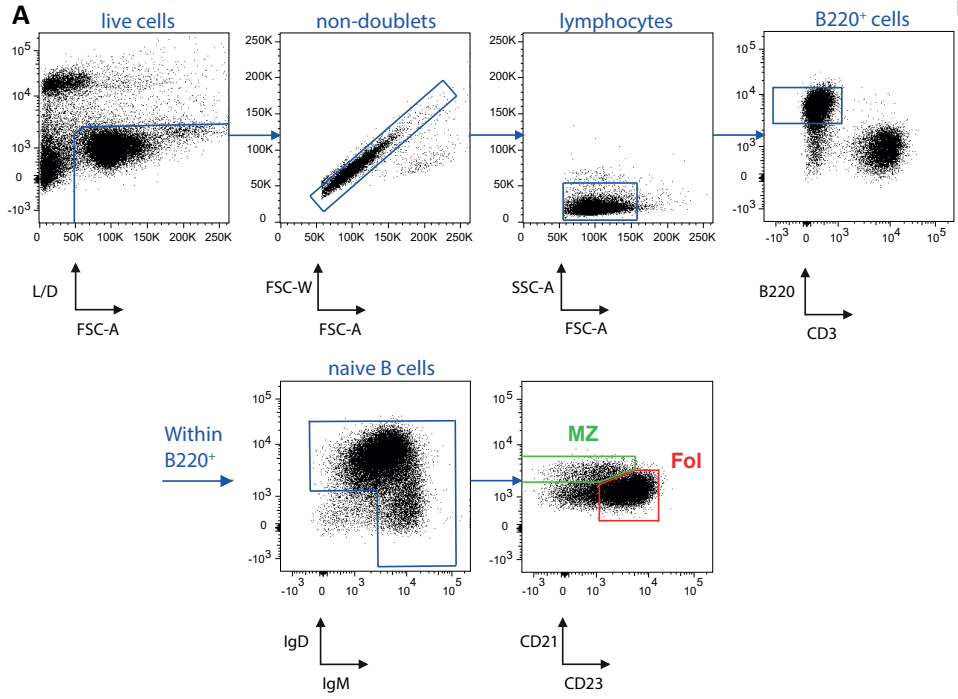


Figure 1. Phosphorylation of BCR molecules in PBMC-derived B cells.

A. Gating strategy for live single lymphocytes from the peripheral blood mononuclear cell (PBMC) fraction. Within lymphocytes, we gated for CD19$^+$ cells (CD19$^+$CD3$^-$). After exclusion of transitional B cells (Trans) and plasmablasts (PB), we gated for naive B cells (CD19$^+$CD38$^-$IgD$^+$CD27$^-$) and IgD-negative memory B cells (CD19$^+$CD38$^-$IgD$^-$CD27$^+$). (B) Histogram overlay for pPLCγ2 in naive B cells after 5 min of anti-IgM stimulation. (C) Histogram overlay for pS6 after 30 min of anti-IgM or recombinant human CD40 ligand (CD40L) and IL-4 stimulation, and for pCD79a after 1 min of anti-IgM in total CD19$^+$ B cells. (D) Histogram overlays of total CD19$^+$ B cells for pSRC-Y418 after 1 min of anti-IgM stimulation and for pSRC-Y507, pPI3K p85 and pSHP-1 after 3 min of anti-IgM stimulation. Vertical lines in histograms are set on unstimulated controls as reference for stimulation, whereby the quantification by geometric mean fluorescence intensity (*g*MFI) is indicated. Only for ribosomal protein S6, which showed a bimodal distribution, phosphorylation is quantified by calculating proportions of positive cells (C). Graphs represent representative histograms of 2-10 individual experiments, each with 2-20 healthy donors per group.

In **Figure 2** we present a parallel phospho-analyses on some previously validated (Rip *et al.*, 2020) and novel targets for murine B cells. We selected live splenic B cells by gating on live B220^{+} cells and subsequently for naïve B cells by IgD and IgM receptor expression (**Figure 2A**). Within naïve B cells, we gated on follicular and marginal zone (MZ) B cells, using CD21 and CD23 (**Figure 2A**). Phosphorylation of PLCγ2 in follicular B cells showed about a 2.5-fold increase upon stimulation with anti-IgM (**Figure 2B**). Likewise, robust phosphorylation of CD79a following anti-IgM stimulation and of S6 following anti-IgM, anti-CD40 or lipopolysaccharide (LPS) stimulation was detected in naïve B cells (**Figure 2C**). Additional BCR targets also displayed responsiveness following anti-IgM stimulation, with an optimal signal at 5 min for pSrc kinases (for both Y424 and Y507) and 1 min for pPI3K p85 and pSHP-1 (**Figure 2D**).



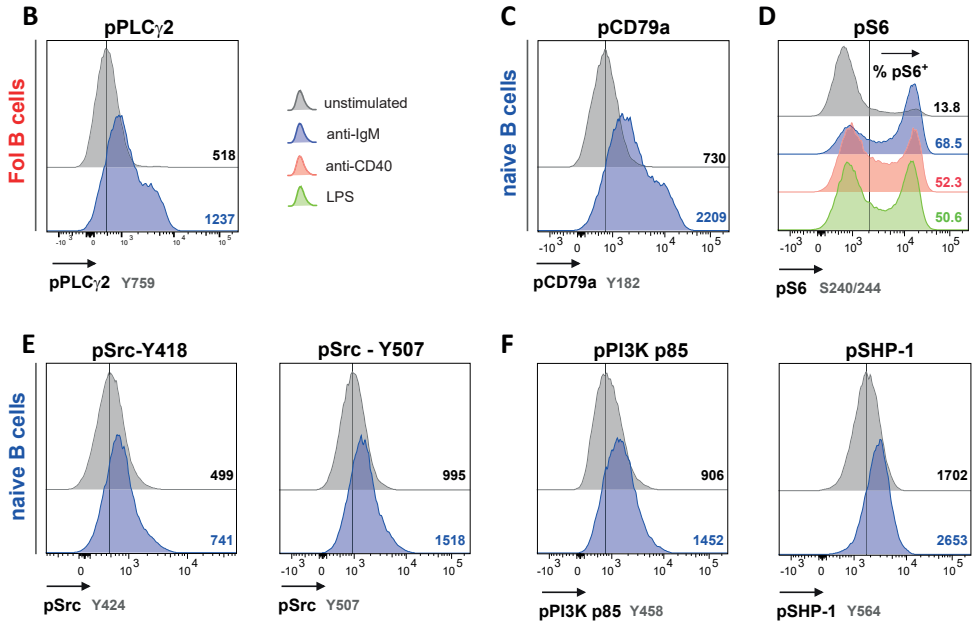


Figure 2. Phosphorylation of BCR molecules in splenic murine B cells.

A. Gating strategy for live, single splenic lymphocytes. Within lymphocytes, we gated for splenic B220^{+} cells (B220^{+}CD3^{-}). After purification for naïve B cells using IgD and IgM, we gated for marginal zone (MZ) B cells (B220^{+}CD21^{+}CD23^{-}) and follicular (Fol) B cells (B220^{+}CD21-CD23^{+}). B. Histogram overlay for pPLCγ2 after 5 min of anti-IgM stimulation in Fol B cells. C. Histogram overlays for pCD79 after 5 minutes of anti-IgM stimulation or pS6 after 3 h of anti-IgM, anti-CD40 or Lipopolysaccharide (LPS) stimulation. D. Histogram overlays of total naive B cells for pSrc-Y424 and pSrc-pY507 after 5 min and for pPI3K p85 and SHP-1 after 1 min of anti-IgM stimulation. Vertical lines in histograms are set on unstimulated controls as reference for stimulation, whereby the quantification by geometric mean fluorescence intensity (*g*MFI) is indicated. Only for ribosomal protein S6, which showed a bimodal distribution, phosphorylation is quantified by calculating proportions of positive cells (C). Graphs represent representative histograms of >3 individual experiments, each with 5 mice per group; wild-type mice were 8-12 weeks old.

NOTES

1. Timing for phosphorylation of other signaling proteins can be are presented in our original paper (**Figures 1-3** and an schematic overview in **Figure S2**) (Rip *et al.*, 2020).
2. It is not required to perform multiple washing steps, since we did not find differences in the quality of the staining between procedures with single or multiple washing steps. Single washing steps are therefore sufficient, unless otherwise indicated within the methods section.
3. The phosphoflow protocol was developed and validated for RPMI1640 medium. We did not investigate effects of other types of medium or supplements such as HEPES or 2-Mercapto-ethanol.
4. Dilutions and timing of the previously validated and additional targets shown in the Data Analysis are included in **Table 2**. Note that both mAb dilution and timing for the phospho-antibody can vary between human and murine samples.
5. Phosphorylation of STAT protein and read-out for the NF-κB signaling pathway should be performed using other protocols, as mentioned in our original paper (**Figures 2-3** and **Figure S1**) (Rip *et al.*, 2020).
6. In downstream signaling pathways in freshly acquired murine B cells are receptive to temperature changes and mechanical stress due to isolation of single cells. Rest these samples at 37 °C prior to stimulation, preferably in an CO_2 incubator. We recommend to rest the samples 3 h, as is critical for optimal detection and sensitivity of the downstream signaling read-outs.
7. The pre-incubation for downstream signaling is not recommended on frozen PBMCs, as this strongly affects viability. We did not encounter this viability issue on frozen splenic suspensions derived from murine tissue.

RECIPES

Solutions are not filtered; media, sera and buffers are sterile, fetal bovine serum (FBS) is heat-inactivated for 1h at 56°C prior to use.

1. Freezing medium
    a. 80% Fetal Bovine Serum
    b. 20% Dimethyl sulfoxide
2. RPMI-2% FCS
    a. RMPI 1640
    b. 2% Fetal Bovine Serum
    3. RPMI-5% FBS
    a. RPMI 1640
    b. 5% Fetal Bovine Serum
4. MACS Buffer
    a. Phosphate-buffered saline
    b. 0.5% Bovine Serum Albumin
    c. 2 mM Ethylenediaminetetraacetic acid solution

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AUTHOR CONTRIBUTIONS

JR designed the research, performed experiments, analyzed the data, and wrote the manuscript. OC and RH contributed to the research design and the writing of the manuscript and supervised the study. All co-authors approved the final manuscript.

COMPETING INTERESTS

The authors declare to have no financial conflicts of interest.

ETHICS

PBMCs were obtained from healthy volunteers (Rotterdam Centrum, the Netherlands). Experimental procedures were approved by the ethics committee of the Erasmus MC.

Prior to animal experiments, all experimental protocols were reviewed and approved by the Erasmus MC Committee of animal experiments (DEC) and the central committee for animal experiments (CCD).

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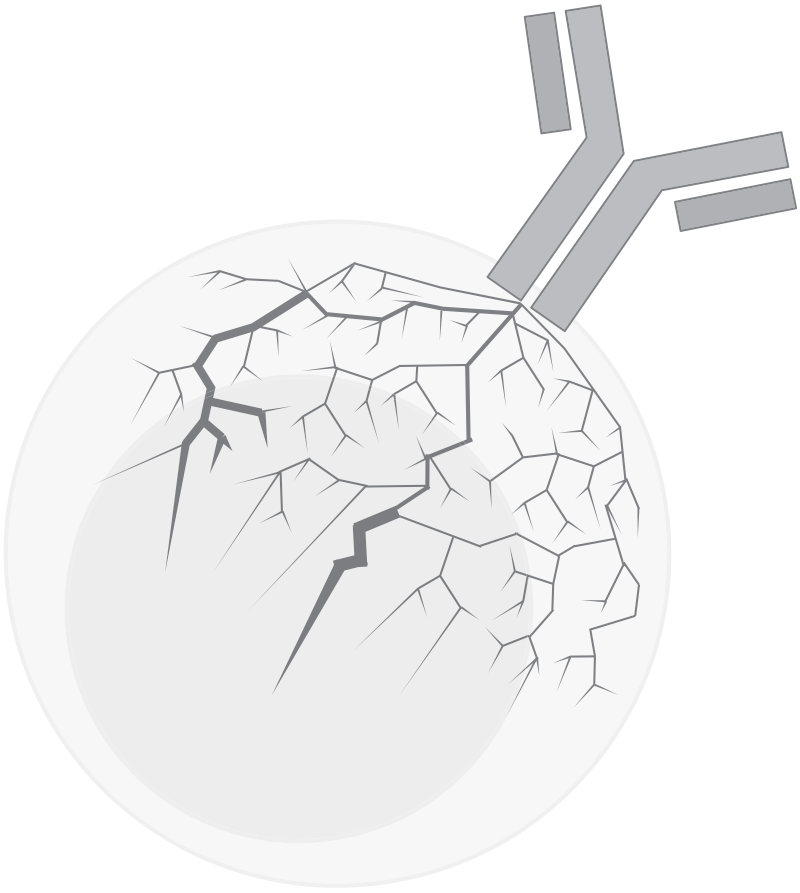

CHAPTER 4

Distinct and overlapping functions of TEC kinase and BTK in B cell receptor signaling

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ABSTRACT

The Tec tyrosine kinase is expressed in many cell types including hematopoietic cells and is a member of the Tec kinase family that also includes Btk. Although the role of Btk in B cells has been extensively studied, the role of Tec kinase in B cells remains largely unclear. It was previously shown that Tec kinase has the ability to partly compensate for loss of Btk activity in B cell differentiation, although the underlying mechanism is unknown. In this report, we confirm that Tec kinase is not essential for normal B cell development when Btk is present, but we also found that Tec-deficient mature B cells showed increased activation, proliferation and survival upon BCR stimulation, even in the presence of Btk. Whereas Tec-deficiency did not affect phosphorylation of PLCγ2 or Ca^{2+} influx, it was associated with significantly increased activation of the intracellular Akt/S6 kinase signaling pathway upon BCR and CD40 stimulation. The increased S6 kinase phosphorylation in Tec-deficient B cells was dependent on Btk kinase activity, as ibrutinib treatment restored pS6 to wild-type levels, although Btk protein and phosphorylation levels were comparable to controls. In Tec-deficient mice in vivo B cell responses to model antigens and humoral immunity upon influenza infection were enhanced. Moreover, aged mice lacking Tec kinase developed a mild autoimmune phenotype. Taken together, these data indicate that in mature B cells, Tec and Btk may compete for activation of the Akt signaling pathway, whereby the activating capacity of Btk is limited by the presence of Tec kinase.

INTRODUCTION

The Tec-family of non-receptor kinases consists of Tec, Btk, Itk, Rlk and Bmx. Within the family, Tec, Btk and Itk are most similar and are characterized by the presence of a pleckstrin homology (PH) domain, a Tec homology (TH) domain, a Src homology (SH)3 and an SH2 domain, and a kinase domain (1). Itk and Btk play important roles during lymphocyte development and activation, and mutations in these molecules are linked to severe immunodeficiencies and malignancies (2). By contrast, the function of Tec in lymphocytes is still not fully clear, although several studies suggest it may have a similar or redundant role to Itk in T-cells and Btk in B cells.

BTK deficiency causes X-linked agammaglobulinemia (XLA) in humans, a severe immunodeficiency characterized by a block in early B cell development at the pre-B cell stage leading to an almost complete absence of mature peripheral B cells (3). In mice, loss of Btk function reduces mature B cell numbers by half, caused by hampered development of mature B cells, which also have a survival disadvantage over wild-type (WT) B cells (4). In contrast, Tec deficiency in B cells does not affect B cell numbers, possibly because of redundancy with Btk (5). However, loss of both Btk and Tec leads to a severe block in B cell development at the pre-B cell stage resembling human XLA, with few mature B cells reaching the periphery (5). These data suggest a complementary role for Tec and Btk in B cell development, whereby Btk can completely compensate for loss of Tec, but Tec can only partially compensate for loss of Btk. It is unclear whether this is due to functional differences between Btk and Tec, or because Btk is more highly expressed in B cells than Tec (6).

Btk is crucial for B cell receptor (BCR) signaling and is involved in several downstream signaling complexes and pathways (7). Upon BCR binding by an antigen, Btk is recruited to the cell membrane by phosphoinositide 3-kinase (PI3K) and phosphorylated by Lyn and Syk. It propagates the BCR signal through its direct substrate phospholipase Cγ2 (PLCγ2), ultimately leading to NFκB activation, providing essential survival, proliferation and differentiation signals to B cells (7). In addition, Btk can activate the Akt pathway, which is involved in cell activation and survival (8). Like Btk, both phosphorylation and activation of Tec kinase are increased upon BCR ligation of human B cells (9). Overexpression of Tec kinase in a Jurkat T cells line induces NFAT activation, however, it is unclear what the role of Tec is in mature B cells, and in which signaling pathways Tec may be involved or redundant to Btk (6). This is currently of particular interest, as Btk inhibitors have recently been shown to be efficacious in the treatment of several B cell malignancies, with limited side effects for patients (7). Therefore, in this study we aimed to further investigate the role of Tec in mature B cells and during immune responses.

We show here that in Tec-deficient mice *in vitro* B cell activation following BCR stimulation was increased. This was associated with enhanced activation of the Akt/S6 kinase pathway upon BCR or CD40 engagement in Tec-deficient B cells, which was dependent on Btk kinase activity. Moreover, Tec-deficient mice manifest increased *in vivo* B cell responses and a mild autoimmune phenotype. Taken together, these data indicate that Btk activity may be limited by competition with Tec kinase.

METHODS

Mice

Btk-deficient (4) and Tec-deficient (5) mice were backcrossed for >10 generations onto a C57BL/6 background. Mice were analysed between 8 and 14 weeks or 34 weeks of age for autoimmunity studies. Mice were bred and kept in the experimental animal facility of the Erasmus Medical Center (EDC) under SPF conditions. All experiments were approved by the Erasmus MC Animal Ethical Committee.

MACS purification and B cell cultures

Bone marrow cells were cultured with 150 Units/mL mouse recombinant IL-7 (R&D) for 5 days, followed by 2 days without rIL-7.

Naïve B cells from spleens were MACS-purified and cultured *in vitro* for 1-4 days as described (10). Cells were stimulated with (combinations of) 10μg/mL F(ab')2 anti-mouse IgM fragments (Jackson ImmunoResearch), 5ng/mL LPS (own production) for cell cycle analysis and varying concentrations (Sigma) for plasmablast cultures, 5ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma), 1μg/mL ionomycin (Sigma), 0.1μg/mL mouse recombinant IL-4 (Peprotech) or anti-CD40 10μg/mL (BD Biosciences, clone 3/23).

Flow cytometry, cell cycle analysis, CSFE labeling and measurement of Ca^{2+} mobilization

Flow cytometry was performed using antibodies and reagents as described (11). For phosphoflow, total spleen cells were stained for extracellular markers and stimulated with (combinations of) anti-IgM $F(ab')_2$ fragments (4 or 25μg/mL), mouse recombinant IL-4 (0.1μg/mL) and anti-CD40 (4 or 20μg/mL) at 37° C. Cells were then fixed with Cytofix™ and permeabilized with Phosflow Perm Buffer III™ (BD Biosciences) and stained for anti-pS6 ribosomal protein (S240/244; Cell Signaling), anti-pAkt (S473; BD) or anti-pPLCγ2 (pY759; BD).

Cell cycle analysis was performed as described (12).

Proliferation analysis was performed by staining cells for 5 minutes at 37° C with 0.05mM carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE, Thermo Fisher Scientific) and subsequent culture for 3 days as described above. Ca^{2+} mobilization was measured as published (13), using 20μg/mL $F(ab')_2$ anti-mouse IgM fragments to stimulate the cells.

All measurements were performed on an LSRII flow cytometer (BD Biosciences) and analysed using FlowJo software (Tree Star).

Quantitative RT-PCR

RNA was isolated using the genElute RNA isolation kit (Sigma) and cDNA was made using the Revert AID H minus first strand cDNA synthesis kit (Thermo Scientific). Germline transcripts of Igκ and Igλ loci were determined using the following primers: κ^0.0.8 and κ^0.1.1 (14), and λ^0.0.1, λ^0.0.2 and λ^0.0.3 (15).

To detect human TEC mRNA, we used a forward primer acatgcttttggctccattc and a reverse primer ggtaccgaagcctggtga; for human BTK we used a forward primer tgttgaaacagtggttcctga and a reverse primer tgctccatttcactggactct. Expression was normalized to Ubiquitin C expression (forward primer atttgggtcgcggttcttg and reverse primer tgccttgacattctcgatggt).

Quantitative PCR was performed using Sybr green master mix (Thermo scientific) and measured on an AB7300 (Applied Biosystems) machine using 7300 software (Applied Biosystems).

DNP-ficoll and TNP-KLH immunization

For T-cell-independent immunization, 50μg DNP(49)-ficoll (Biosearch Technologies) in PBS was injected intraperitoneally. Serum was collected at baseline and 2, 4 and 7 days after immunisation. For T-cell-dependent immunization, 100μg TNP(29)-KLH (Biosearch Technologies) and 0.8mg Imject aluminum hydroxide adjuvant (Thermo Scientific) in PBS was injected intraperitoneally in mice at day 0, followed by a boost immunization with 100μg TNP(29)-KLH in PBS 35 days later. Mice were sacrificed 7 days after the boost.

Ibrutinib treatment

Mice were treated *in vivo* with ibrutinib as described (10) and sacrificed 3 hours later for analysis. For *in vitro* studies, 1μM ibrutinib was added to the culture medium.

Influenza virus infection

10^4 X-31 influenza virus particles diluted in PBS were given intra-nasally (16). Anti-hemagglutinin (HA) inhibition assay was performed on serum collected 15 and 25 days after infection (17).

Immunohistochemistry

Cryostat sections (6μm) were cut from OCT compound embedded tissues, fixed in acetone and processed as described (18).

Ig subclass concentrations and autoreactivity

Isotype-specific serum antibody levels (19) and anti-DNA IgM and IgG autoantibody levels (11) were measured as described. Ig autoreactivity was analysed by HEp2-staining as described (10).

RESULTS

Development of Tec-deficient B cells is comparable to wild type B cells

To confirm that B cell development in the bone marrow (BM) of Tec-deficient mice is normal, as previously described (5), we analyzed BM cells of wild-type (WT), Tec-deficient ($Tec^{-/-}$), Btk-deficient ($Btk^{-/-}$) and Tec/Btk double-deficient ($Tec/Btk^{-/-}$) mice on the C57BL/6 background by flow cytometry.

In the BM, total cell numbers were comparable in all mouse groups, but total B-lineage cell numbers were lower in $Tec/Btk^{-/-}$ mice (**Figure S1A-S1B**). Similar to findings on a mixed background (5), $Tec/Btk^{-/-}$ mice showed a developmental block at the large pre-B cell stage (**Figure S1C**). $Btk^{-/-}$ B cells, but not $Tec^{-/-}$ B cells, showed a partial developmental block at the large pre-B cell stage (as described previously (12)). This indicates that lack of Tec could be completely compensated by Btk.

In $Tec^{-/-}$ mice pre-BCR function was unaffected, as analyzed by modulation of cell surface markers, germline transcription over unrearranged Ig L chain loci and developmental progression of pre-B cells *in vitro* (**Figure S1D-S1F**) indicating that lack of Tec could be completely compensated by Btk.

Finally, since we found that Btk cooperates with the linker protein Slp65 as a tumor suppressor at the pre-B cell stage, we analyzed the effects of Tec expression on pre-B cell tumor formation. $Btk^{-/-}$ mice do not develop pre-B cell tumors, but the incidence of pre-B cell lymphomas is higher in $Slp65/Btk^{-/-}$ mice than $Slp65^{-/-}$ mice (20). However, we did not observe tumors in $Tec/Btk^{-/-}$ mice, nor differences in pre-B cell tumor frequencies between $Tec/Btk/Slp65^{-/-}$ mice and $Slp65/Btk^{-/-}$ mice (data not shown). Therefore, Tec does not have a tumor suppression function at the pre-B cell stage that may be masked by the expression of Btk.

Together, these data suggest that, although Tec and Btk have complementary roles in pre-BCR-mediated developmental progression of pre-B cells (5), B cell development is normal in $Tec^{-/-}$ mice.

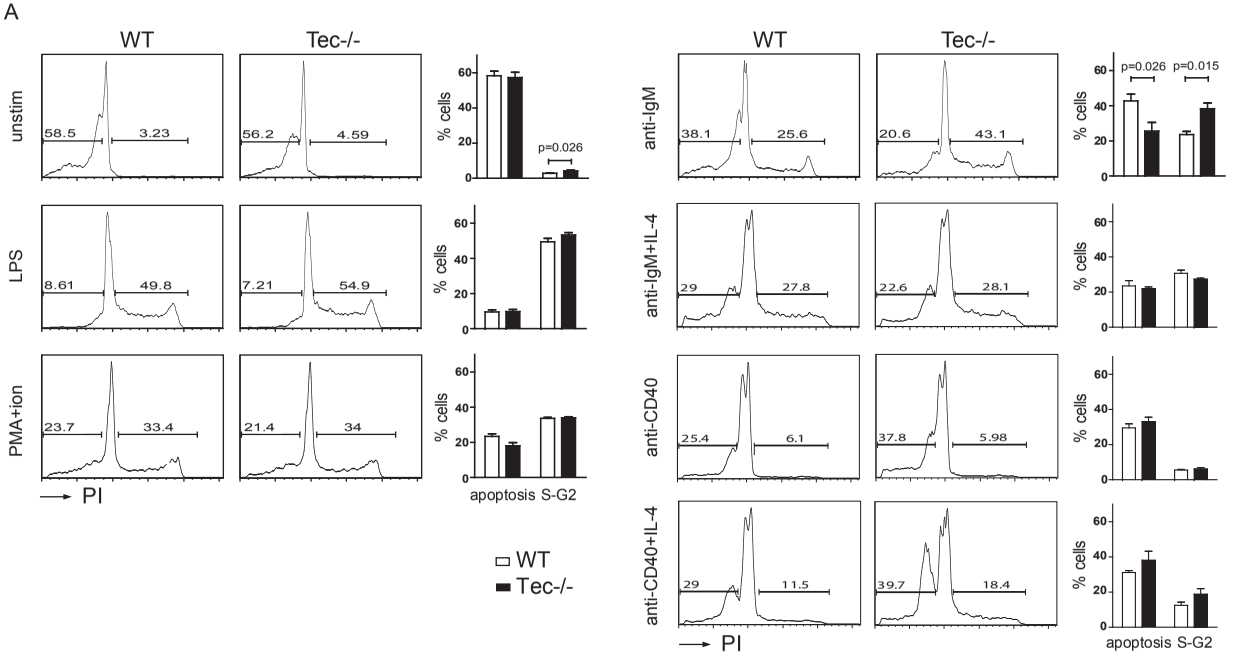
Survival, proliferation and activation are enhanced in mature Tec$^{-/-}$ B cells *in vitro*

Total splenocyte and B cell numbers were lower in Btk$^{-/-}$ and Tec/Btk$^{-/-}$ mice, compared to WT and Tec$^{-/-}$ mice (**Figure S1A-S1B**). Moreover, we found a relative increase in CD21^{+}CD23^{low} marginal zone B cells and, as previously shown (5), a decrease in mature IgD^{high}IgM^{low} B cells in the spleens of Tec/Btk$^{-/-}$ mice (**Figure S1G** and data not shown). Nevertheless, in these analyses we did not detect differences in the B cell compartments in the spleen or peritoneal cavity between Tec$^{-/-}$ and WT mice (Figure S1G and data not shown).

Next, we compared survival, proliferation and activation of mature B cells in WT and Tec$^{-/-}$ mice. MACS-purified naïve splenic B cells were cultured with either LPS (5ng/mL), a combination of PMA (5ng/mL) and ionomycin (1μg/mL), anti-IgM F(ab)$_2$ fragments (10μg/mL) with or without recombinant IL-4 (rIL-4, 0.1μg/mL) or anti-CD40 (10μg/mL) with or without rIL-4. After 2 days of culture propidium iodide (PI) staining revealed that unstimulated Tec$^{-/-}$ cultures had significantly more proliferating cells than unstimulated WT cultures (**Figure 1A**). In addition, following anti-IgM stimulation, Tec$^{-/-}$ B cells had increased proportions of proliferating cells (~43%, compared with ~26% in wild-type B cells) and decreased proportions of apoptotic cells (21%, compared with ~38% in wild-type B cells) (**Figure 1A**). Addition of IL-4 to the cultures decreased apoptosis in WT, but not in Tec$^{-/-}$ B cell cultures, suggesting the beneficial effect of Tec deficiency on cell survival is mediated through similar downstream pathways, possibly the Akt pathway. The other stimulations showed no significant differences. CFSE labeling confirmed there were more cell divisions upon anti-IgM stimulation, with or without rIL-4 (**Figure 1B**). Anti-CD40-stimulated Tec$^{-/-}$ B cells showed slightly increased cell division in Tec-deficient B cells, but the other stimulation conditions showed no differences (**Figure 1B** and data not shown).

We further analyzed the B cell activation status by flow cytometry after 1 and 3 days of activation *in vitro*. After 3 days in culture, expression of activation markers CD25 and CD69 was increased particularly in Tec$^{-/-}$ B cells cultured with anti-IgM and rIL-4 or with anti-CD40 and rIL-4, as was the cell size when measured by forward scatter (FSC), showing an enhanced activation status of the Tec$^{-/-}$ B cells (**Figure 1C**). Similar but less pronounced increases were found at day 1 (data not shown).

In summary, Tec-deficient mature B cells show increased activation, proliferation and survival upon *in vitro* BCR stimulation with anti-IgM or anti-CD40, particularly in combination with rIL-4, compared with WT B cells.


A
WT
Tec-/-
unstim
LPS
PMA+ion
58.5
3.23
56.2
4.59
8.61
49.8
7.21
54.9
23.7
33.4
21.4
34
PI
% cells
p=0.026
apoptosis S-G2
WT
Tec-/-
anti-IgM
anti-IgM+IL-4
anti-CD40
anti-CD40+IL-4
38.1
25.6
20.6
43.1
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29
27.8
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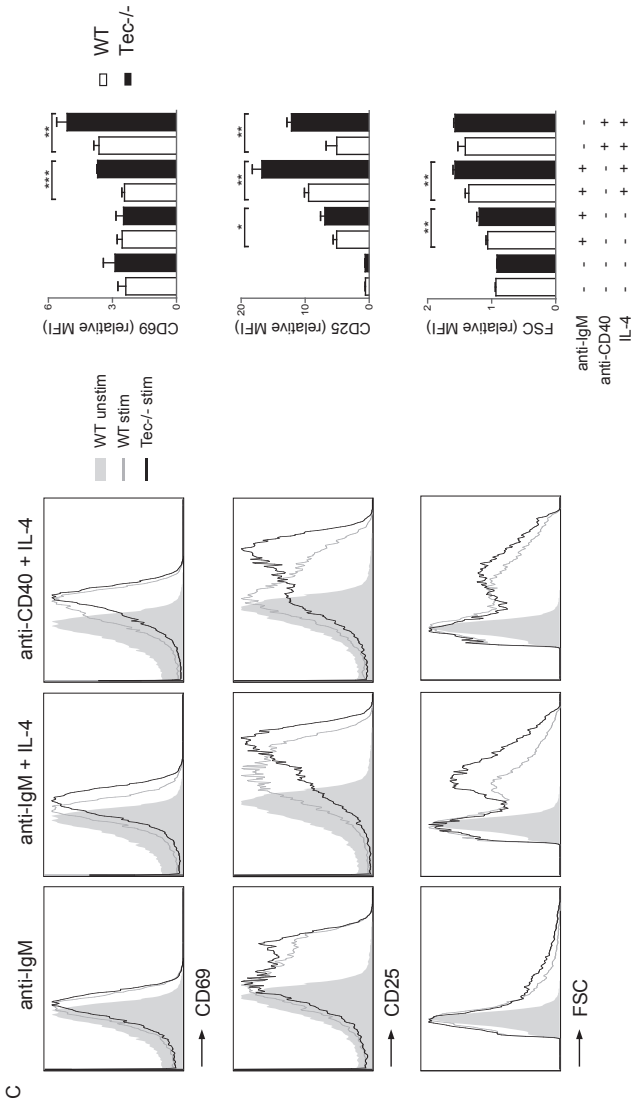


Figure 1. Enhanced survival and activation of Tec$^{-/-}$ B cells.

[A-C] MACS-sorted naïve splenic B cells from mice aged 8-14 weeks analyzed by flow cytometry. [A] Cell cycle analysis by DNA content using propidium iodide (PI) of B cells stimulated for 2 days with or without indicated stimuli shown as histrograms (*left*) and quantified as average values ± SEM (*right*). [B] Proliferation by CFSE analysis of purified B cells stimulated for 3 days with or without indicated stimuli. Numbers indicate cell divisions. [C] Surface expression of activation markers after stimulation with or without indicated stimuli for 3 days, shown as histogram overlays of the indicated genotypes (*left*) or quantified as relative MFI to unstimulated WT cells after 1 day in culture (set to 1, *right*). Graphs represent 2-5 individual experiments and n=8-16 mice per group; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U test

BCR-dependent PLCγ activation is not affected in Tec$^{-/-}$ B cells

To study BCR signaling in Tec$^{-/-}$ B cells, we measured tyrosine phosphorylation of PLCγ2 at position Y759 (pPLCγ2), which is a major phosphorylation target of Btk and Tec (21) by flow cytometry. We found no difference in pPLCγ2 between WT and Tec$^{-/-}$ cells upon anti-IgM stimulation after 5 minutes (peak of pPLCγ2 expression, **Figure 2A**) nor for up to 60 minutes (**Figure 2B**), with either a suboptimal low (4μg/mL) or an optimal high (25μg/mL) of anti-IgM. Accordingly, calcium influx, which occurs downstream of PLCγ2, was not increased in Tec$^{-/-}$ B cells compared to WT B cells. Furthermore, there was no change in Btk-dependence of calcium influx, as shown by *in vivo* treatment with ibutinib, a Btk inhibitor that irreversibly binds to the Btk kinase domain, thereby inhibiting its function (22) (**Figure 2C**). In addition, we found no differences in pBtk (Y223) or Btk protein levels (data not shown).

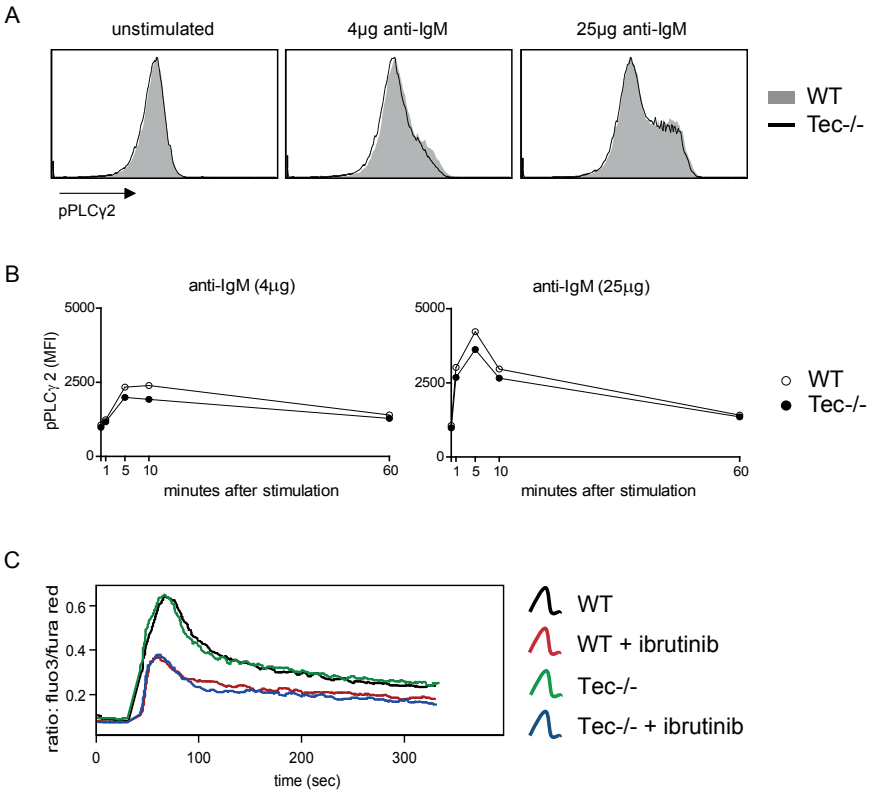


Figure 2. Loss of Tec expression does not affect the PLCγ2/Ca^{2+} pathway in B cells.
[A-B] Phospho-PLCγ2 expression in B cells upon 4 or 25μg/mL F(ab')$_2$ anti-IgM stimulation in WT and Tec$^{-/-}$ mice, shown as histogram overlays [A] and quantified over time [B]. [C] Ca^{2+} influx in B cells from WT and Tec$^{-/-}$ mice 3 hours after *in vivo* treatment with ibrutinib, upon 25μg/ml F(ab')$_2$ anti-IgM stimulation *in vitro*. Signals were normalized for maximum influx following stimulation with ionomycin. Graphs represent 1-4 individual experiments and n=4-12 mice per group.

pAkt and pS6 expression are increased in Tec$^{-/-}$ B cells upon BCR and CD40 stimulation

As we did not detect differences in calcium influx, we next measured serine phosphorylation of Akt (S473), which is also activated downstream of BCR signaling, but in a pathway separate from PLCγ2, as well as phosphorylation of S6 kinase (S240/244), a downstream target of Akt.

Kinetics experiments with different stimuli showed an early peak of pAkt and pS6 expression in B cells after 1-3 hours, but a higher second peak after 24 hours of stimulation (**Figure S2A-B**). pAkt expression was increased after 24 hours of stimulation with both a low dose (4μg/mL) and a high dose (25μg/mL) of anti-IgM in Tec$^{-/-}$ B cells, compared to wild-type B cells (**Figure 3A**). Addition of rIL-4 to a low dose of anti-IgM did not induce differences in pAkt expression, however, both a low dose of anti-CD40 (4μg/mL) and a high dose (20μg/mL) in combination with rIL-4 induced robust upregulation of pAkt expression which was higher in Tec$^{-/-}$ B cells than in WT B cells (**Figure 3A**). Correspondingly, phosphorylation of S6 was also increased in Tec$^{-/-}$ B cells compared to WT B cells following 24h of BCR or anti-CD40 with rIL-4 stimulation (**Figure 3B**). These data show that, unlike the PLCγ2/calcium flux pathway, loss of Tec expression leads to enhanced activation of the Akt/S6 pathway.

Increased Akt and ribosomal S6 phosphorylation in Tec$^{-/-}$ B cells is dependent on Btk kinase activity

As we found increased activation of the Akt/S6 pathway, but not of the PLCγ2/Ca^{2+} pathway in Tec$^{-/-}$ B cells, we hypothesized that Tec competes with Btk in the Akt pathway. In this model, Tec kinase would be less effective in activating Akt and S6, but not involved in the PLCγ2 pathway when Btk is present.

To test this hypothesis, we performed phosphoflow analysis on B cells stimulated with anti-IgM (4μg/mL) and rIL-4, in the absence or presence of ibritinib *in vitro* (**Figure 3C**), or on B cells from mice that were treated *in vivo* for 3 hours prior to the experiment and stimulated with anti-IgM (25μg/mL) *in vitro* (**Figure 3D**). This allowed us to study the effect of Btk kinase activity on the Akt pathway in a setting where mature B cells are present, as opposed to Tec/Btk$^{-/-}$ mice in which mainly IgM^{high}IgD^{low} immature/transitional B cells are present. After 24 hours in culture, pAkt and pS6 expression were higher in Tec$^{-/-}$ B cells compared to WT B cells (**Figure 3C-D**). After ibrutinib treatment *in vitro*, pAkt and pS6 expression decreased to similar levels in Tec$^{-/-}$ and WT B cells (**Figure 3C**). Furthermore, after *in vivo* treatment, pAkt decreased in Tec$^{-/-}$ B cells and pS6 expression dropped to placebo treated WT levels (p=0.69; **Figure 3D**).

In summary, these data show that in the absence of Tec, phosphorylation of Akt and ribosomal S6 is increased, whereby this increase is dependent on Btk kinase activity. We conclude that under WT conditions, Tec and Btk may compete, possibly for binding with Akt, but that Tec is less capable of efficiently activating the Akt pathway.

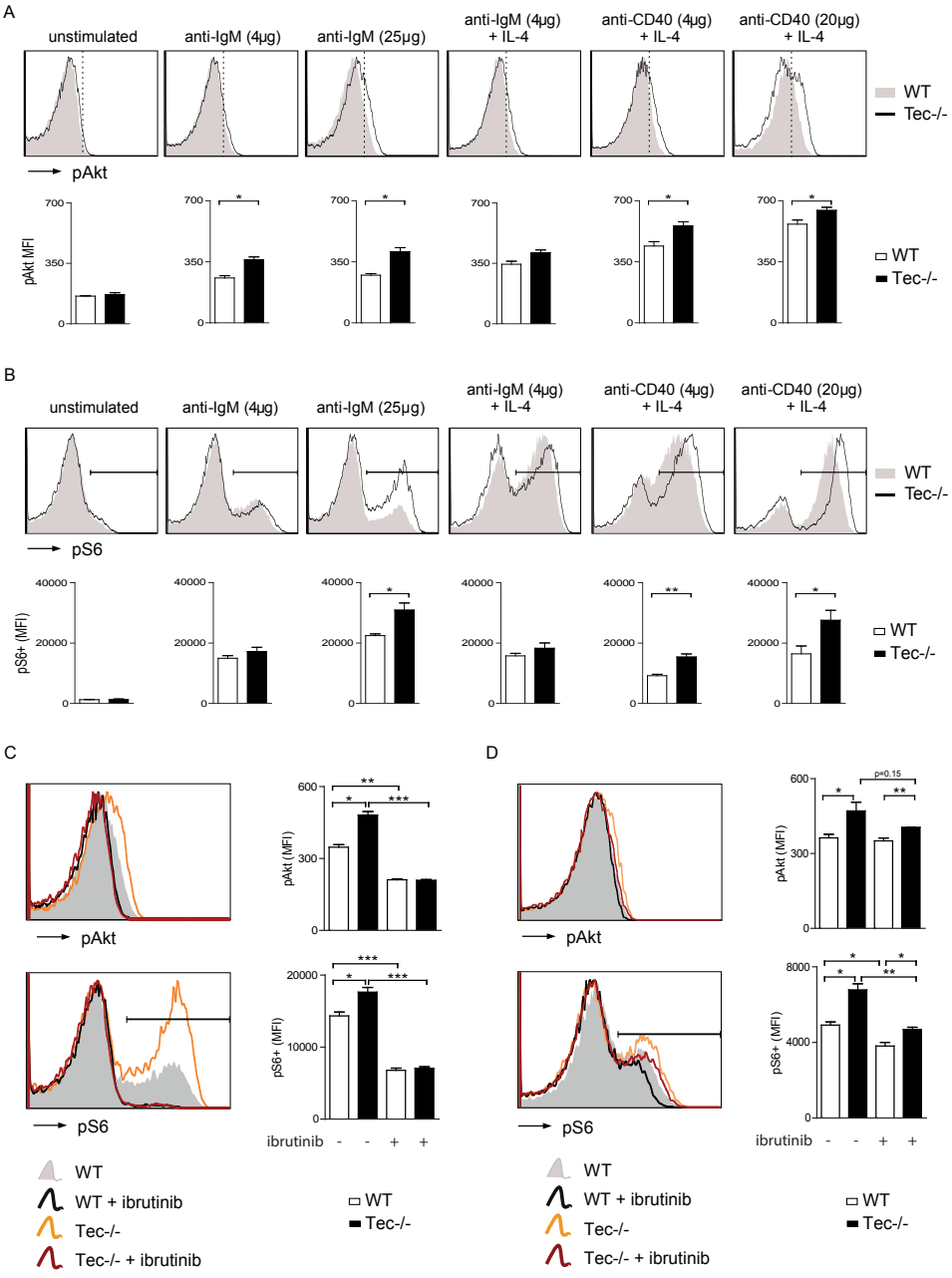


Figure 3 | Increased activation of the Akt/S6 pathway in Tec$^{-/-}$ mice.
[A] Histogram overlays (*top*) and quantification (*bottom*) of phospho-Akt expression in unstimulated B cells or following 24 hours of stimulation with the indicated stimuli. [B] Histogram overlays (*top*) and quantification (*bottom*) of phospho-S6 expression in unstimulated B cells or following 24 hours of stimulation with the indicated stimuli. [C] Phospho-Akt and phospho-S6 expression in B cells upon 24 hour *in vitro* stimulation with 25 µg/mL F(ab')$_2$ anti-IgM,

with or without ibrutinib, shown as histogram overlays (*left*) and quantification (*right*). [D] Phospho-Akt and phospho-S6 expression in B cells (from mice 3 hours after *in vivo* treatment with ibrutinib) upon *in vitro* stimulation with 25μg/mL $F(ab')_2$ anti-IgM, shown as histogram overlays (*left*) and quantification (*right*). Graphs represent 1-4 individual experiments and n=4-12 mice per group; *p<0.05, **p<0.01 by Mann-Whitney U test.

Increased activation of $Tec^{-/-}$ B cells *in vivo* after immunization with model antigens

To study the effects of increased BCR-mediated activation of $Tec^{-/-}$ B cells *in vivo*, we immunized WT and $Tec^{-/-}$ mice with model antigens. First, we performed a T-cell-independent DNP-ficoll immunization. In $Tec^{-/-}$ mice, DNP-specific IgM and IgG3 antibodies in serum were both significantly increased 7 days after immunization, compared to WT mice (**Figure 4A**).

Next, we immunized mice with the T-cell-dependent TNP-KLH antigen in alum. Seven days after the boost (day 42 after primary immunization), we found that both total and high affinity anti-TNP antibodies were significantly increased in $Tec^{-/-}$ mice (**Figure 4B**). Ratios between high-affinity and total anti-TNP antibodies were comparable between $Tec^{-/-}$ and WT mice (data not shown), indicating that affinity maturation was not affected in $Tec^{-/-}$ mice. Absolute numbers of germinal center (GC) B cells and follicular T helper (Tfh) cells ($CD3^+CD4^+CXCR5^+PD1^{hi}$) in the spleen on day 42 were not different between $Tec^{-/-}$ and WT mice (**Figure 4C** and data not shown). However, absolute numbers of total IgM and IgG plasma cells (PC) were significantly increased in the spleens, but not BM, of $Tec^{-/-}$ mice, suggesting that only short-lived but not long-lived PC were affected in $Tec^{-/-}$ mice (**Figure 4C**).

Because Tec is also expressed in DCs and T cells, we wanted to determine whether B cell-intrinsic effects of Tec-deficiency contributed to the increase in TNP-specific Ab formation and the increase in splenic PCs upon *in vivo* immunization. To this end, we cultured MACS purified B cells for 4 days with decreasing doses of LPS (mimicking a T-cell independent stimulus) or LPS and rIL-4 (mimicking T-cell co-stimulation), to induce IgM, IgG3 and IgG1 expressing plasma blasts, respectively. Proportions of IgM^+ plasmablasts were comparable upon LPS stimulation, but proportions of $IgG3^+$ plasmablasts were significantly increased in $Tec^{-/-}$ cultures with a suboptimal LPS (25ng/mL) concentration and $IgG1^+$ plasmablasts were significantly increased in $Tec^{-/-}$ cultures even at high LPS concentrations (**Figure 4D**).

Together, these data show that B cell activation *in vivo* is enhanced in $Tec^{-/-}$ mice, both during a T-cell-independent and a T-cell-dependent B cell response, whereby B cell-intrinsic effects of loss of Tec (at least partially) contributes to the enhanced B cell responses.

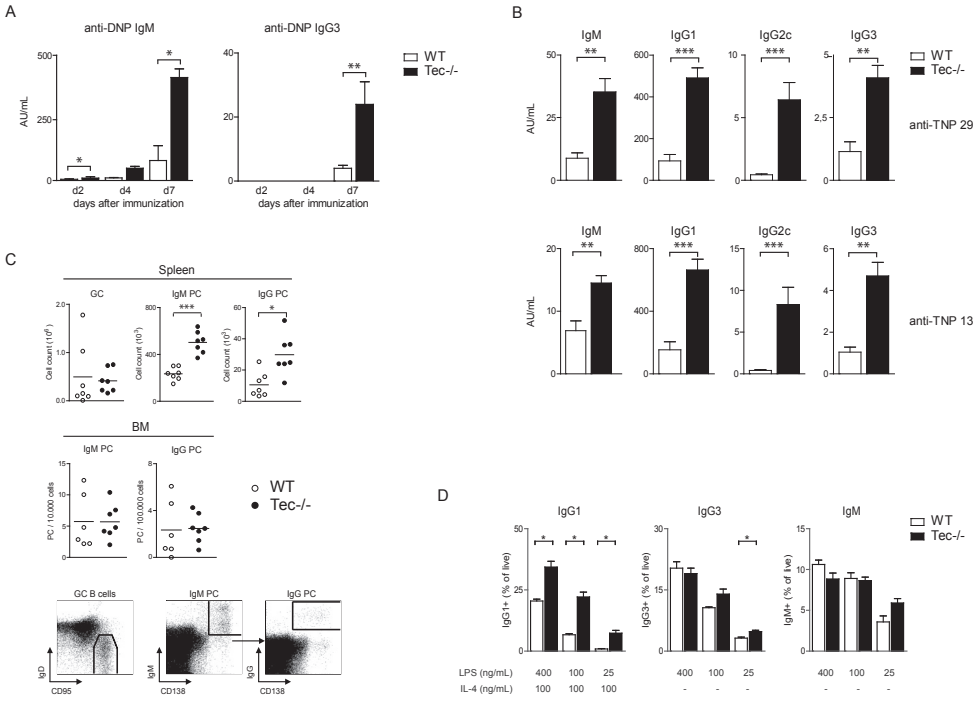


Figure 4. Enhanced *in vivo* humoral immunity in Tec$^{-/-}$ mice.
[A] DNP-specific IgM and IgG3 antibodies in serum of mice 2, 4 or 7 days after immunization with the T-cell independent antigen DNP-ficoll. [B] TNP-specific IgM, IgG1, IgG2c and IgG3 high affinity (ELISA using low-density, TNP13, hapten-KLH coupling) and total affinity (ELISA using high-density, TNP29, hapten-KLH coupling) antibodies in serum 7 days after TNP-KLH boost at day 35 after primary immunization with the T-cell dependent antigen TNP-KLH. [C] Germinal center (GC) B cell and plasma cell (PC) numbers in spleen and bone marrow (BM) at day 7 after boost immunization. Gating strategy is shown in representative flow plots (*bottom*). GC B cells were characterized as CD19^{+}IgD^{-}CD95^{+}, IgM^{+} PC as CD11b^{-}CD138^{+}IgM^{+} and IgG^{+} PC as CD11b^{-}CD138^{+}IgM^{-}IgG^{+}. [D] Plasmablast formation of naïve B cells after 4 days of culture with decreasing concentrations of LPS or LPS and rIL-4, measured by flow cytometry. N=5-7 mice per group; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U test.

Higher anti-virus specific antibody levels in serum upon influenza infection in Tec$^{-/-}$ mice

To study B cell responses in Tec$^{-/-}$ mice in a more physiological setting, we performed an infection with H3N2 influenza virus (X31 strain (17)) in Tec$^{-/-}$ and WT mice. Weight loss after infection was moderately decreased in Tec$^{-/-}$ mice (**Figure S3A**). At day 25 after infection, PC and GC B cells, marking the formation of inducible bronchus associated lymphoid tissue (iBALT), were found in the lungs of WT mice (**Figure 5A**). Although some Tec$^{-/-}$ mice showed a slight increase in GC B cells, there was no difference in PC numbers in the lungs (**Figure 5A**). PC and GC B cell numbers in the draining lymph nodes were also comparable between WT and Tec$^{-/-}$ mice at 7 or 15 days post infection (**Figure 5A**). Accordingly, histological analysis of iBALT formation in the lungs 25 days after infection showed no difference in number or size of GL7^{+} B cell clusters (**Figure 5B**).

Next, we measured serum antibody titers for X-31 and heterotypical antibodies directed against related H3N2 influenza viruses (England/72, Port Chalmers/73 and Victoria/75). Both 15 and 25 days after infection we found higher titers of X-31 specific antibodies, and heterotypical antibodies in serum from Tec$^{-/-}$ compared to WT mice (**Figure 5C**), but not in total serum antibodies (**Figure S3B**). Because we previously found that increased Btk activity in B cells is associated with local autoantibody production during influenza infection (10), we performed HEp2 reactivity analyses. We found no IgM autoantibodies in the BAL fluid and detected low levels of IgG autoantibodies in both WT and Tec$^{-/-}$ mice (**Figure S3C**).

These findings indicate that both the X-31 influenza virus-specific humoral response and the non-X31 specific antiviral response is enhanced in Tec$^{-/-}$ mice, suggesting that B cells are more activated in Tec$^{-/-}$ mice upon influenza virus infection.

Aging Tec$^{-/-}$ mice develop a spontaneous mild autoimmune phenotype

To investigate whether the enhanced BCR signaling in the absence of Tec is associated with autoimmunity, we aged WT and Tec$^{-/-}$ mice up to ~34 weeks and analyzed the mice for signs of B cell activation and autoantibody formation.

Although we did not find differences in the numbers of GC B cells in the spleens of Tec$^{-/-}$ mice (**Figure 6A**), the co-stimulation markers CD80 and PDL1 (but not CD86) were upregulated on these cells compared to WT controls, suggesting they were more activated. Accordingly, we found a significant increase in IgM^{+}, IgG^{+} and IgA^{+} PC in the spleens from Tec$^{-/-}$ mice (**Figure 6B**). Immunohistological analysis confirmed the increase in PC, which were present in the white pulp in the spleens of Tec$^{-/-}$ mice, but no histological abnormalities were observed.

Already at 10 weeks of age, IgG1 and IgG2c antibody levels in serum of Tec$^{-/-}$ mice were slightly increased compared to WT mice (**Figure S4**). In aged mice, we found higher serum levels of DNA-specific IgM antibodies in Tec$^{-/-}$ than in WT mice, and more Tec$^{-/-}$ mice showed DNA-specific IgG antibodies (5/17 Tec$^{-/-}$ versus 1/22 WT) (**Figure 6C**). In HEp2 reactivity assays, 13/21 sera from Tec$^{-/-}$ mice showed IgM staining, whereas IgM autoantibodies were not detectable in WT mice (**Figure 6D**). For IgG autoantibodies, we found staining in 2/16 WT mice, but in 15/21 Tec$^{-/-}$ mice (**Figure 6D**).

The presence of GC and IgG auto-antibodies suggested involvement of T-cells in the autoimmune phenotype in Tec$^{-/-}$ mice. However, we found no significant differences in memory phenotype of CD4^{+} T-cells (CD44^{hi}CD62L^{lo}), pro-inflammatory cytokine production by CD4^{+} T-cells (IFNγ, IL-4, IL-6), Tfh cell numbers (CXCR5^{hi}PD1^{hi}) or ICOS expression by Tfh cells (data not shown). Although kidneys from Tec$^{-/-}$ mice showed slightly more IgM and IgG immune depositions in the glomeruli, we did not find lymphocyte infiltrates in kidneys, lungs or salivary glands in these mice (data not shown).

Together, these data show that aging Tec$^{-/-}$ mice develop a spontaneous mild autoimmune phenotype.

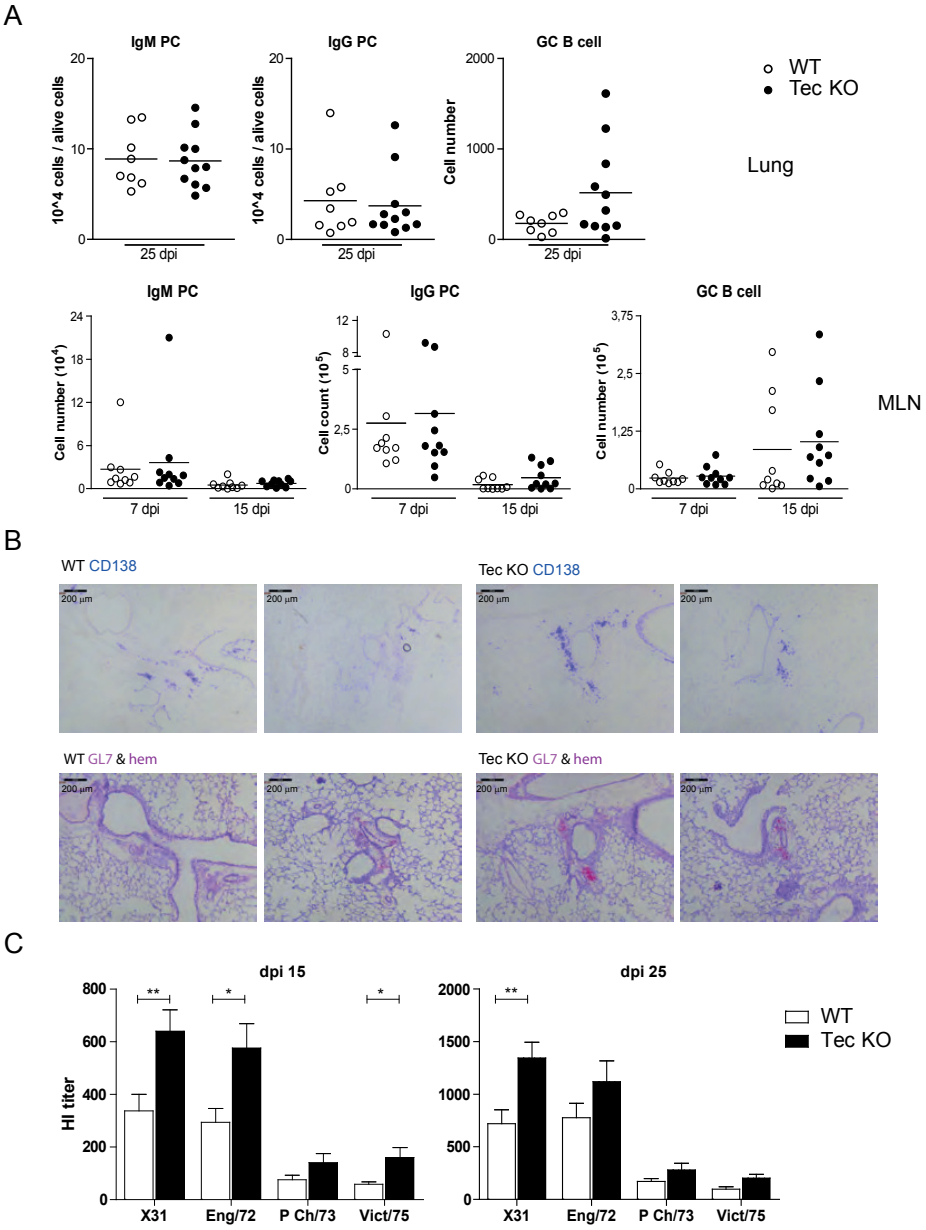


Figure 5. Enhanced humoral immunity in Tec$^{-/-}$ mice after influenza infection.
[A] IgM and IgG plasma cell (PC) and germinal center (GC) B cell numbers in lungs and mediastinal lymph nodes (MLN) on day 7, 15 and 25 after influenza infection in the indicated mouse groups. GC B cells were characterized as CD19^{+}IgD^{-}CD95^{+}, IgM^{+} PC as CD11b^{-}CD138^{+}IgM^{+} and IgG^{+} PC as CD11b^{-}CD138^{+}IgM^{-}IgG^{+}. [B] Representative immunohistochemical analysis of CD138^{+} plasma cells (blue) and GL7^{+} GC B cells (brown), counterstained with hematoxylin in lungs from WT and Tec$^{-/-}$ mice. [C] Haemagglutinin inhibition assay titers for X-31, Eng/72, P Ch/73 and Vict/75 virus-specific antibodies in serum of mice 15 or 25 days after infection. N= 8-10 per group; *p<0.05, **p<0.01 by Mann-Whitney U test.

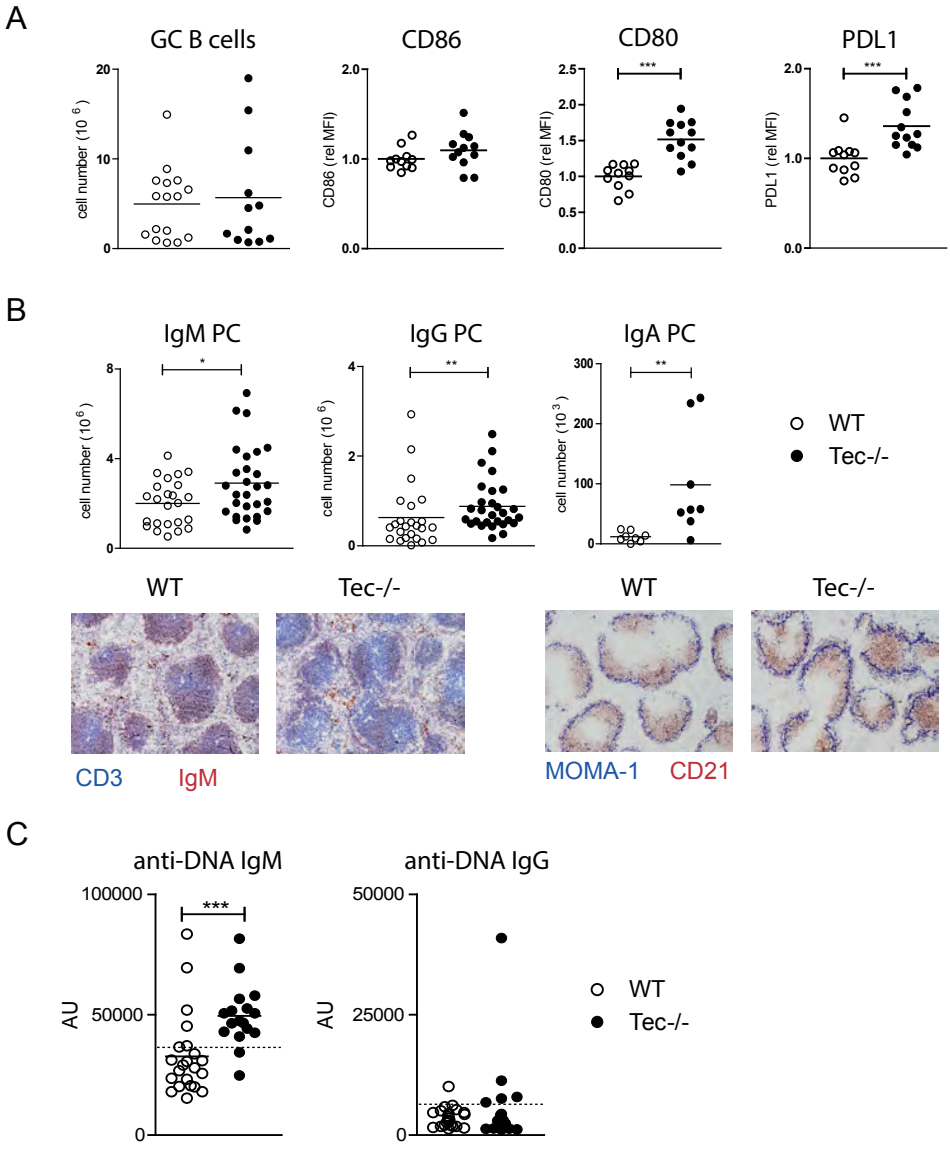
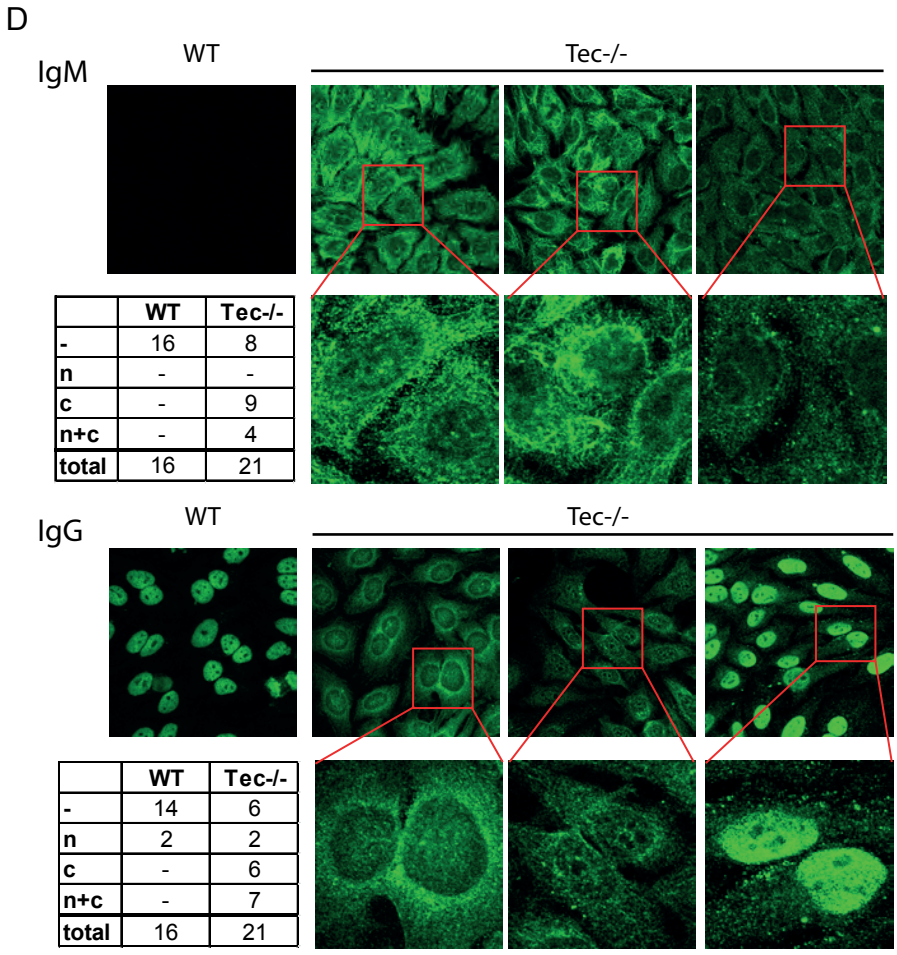


Figure 6. Mild autoimmune phenotype in Tec$^{-/-}$ mice.



	WT	Tec-/-
-	16	8
n	-	-
c	-	9
n+c	-	4
total	16	21

	WT	Tec-/-
-	14	6
n	2	2
c	-	6
n+c	-	7
total	16	21

Figure 6. Mild autoimmune phenotype in Tec$^{-/-}$ mice.
[A] Flow cytometric analysis of GC B cell numbers and CD86, CD80 and PDL-1 expression in spleens from WT and Tec$^{-/-}$ mice. [B] IgM, IgG and IgA plasma cell numbers in the spleen. Immunohistochemistry of spleens showing (left panel) CD3 in blue (T cells) and IgM in red (in follicles staining B cells and extra-follicular staining IgM plasma cells) and (right panel) MOMA1 in blue (macrophages) and CD21 in red (marginal zone B cells). [C] anti-DNA specific IgM and IgG antibodies in serum from 30-40-week-old WT and Tec$^{-/-}$ mice; each symbol represents an individual mouse; detection limit of assay is depicted by a dotted line. [D] Representative pictures of Hep-2 slides showing IgM and IgG auto-antibodies in serum from 30-40-week-old mice. n=nuclear; c=cytoplasmic. Data are derived from 7-28 mice analyzed per group. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U test.

TEC expression levels correlate with BTK expression levels in CLL B cells

To study the relationship between TEC and BTK in human B cells, we sorted three peripheral B cell subsets from healthy donors: naïve mature B cells ($CD19^+CD27^-IgD^+$), natural effector B cells ($CD19^+CD27^-IgM^+$) and IgG memory B cells ($CD19^+CD27^+IgG^+$), and measured TEC and BTK RNA expression by qPCR. We found expression of TEC and BTK in all three B cell subsets, whereby the lowest TEC expression was seen in natural effector B cells (**Figure 7A**).

Next, we analyzed B cells from chronic lymphatic leukemia (CLL) patients, as BTK plays a crucial role in survival of these cells (7). In CLL cells, which most closely resemble natural effector B cells, expression of BTK and TEC were correlated (**Figure 7B**). In most patients, the expression of BTK and TEC was higher than in natural effector B cells from healthy controls, particularly in unmutated CLL patients. Finally, we measured BTK and TEC RNA expression in several human tumor B cell lines and found that in part of the cell lines BTK and TEC expression correlated (high in RAJI and RS4;11 and low in LP1 and JVM-2; **Figure 7C**). However, in the BTK^{low} plasma cell leukemia U226 (23) substantial TEC expression was found and the BTK^{hi} precursor B cell acute lymphoblastic leukemia BV173 TEC expression was very low (**Figure 7C**).

These data indicate that BTK and TEC expression correlated in human CLL, but that in leukemia cell lines the two genes may be differentially expressed.

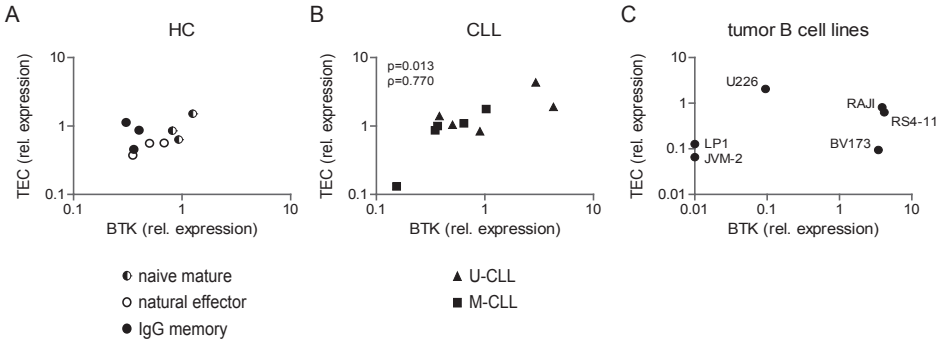


Figure 7. BTK and TEC expression are correlated in CLL B cells.
[A-C] RT-PCR analysis of the correlation between BTK and TEC RNA expression in human healthy naïve mature B cells ($CD19^+IgD^+CD27^-$), natural effector B cells ($CD19^+CD27^-IgM^+$) and IgG memory B cells ($CD19^+CD27^+IgG^+$) [A], unmutated and mutated CLL B cells (U-CLL and M-CLL) [B] and several tumor B cell lines [C]. Expression is relative to Ubiquitin C expression. Correlation was analyzed by Spearman test.

DISCUSSION

Previous studies have shown that Btk and Tec may have redundant roles in B cell activation, and that Tec can partially compensate for loss of Btk (5). Here we show that in pre-BCR signaling, but not in BCR signaling, lack of Tec could be completely compensated by Btk. We provide evidence that loss of Tec leads to increased B cell activation through enhanced activation of the Akt pathway, whereby increased phosphorylation is dependent on Btk kinase activity. Tec-deficient mature B cells show increased activation, proliferation and survival upon anti-IgM and anti-CD40 with rIL-4 stimulation in vitro. Moreover, Tec-deficient mice have enhanced humoral immunity upon immunization or viral infection and develop a mild autoimmune phenotype upon aging. Importantly, these data provide evidence supporting a role for Tec in limiting B cell activation through competition with Btk.

Tec is phosphorylated upon BCR stimulation, and in Jurkat T-cells increased Tec expression induced NFAT activation (6, 9). Surprisingly, we found that loss of Tec did not affect BCR-dependent PLCγ2 phosphorylation or Ca^{2+} influx, but increased BCR signaling through enhanced activation of the Akt pathway. This activation was dependent on Btk kinase activity, as ibrutinib strongly reduced phosphorylation of Akt and ribosomal S6. We found no increase in Btk protein levels, or in phosphorylation of Btk at the Y223 auto-phosphorylation site. Therefore, we propose a model in which competition for binding of downstream signaling molecules between Btk and Tec limits B cell activation in WT B cells. In line with this, we found that transgenic mice with B cell-specific overexpression of Btk protein (10) show increased phosphorylation of Btk at Y551 in the kinase domain and increased phosphorylation of S6 kinase (OBC and RWH, unpublished observation). The finding that the effects of loss of Tec were limited to the Akt/S6 pathway, and not observed in the PLCγ2/Ca^{2+} pathway, clearly indicate a differential role for Tec in these two pathways in which Btk acts as a key player.

It has been demonstrated that Btk can directly interact with and phosphorylate Akt in DT40 chicken B cells (8). However, phosphorylation of Akt has also been shown to depend on PI3K and Syk, but does not require Btk (24, 25). In addition, it was reported that in mature B cells Btk can enhance PI3K activity by bringing PIP5K to the plasma membrane and stimulating the production of the PI3K substrate PIP_2 by PIP5K (26). Through this indirect mechanism, Btk would have the capacity to enhance the activity of PI3K and thus phosphorylation of the downstream target Akt. However, this mechanism is not very likely, since the stimulation of PIP2 production by BTK was shown to be independent of its kinase activity (26) and we show that increased phosphorylation of Akt and S6 in $Tec^{-/-}$ cells is dependent on Btk kinase activity. This is in line with data showing downregulation of Akt phosphorylation upon ibrutinib treatment *in vitro* in several lymphoma and *in vivo* in chronic lymphatic leukemia (CLL) patients (27-31). Furthermore, we found that BTK and TEC RNA expression was increased in CLL B cells and that these levels are correlated. These data support the model that Tec and Btk compete for binding of Akt, but that activation of Akt is more efficient by Btk.

Model antigen immunizations showed that humoral immunity was enhanced in $Tec^{-/-}$ mice, but affinity maturation in the GC was not affected. In addition, we found no increase in long-lived PC in the BM, suggesting that selection into the memory compartment is normal. Increased activation of Akt promotes cell proliferation and survival (32). Indeed, we found that proliferation upon BCR stimulation was increased in $Tec^{-/-}$ B cells. Therefore, it is likely that more B cells, including autoreactive B cells, survive in Tec-deficient mice, leading to enhanced humoral immunity and development of a mild autoimmune phenotype. As our mice are fully Tec-deficient, we cannot exclude that the lack of Tec in other cells, such as T-cells, contributes to the development of autoimmunity. However, we did not find any apparent differences in $CD4^+$ T-cell cytokine production or Tfh differentiation. In addition, we found enhanced plasmablast formation in a pure B cell culture upon LPS and rIL-4 stimulation, but not with LPS alone. We therefore conclude that the phenotype observed in $Tec^{-/-}$ mice is likely driven in part by a B cell intrinsic increase in activation. The lack of a clear B cell phenotype in Tec-deficient mice on a C57BL/6 x 129 background in previous studies (5) may be attributed to the mixed background of those mice. We have previously shown that transgenic mice overexpressing human BTK only displayed an autoimmune phenotype when backcrossed onto the C57BL/6 background (10).

These data provide new insight in the role for Tec in B cell activation. We show that Tec is involved in the Akt pathway, where it may compete with Btk, but is not involved in activation of the PLCγ2 pathway. This is of particular interest in light of the current efforts to develop blocking agents that affect B cell activation in B cell malignancies. Loss or downregulation of Tec may contribute to the development of tumors through enhanced activation of Akt. Ibrutinib has shown excellent therapeutic efficacy in CLL and other B cell malignancies (33, 34). However, acquired resistance to ibrutinib, involving mutations of the ibrutinib binding site or in the *Plcg2* gene, has been reported (35, 36). Although second-generation Btk inhibitors with promising efficacy profiles have been developed (37, 38), it is expected that combination regimens will result in long-lasting responses as they may overcome resistance. Indeed combining ibrutinib with a PI3K or Akt pathway inhibitor was shown to have synergistic effects (36, 39-42).

In summary, our data showing that in primary mouse B cells Tec kinase acts to limit the activating capacity of Btk contributes to a better understanding of the differential roles of Btk and Tec. This is important for the development and improved application of BCR-signal blocking agents, in the treatment of B cell malignancies as well as autoimmune disorders.

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CONFLICT OF INTEREST DISCLOSURES

The authors declare no conflict of interest

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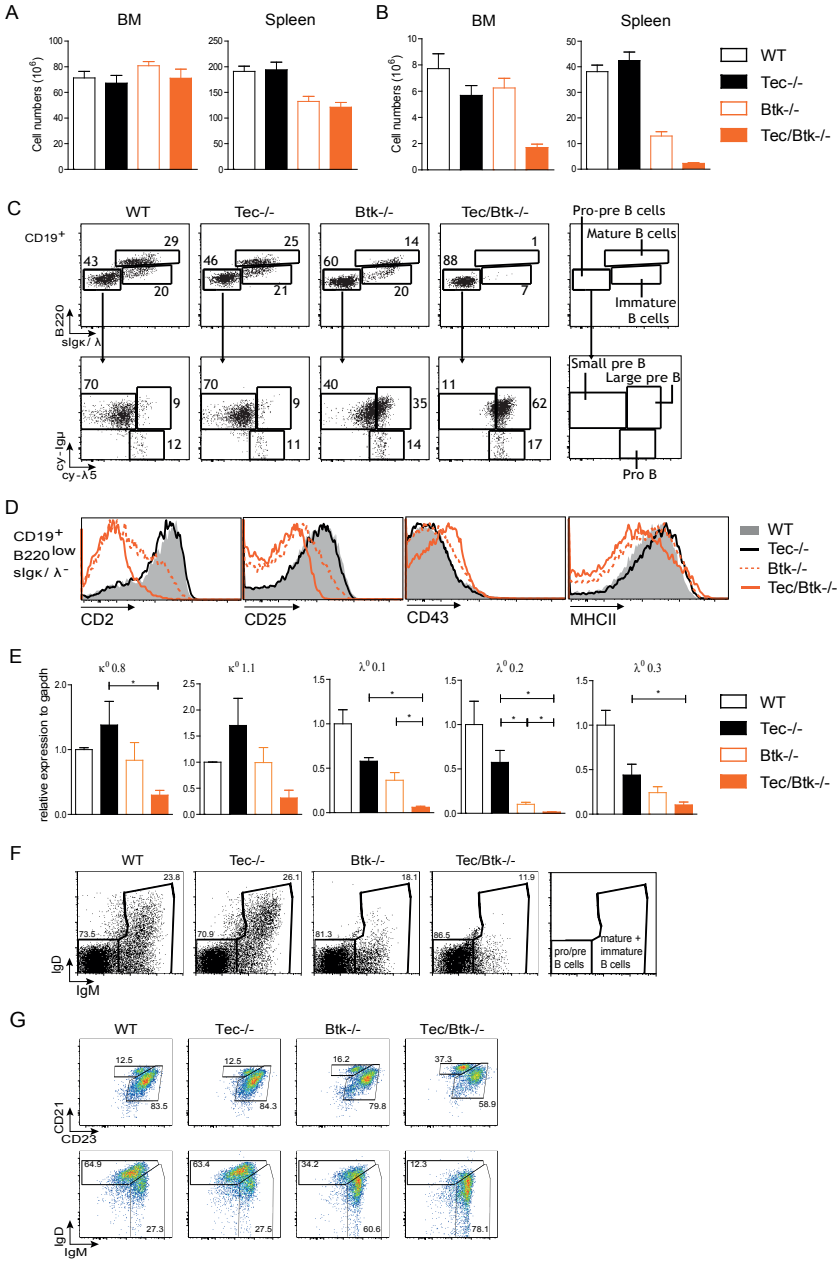
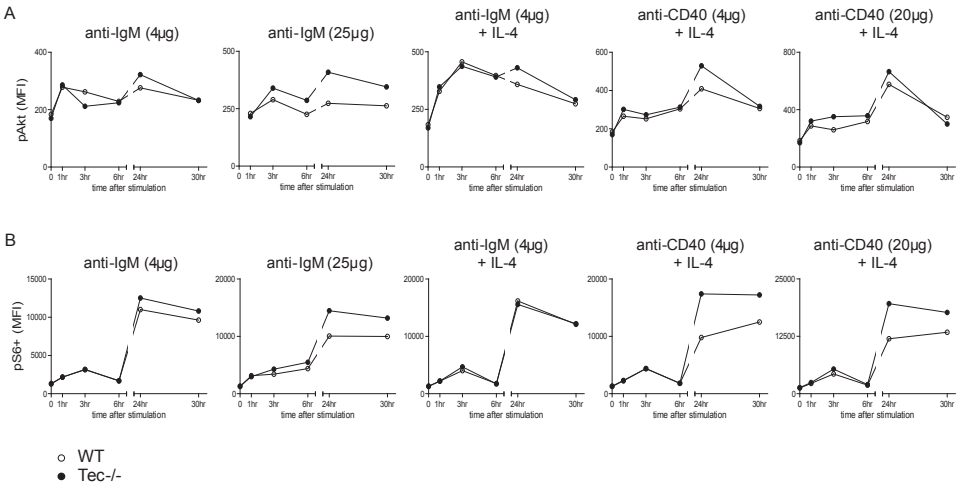
SUPPLEMENTAL MATERIAL

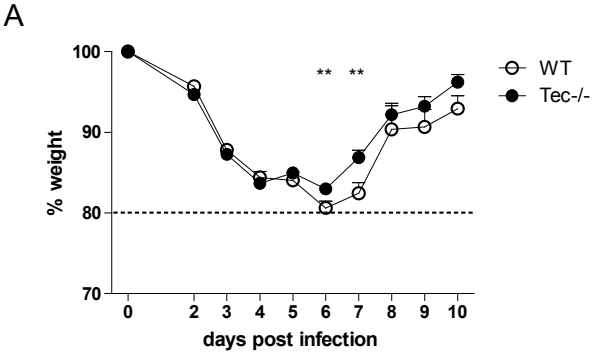


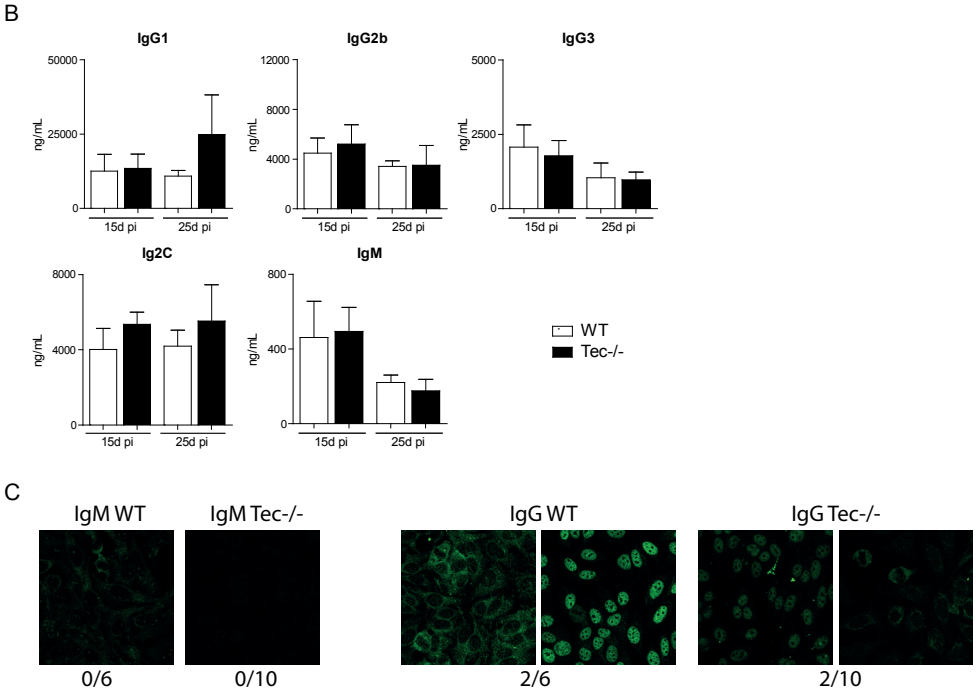
Supplementary figure 1. [A] Absolute cell numbers in BM (one femur) and spleen. [B] Absolute B-cell numbers in BM and spleen. [C] CD19+ B-cells were gated and analyzed for B220 and surface Ig L chain. B220low Igκ/λneg pro/pre-B-cells were gated and subsequently analyzed for cytoplasmic (cy) Igμ and λ5 surrogate light chain. [D] Surface expression levels of CD2, CD25, CD43 and MHCII on pre-B-cells from the indicated mice, shown as histogram overlays. [E] Quantitative RT-PCR measurement of the different germline transcripts in the Igκ and Igλ

light chain loci present in sorted B220low Igκ/λneg pro/pre-B-cells from the indicated mice. Data are presented as average values ± SEM. [F] Flow cytometric analysis of surface IgD and IgM expression in CD19+ B-lineage cells upon 5 days of culture of total BM cells with rIL-7 and 2 subsequent days in the absence of IL-7. [G] Analysis of Follicular B-cells (CD21+CD23+) and IgM/IgD expression in gated CD19+B220+ cell fractions in spleens of indicated mice. N=4 per group; *p<0.05 by Mann-Whitney U test.

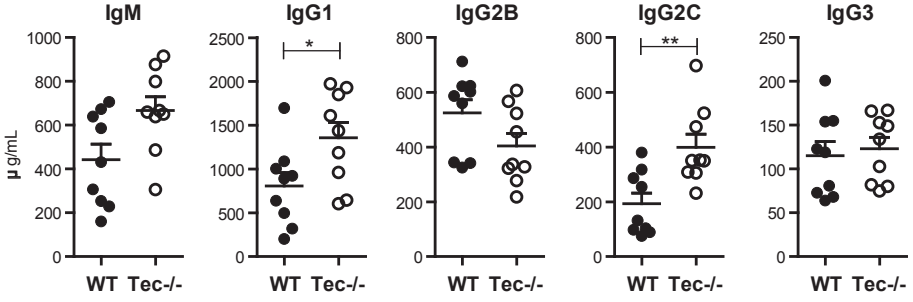


Supplementary figure 2. [A-B] Flow cytometry analysis of phospho-Akt [A] and phospho-S6 [B] expression in WT and Tec$^{-/-}$ B cells at several time-points after in vitro stimulation with indicated stimuli.

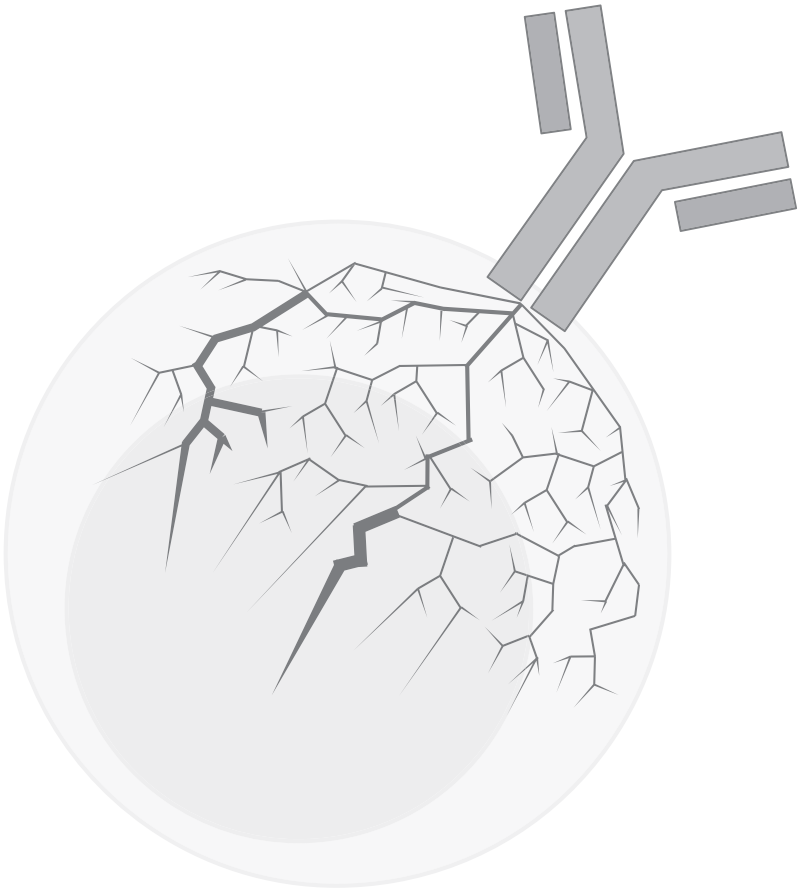




Supplementary figure 3. [A] Weight loss upon X31 influenza infection of WT and Tec$^{-/-}$ mice. [B] Total Ig serum levels of influenza infected WT and Tec$^{-/-}$ mice 15 and 25 days post infection (dpi). [C] HEp2 reactivity of IgM or IgG antibodies in BAL fluid of WT and Tec-/- mice 25 dpi. Shown are representative examples from WT n=6; Tec$^{-/-}$ n=10; **p<0.01 by Mann-Whitney U test.



Supplementary figure 4. Ig serum levels in 8-10 week old mice. *p<0.05 and **p<0.01 by Mann-Whitney U test.



CHAPTER 5

Bruton's tyrosine kinase inhibition induces rewiring of proximal and distal B cell receptor signaling in mice

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Simar Pal Singh[#], Jennifer A.C. van Hulst, Rudi W. Hendriks
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Under revisionn

ABSTRACT

Bruton′s tyrosine kinase (Btk) is a crucial signaling molecule in B-cell receptor (BCR) signaling and a key regulator of B cell differentiation and function. Btk inhibition has shown impressive clinical efficacy in various B cell malignancies. However, it remains unknown whether inhibition additionally induces changes in BCR signaling due to feedback mechanisms, a phenomenon referred to as BCR rewiring. In this report, we studied the impact of Btk activity on major components of the BCR signaling pathway in mice. As expected, NF-κB and Akt/S6 signaling was decreased in Btk-deficient B cells. Unexpectedly, phosphorylation of several proximal signaling molecules including CD79a, Syk and PI3K, as well as the key Btk-effector PLCγ2 and the more downstream kinase Erk were significantly increased. This pattern of BCR rewiring was essentially opposite in B cells from transgenic mice overexpressing Btk. Importantly, prolonged Btk inhibitor treatment of wild-type mice or mice engrafted with leukemic B cells also resulted in increased phosho-CD79a and phospho-PLCγ2 in B cells. Our findings show that Btk enzymatic function determines phosphorylation of proximal and distal BCR signaling molecules in B cells. We conclude that Btk inhibitor treatment results in rewiring of BCR signaling, which may affect both malignant and healthy B cells.

INTRODUCTION

Bruton's tyrosine kinase (BTK) is a critical component of the B-cell receptor (BCR) signaling pathway that is crucial for development, activation and differentiation of B cells, and supports B-cell survival and progression of disease in various B-cell malignancies[1, 2] . In chronic lymphocytic leukemia (CLL) and mantle-cell lymphoma (MCL) constitutive BCR signaling promotes clonal activation and accumulation of malignant B cells[3]. Targeting BTK activity with ibrutinib, a BTK inhibitor that covalently binds Cys481 in its kinase domain, has demonstrated impressive clinical efficacy in CLL and MCL[4, 5]. Second-generation BTK inhibitors, such as acalabrutinib, show superior efficacy with limited toxicity and off-target side effects[6]. Recently, a study showed that BTK inhibitors can effectively treat other B-cell-mediated diseases as treatment with evobrutinib decreased the formation of active lesions in multiple sclerosis patients[7].

Functional BTK protein is essential for B cells, as loss-of-function mutations in the *BTK* gene lead to the inherited immunodeficiency disease X-linked agammaglobulinemia (XLA). In these patients B-cell development is arrested at the pre-B cell stage, leading to an almost complete absence of peripheral B cells and circulating antibodies[8, 9]. Although in Btk-deficient mice peripheral B-cell maturation and activation is significantly affected[10-12], the B-cell developmental block in the bone marrow is mild. However, combined deficiency of Btk and its family member Tec or the B-cell linker molecule SLP-65 results in a severe block in B cell development[13, 14]. Using an inducible knock-out model it was recently shown that Btk is not required for maintenance of the existing follicular B cell pool, although B cells did not proliferate upon anti-IgM stimulation[15].

Treatment of CLL and MCL patients with ibrutinib or acalabrutinib strongly decreases disease activity[3-6, 16], but in some patients prolonged exposure to BTK inhibitors is associated with development of resistance. Therapy resistance may originate either from genetic mutations in signaling molecules, acquired changes in BCR signaling or upregulation of compensatory pathways that provide proliferation or survival signals. In this context, patients relapsing upon inhibition therapy showed mutation of the BTK inhibitor binding site Cys481, or a gain-of-function mutation in the BTK-downstream effector PLCγ2[17]. Acquired mutations in various other genes, such as *MYD88*, *TP53*, *SF3B1*, and *CARD11*, were also identified. Inhibition of driver oncogene function is associated with rapid rewiring of signal transduction pathways in many tumor types[18]. This phenomenon mirrors regular feedback mechanisms in non-malignant cells, whereby kinase activation engages homeostatic mechanisms that inactivate upstream signaling[19]. For example, a significant reduction in total BTK protein expression was observed in CLL cells from ibrutinib-treated patients or in diffuse large B cell lymphoma lines chronically exposed to BTK-inhibition[17]. Conversely, BTK protein levels are upregulated upon stimulation of mature cells through the BCR, CD40 or Toll-like receptors[20, 21].

It remains unknown whether the observed rewiring reflects selection of tumor cells from a heterogeneous population or inhibitor-induced molecular changes, for example by epigenetic modifications. Indeed, ibrutinib treatment was associated with substantial genome-

wide changes in chromatin accessibility and histone modifications[22]. Furthermore, the effects of Btk-deficiency or long-term kinase inhibition on BCR signaling of non-leukemic mature B cells remain poorly characterized.

In this report, we aimed to explore the consequences of *in vivo* Btk-kinase inhibition on the phosphorylation status of key BCR signaling molecules in splenic B cells in mice. Remarkably, Btk-deficiency and prolonged *in vivo* acalabrutinib treatment was associated with increased proximal BCR signaling and divergent downstream signaling in follicular B cells, but not in transitional and marginal zone (MZ) B cells. These findings provide evidence that BTK inhibition induces complex rewiring of BCR signaling.

RESULTS

Btk-deficient B cells show aberrant anti-IgM-induced activation of distal BCR signaling proteins

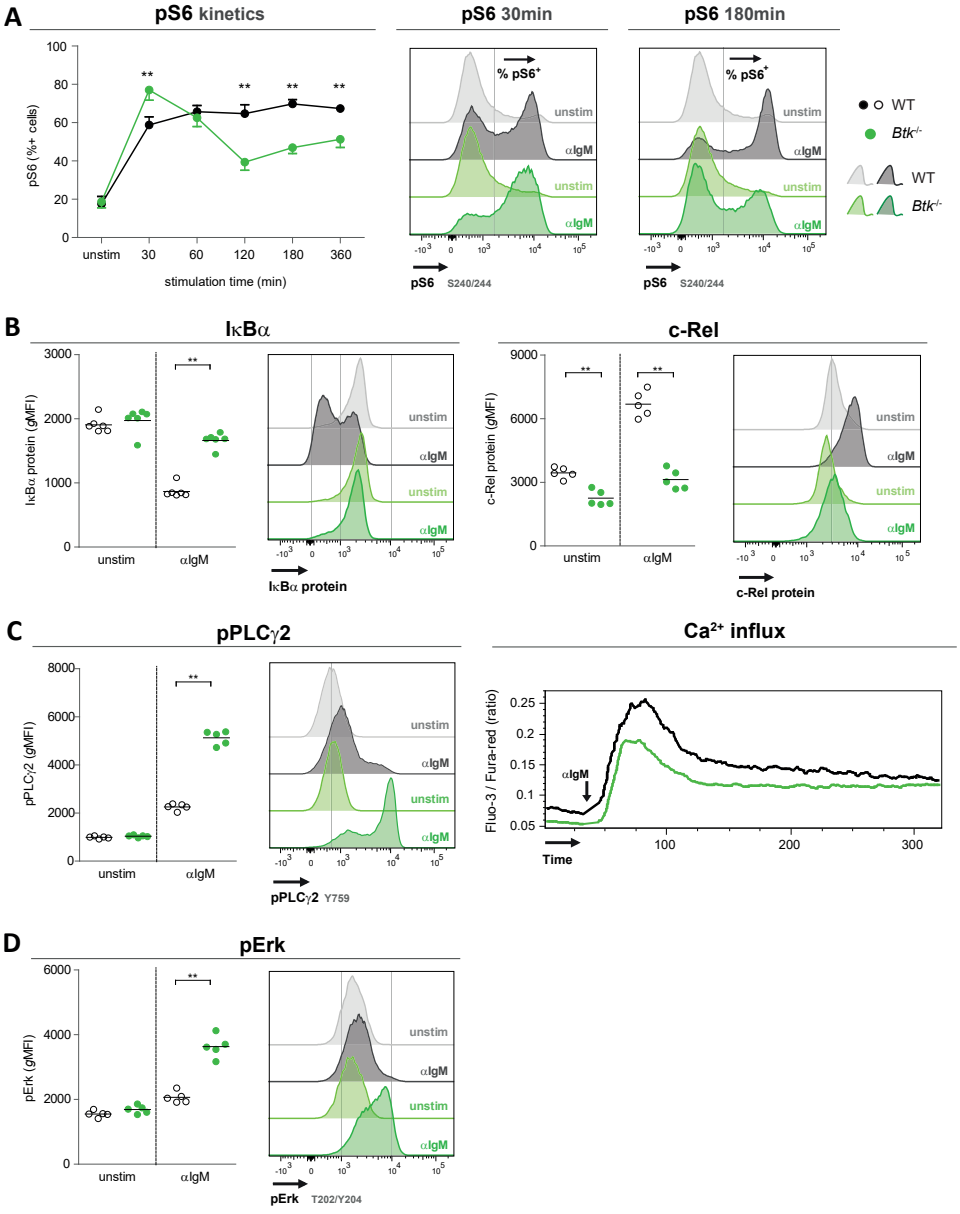
First, we aimed to confirm that Btk is crucial for BCR signaling and compared unstimulated and anti-IgM-stimulated splenic $B220^{+}CD21^{-}CD23^{+}$ follicular B cells from Btk-deficient (*Btk*$^{-/-}$) and wild-type (WT) mice (for gating strategy: see **Suppl. Fig. 1**). The main BCR downstream pathways, Akt, NF-κB, PLCγ2 and MAPK, were analyzed by flow cytometry.

We first quantified S240/244 phosphorylation of the ribosomal protein S6 as a measure of activation of the Akt pathway. No differences were detected between unstimulated *Btk*$^{-/-}$ and WT follicular B cells, but *Btk*$^{-/-}$ follicular B cells displayed increased S6 phosphorylation after 30 min and decreased S6 phosphorylation after 120-360 min of anti-IgM stimulation, compared with WT B cells (**Figure 1A**). Accordingly, *in vitro* culture of splenic B cells with acalabrutinib showed that S6 phosphorylation at 120-180 min was highly dependent on Btk kinase activity (**Suppl. Fig. 2A**).

As expected[23, 24], anti-IgM-driven NF-κB activation was decreased in *Btk*$^{-/-}$ follicular B cells, as evidenced by reduced degradation of the inhibitory protein IκBα and reduced expression of c-Rel protein (**Figure 1B**). Although Ca^{2+} influx was reduced upon anti-IgM stimulation, phosphorylation of the key Btk-effector PLCγ2 (Y759) was increased in *Btk*$^{-/-}$ B cells, compared with WT B cells (**Figure 1C**). Strikingly, anti-IgM-induced pErk (T202/Y204) was significantly higher in *Btk*$^{-/-}$ B cells than in WT B cells (**Figure 1D**).

In line with the strongly reduced activation of the Akt/S6 and NF-κB pathways, the induction of the anti-apoptotic proteins Mcl-1 and Bcl-XL by these two pathways respectively[25, 26], was significantly impaired in *Btk*$^{-/-}$ follicular B cells upon 22 h stimulation with anti-IgM (**Figure 1E**).

Taken together, these findings indicate that Btk-deficiency differentially affected the main distal BCR signaling pathways. Whereas NF-κB and Akt signaling was hampered, pErk and pPLCγ2 expression was remarkably increased in anti-IgM-stimulated *Btk*$^{-/-}$ B cells.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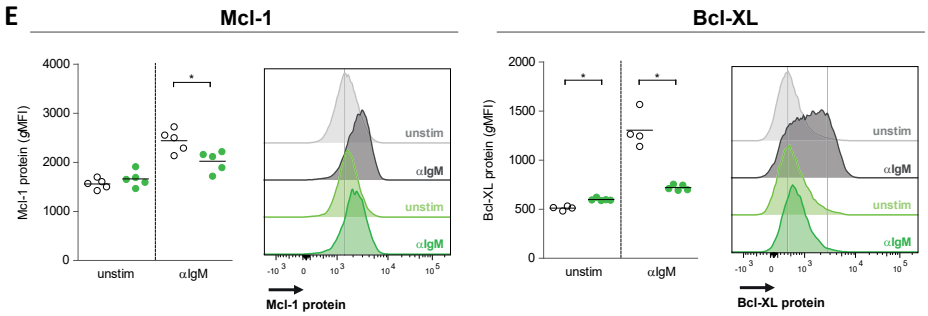


Figure 1. BCR signaling is rewired in Btk-deficient follicular B cells.
(A) Ribosomal protein S6 phosphorylation kinetics represented by percentage of positive cells in follicular splenic B cells after 30, 60, 120, 180 and 360 min incubation with anti-IgM stimulation. (B) Quantification by geometric mean fluorescence intensity (*g*MFI) for IκBα protein degradation after 1 h and c-Rel protein after 22 h of anti-IgM stimulation of follicular splenic B cells. (C) *g*MFI for pPLCγ2 after 5 min of anti-IgM stimulation (*left*) and Ca^{2+} influx measurement (*right*) in $B220^{+}$ B cells upon stimulation with 25 µg anti-IgM in WT and *Btk*$^{-/-}$ mice, representative for five mice analyzed. (D) *g*MFI for Erk phosphorylation after 5 min of anti-IgM stimulation. (E) Quantification by *g*MFI for Mcl-1 and Bcl-XL after 22 h of anti-IgM stimulation. Representative histogram overlays are depicted on the right of all panels. Symbols represent individual mice and lines indicate mean values. Graphs represent three to four individual experiments, each with 5-6 mice per group; Btk-deficient (*Btk*$^{-/-}$) mice and wild-type (WT) controls were 8-12 weeks old; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

Btk-deficient B cells show increased anti-IgM-induced phosphorylation of proximal BCR signaling molecules

To more comprehensively study the effects of Btk-deficiency, we investigated phosphorylation of key BCR proximal signaling molecules in splenic follicular B cells. Upon BCR engagement, the signaling cascade starts with phosphorylation of a Src-family kinase, most probably Lyn. Both at the activating Y424 and the inhibitory Y507 site[27], Src phosphorylation was slightly reduced in unstimulated *Btk*$^{-/-}$ follicular B cells. Very surprisingly, phosphorylation at both pSrc sites was significantly increased upon anti-IgM stimulation in *Btk*$^{-/-}$ follicular B cells compared with WT controls (**Figure 2A; Suppl. Fig. 2B**).

While basal pCD79a (Y182) was only slightly increased in *Btk*$^{-/-}$ follicular B cells, the level of anti-IgM-induced pCD79a was remarkably higher in *Btk*$^{-/-}$ B cells than in WT B cells (**Figure 2B**). This phenomenon was not due to high CD79a protein expression in *Btk*$^{-/-}$ follicular B cells. On the contrary, in *Btk*$^{-/-}$ follicular B cells CD79a expression was somewhat lower, yet CD79b expression was higher than in WT B cells (**Suppl. Fig. 3A**).

Like pCD79a, also anti-IgM-induced phosphorylation of Syk (Y348), SLP-65 (Y84) and the p85 PI3K subunit (Y458) was significantly increased in *Btk*$^{-/-}$ follicular B cells (**Figure 2C**). Protein levels of Syk and PLCγ2, however, were decreased or unchanged in *Btk*$^{-/-}$ follicular B cells (**Suppl. Fig. 3A**). Unstimulated *Btk*$^{-/-}$ follicular B cells displayed reduced phosphorylation of SHP-1 at Y564, a phosphatase with the capacity to dephosphorylate BCR downstream targets and is recruited by inhibitory receptors such as CD5, CD22 and

Siglec-G[28-30]. Upon anti-IgM stimulation, however, pSHP-1 was similar in *Btk*$^{-/-}$ and WT follicular B cells (**Figure 2D**), indicating that the increased anti-IgM-induced phosphorylation of proximal BCR signaling molecules in *Btk*$^{-/-}$ B cells cannot be explained by reduced SHP-1 phosphatase activity.

In summary, anti-IgM-induced phosphorylation of key proximal BCR signaling molecules was significantly higher in Btk-deficient than in WT follicular B cells, indicating that the absence of Btk is associated with rewiring of BCR signaling.

Limited BCR rewiring in Btk-deficient splenic transitional and marginal zone B cells

As we previously found differences in basal PLCγ2 phosphorylation across transitional, follicular and MZ B cells in the spleen of WT mice[31], we next investigated basal phosphorylation and BCR signaling responsiveness in WT mice in these three splenic B cell subpopulations. Compared to follicular B cells, basal phosphorylation of CD79a, Syk, PLCγ2, Erk and S6 was similar or only slightly higher in T1 transitional B cells (**Figure 3A;** see for gating: **Suppl. Fig. 1**). However, upon BCR stimulation transitional T1 B cells showed an enhanced responsiveness to BCR engagement compared to follicular B cells for CD79a, Syk, PLCγ2 and Erk, whereas anti-IgM-induced pS6 was reduced (**Figure 3A**). In contrast, in MZ B cell basal phosphorylation of Syk, PLCγ2, Erk and S6 was significantly increased, compared to follicular B cells. Upon BCR stimulation, MZ B cells exhibited increased phosphorylation of CD79a, Syk, PLCγ2 and Erk when compared with follicular B cells (**Figure 3A**). However, the proportions of pS6-positive cells following anti-IgM stimulation were comparable between MZ and follicular B cells (**Figure 3A**). Essentially, these findings imply that - except for pS6 - T1 transitional B cells are more responsive to BCR signaling and that MZ B cells show a pre-activated basal signaling, when compared to follicular B cells.

Next, we investigated whether BCR signaling was altered in *Btk*$^{-/-}$ T1 and MZ B cells. Whereas we observed limited differences between *Btk*$^{-/-}$ and WT T1 B cells in basal phosphorylation of CD79a, Syk and Erk, phosphorylation of Src-Y424, Src-Y507, PLCγ2 and S6 was significantly lower in *Btk*$^{-/-}$ T1 B cells (**Figure 3B**). BCR stimulation of *Btk*$^{-/-}$ T1 B cells resulted in higher pCD79a, similar pSrc-Y424, pSrc-Y507, pSyk, pPLCγ2 and pErk, and reduced pS6, compared with WT T1 B cells.

In *Btk*$^{-/-}$ MZ B cells basal phosphorylation of all signaling molecules was significantly lower than in WT MZ B cells, except for Syk (**Figure 3B**). Differences were generally moderate, but considerable for pSrc-Y424, pSrc-Y507 and pS6. BCR stimulation of *Btk*$^{-/-}$ MZ B cells resulted in significantly higher pCD79a, similar pSrc-Y424, pSrc-Y507, pSyk, pPLCγ2 and pErk, and reduced pS6, compared with WT MZ B cells, paralleling our findings in T1 B cells.

Taken together, we observed complex BCR signaling differences across different splenic B cell subpopulations. Compared with Btk-deficient follicular B cells both splenic transitional and MZ B cells showed only limited BCR rewiring, characterized by modestly increased pCD79a and decreased pS6.

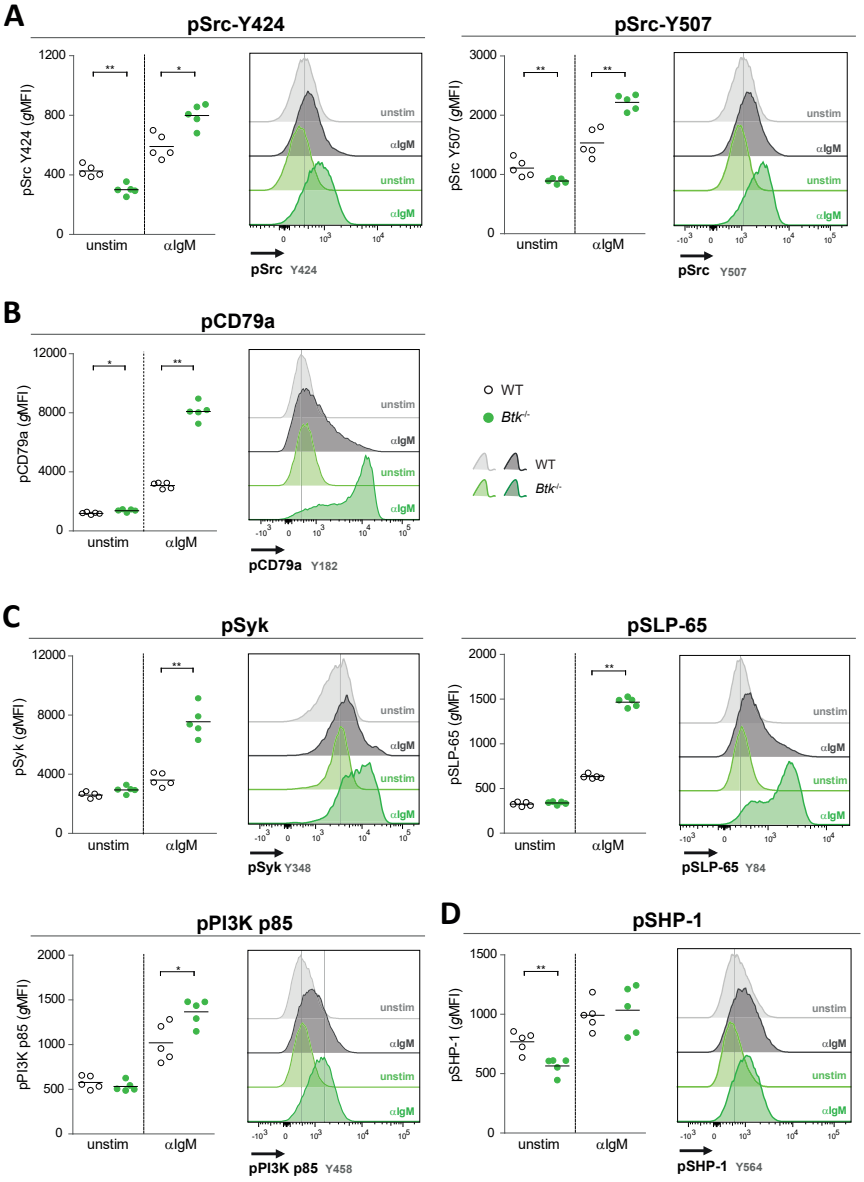


Figure 2. Proximal BCR signaling is strongly increased in Btk-deficient follicular B cells.
(A) Quantification by geometric mean fluorescence intensity (*g*MFI) for either 5 min for pSrc-Y424 or pSrc-Y507 of anti-IgM stimulation and gated for follicular B cells. (B) Quantification *g*MFI for pCD79a after 5 min of anti-IgM stimulation (left) and expression of CD79a surface protein (*middle*) and CD79b total protein (*right*) in Btk$^{-/-}$ follicular B cells compared to WT counterparts. (C-D) Quantification (*g*MFI) after 5 min of anti-IgM stimulation for (C) pSyk and pSLP-65, 1 min for pPI3K p85 and (D) 3 min for pSHP-1 in follicular B cells. Representative histogram overlays are depicted on the right. Symbols represent individual mice and lines indicate mean values. Graphs represent three to four individual experiments, each with 4-6 mice per group; Btk-deficient (*Btk*$^{-/-}$) mice and wild-type (WT) controls were 8-12 weeks old; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

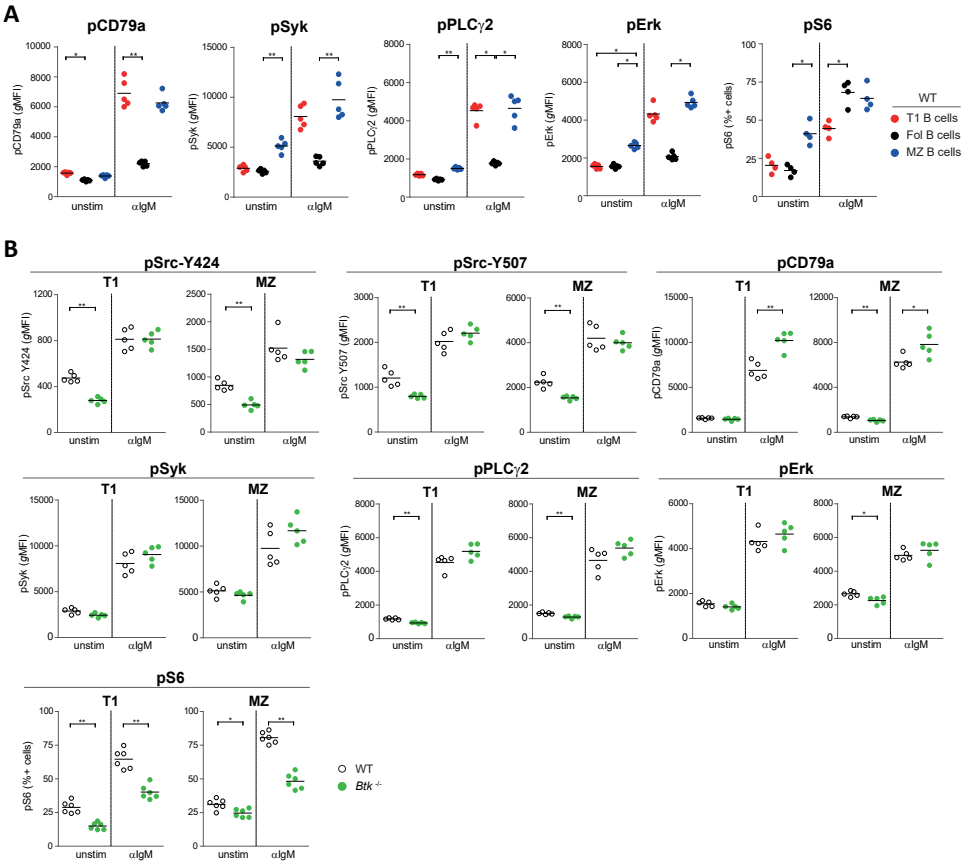


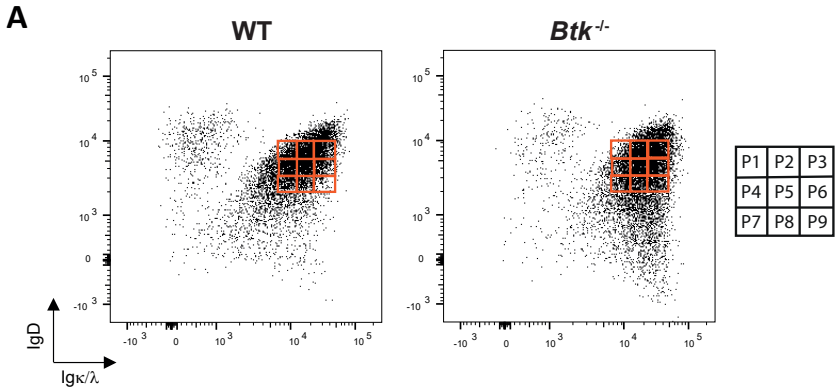
Figure 3. Limited rewiring of BCR signaling in Btk-deficient marginal zone and transitional type 1 B cells. (A) Quantification by geometric mean fluorescence intensity (*g*MFI) after 5 min of anti-IgM stimulation for pCD79a, pSyk, pPLCγ2 and pErk or by percentage of positive cells after 3 h stimulation with anti-IgM for pS6 in transitional type 1 (T1), marginal zone (MZ) and follicular (Fol) B cells from wild-type (WT) mice. (B) Quantification by *g*MFI after 5 min of anti-IgM stimulation for pSrc-Y424, pSrc-Y507, pCD79a, pSyk, pPLCγ2 and pErk or by percentage of positive cells after 3 h stimulation with anti-IgM for pS6 in T1 and MZ B cells from the indicated mice. Symbols represent individual mice and lines indicate mean values. Graphs represent two to three individual experiments, each with 4-6 mice per group; Btk-deficient (*Btk*$^{-/-}$) mice and wild-type (WT) controls were 8-12 weeks old; *p<0.05, **p<0.01 by Kruskal-Wallis with Dunn's multiple comparison posthoc test (A) or Mann-Whitney *U* test (B).

Enhanced upstream BCR signaling in Btk-deficient B cells is independent of differences in IgM/IgD profile between *Btk*$^{-/-}$ and WT B cells

As Btk-deficient B cells have increased IgM expression and fail to fully upregulate IgD[10, 32, 33], we investigated whether an altered IgM/IgD expression profile contributed to the strong increase in proximal signaling in *Btk*$^{-/-}$ B cells. To compare follicular B cells with similar BCR expression levels between *Btk*$^{-/-}$ and WT mice, we gated for nine different populations (P1-9) with variable expression for IgD and Igκ/λ light chain (**Figure 4A**).

All nine gated populations showed higher CD79a phosphorylation in anti-IgM-stimulated Btk$^{-/-}$ B cells than in WT B cells, showing that increased pCD79a in *Btk*$^{-/-}$ follicular B cells is independent of the IgM/IgD profile (**Figure 4B**). Increasing Igκ/λ expression, while retaining similar IgD expression resulted in increased pCD79a signaling of WT B cells and even more so in *Btk*$^{-/-}$ follicular B cells (most evident in the P7-P8-P9 comparison). The IgM/IgD expression profile (with similar total Ig surface expression, inferred from Igκ/λ signals) appeared to control the magnitude of the pCD79a response in *Btk*$^{-/-}$ B cells: pCD79a expression further increased in *Btk*$^{-/-}$ but not in WT follicular B cells with lower IgD expression values, e.g. in a P2-P5-P8 comparison (**Figure 4B,C**). Similar results were found for pPLCγ2 (**Figure 4D,E**). Lastly, we stimulated B cells with F(ab')$_2$-Igκ light chain fragments to check whether signaling is increased in *Btk*$^{-/-}$ B cells when stimulating both the IgD and IgM BCR and found that this was indeed the case (**Figure 4F**).

Collectively, these data show that IgM and IgD expression differences between *Btk*$^{-/-}$ and WT B cells cannot explain the increased BCR responsiveness and rewiring in *Btk*$^{-/-}$ follicular B cells.



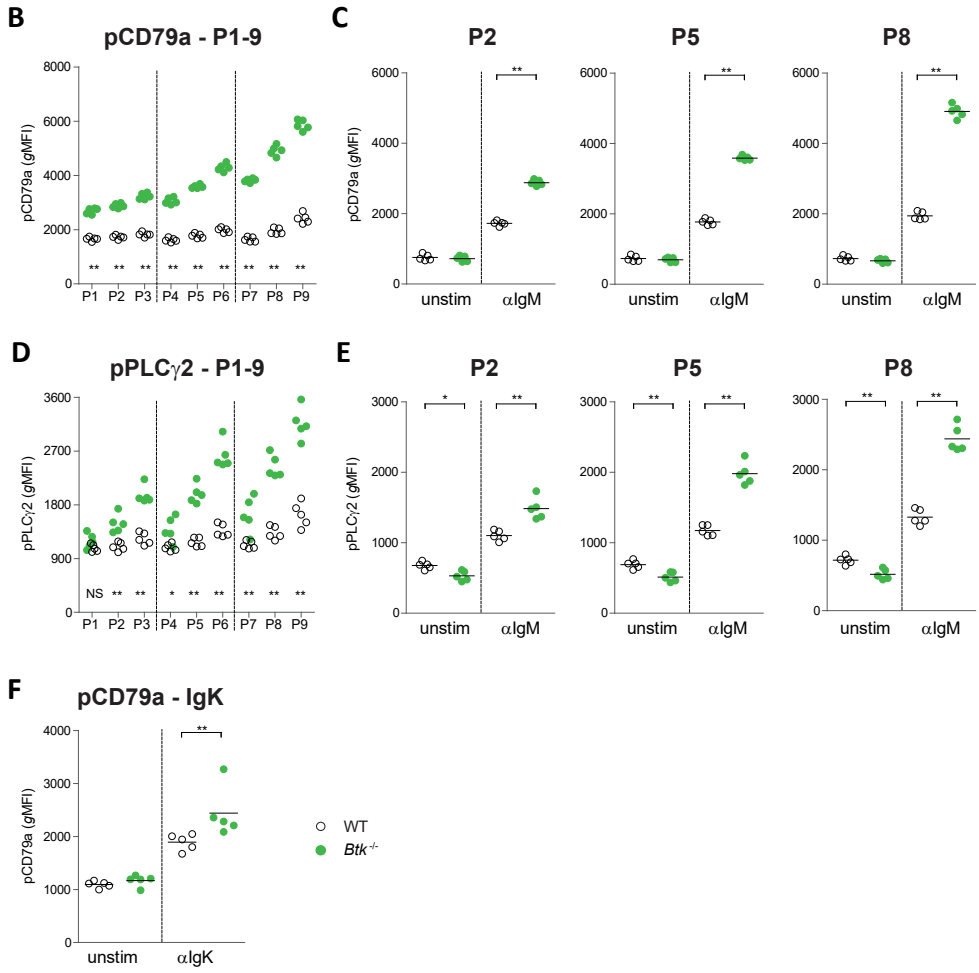


Figure 4. Enhanced upstream BCR signaling in Btk-deficient B cells is independent of differences in IgM/IgD profile.

(A) Gating strategy for nine populations (P1-P9) differing in BCR expression based on IgD and light chain expression levels. The expression of IgD and the light chain measured by geometric mean fluorescence intensity (*g*MFI) was similar between Btk-deficient (*Btk*$^{-/-}$) and wild-type (WT) follicular B cells in these 9 gated populations. (B-C) Quantification by *g*MFI after 5 min of anti-IgM stimulation for pCD79a (B-C) and pPLCγ2 (D-E), showing P1-9 for both Btk$^{-/-}$ and WT follicular B cells in one graph (B,D) or specifically P2, P5 and P8 including unstimulated controls (C,E). (F) Quantification by *g*MFI after 5 min of anti-κ light chain (anti-IgK) stimulation for pCD79a. Graphs represent two to three individual experiments, each with five mice per group; Symbols represent individual mice and lines indicate mean values; *Btk*$^{-/-}$ and WT mice were 8-12 weeks of age; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

Proximal BCR signaling is enhanced in follicular B cells overexpressing Btk

Given that Btk-deficiency strongly affected phosphorylation of both proximal and distal BCR signaling molecules, we investigated whether overexpression of Btk protein had opposite effects. Indeed, when we analyzed B cells from CD19-hBtk transgenic mice with B-cell-specific overexpression of human BTK, we found that CD79a phosphorylation was significantly decreased both in unstimulated and anti-IgM stimulated follicular B cells (**Figure 5A**). Also, a trend of decreased BCR responsiveness to anti-IgM stimulation was observed by calculating the ratio geometric mean fluorescence intensity (*g*MFI) between stimulated and unstimulated follicular B cells for pPLCγ2 and pBtk, although this was not significant (**Figure 5B,C**). This could not be explained by decreased protein levels, because expression of CD79a was only slightly reduced and levels of other BCR signaling protein including pPLCγ2 were essentially unchanged compared to WT B cells (**Suppl. Fig. 3B**). Also, CD19-hBtk follicular B cells showed increased pErk in unstimulated and anti-IgM stimulated B cells, whereby BCR responsiveness was comparable to WT counterparts (**Figure 5D**).

Other distal signaling pathways did show the expected pattern: in CD19-hBtk B cells anti-IgM-induced NF-κB activation was enhanced, since IκBα degradation was increased (**Figure 5E**). As previously reported[34], anti-IgM-induced activation of the Akt/S6 signaling pathway was enhanced (**Figure 5F**).

Taken together, these findings show that increased Btk expression and Btk-deficiency had essentially opposite effects on BCR signaling, perhaps with the exception of Erk phosphorylation.

Single-dose *in vivo* Btk inhibition mainly affects distal BCR signaling molecules

Next, we aimed to study whether inhibition of Btk kinase activity would cause similar rewiring of BCR signaling as observed in *Btk*$^{-/-}$ follicular B cells. We treated WT mice *in vivo* with acalabrutinib or vehicle control by oral gavage. After three hours of treatment, anti-IgM-induced calcium signaling was strongly decreased in follicular B cells from acalabrutinib-treated mice compared with these cells from vehicle-treated control mice (**Figure 6A**). The proximal signaling molecules analyzed essentially showed somewhat decreased basal signaling in follicular B cells after acalabrutinib treatment (**Figure 6B**). Upon anti-IgM stimulation phosphorylation of CD79a was significantly decreased in follicular B cells from acalabrutinib-treated mice, but other proximal signaling molecules retained similar levels of anti-IgM-induced phosphorylation, compared follicular B cells from vehicle-treated mice (**Figure 6B**).

By contrast, analysis of distal BCR signaling molecules revealed that Erk and pS6 phosphorylation and IκBα degradation were significantly reduced in anti-IgM stimulated B cells from acalabrutinib-treated mice, compared with control mice (**Figure 6C**).

In conclusion, in *vivo* acalabrutinib administration strongly affected distal mediators of the BCR signaling cascade within three hours, as expected. Moreover, Btk inhibition had subtle upstream effects, particularly resulting in reduced phosphorylation of the very proximal CD79a BCR signaling molecule.

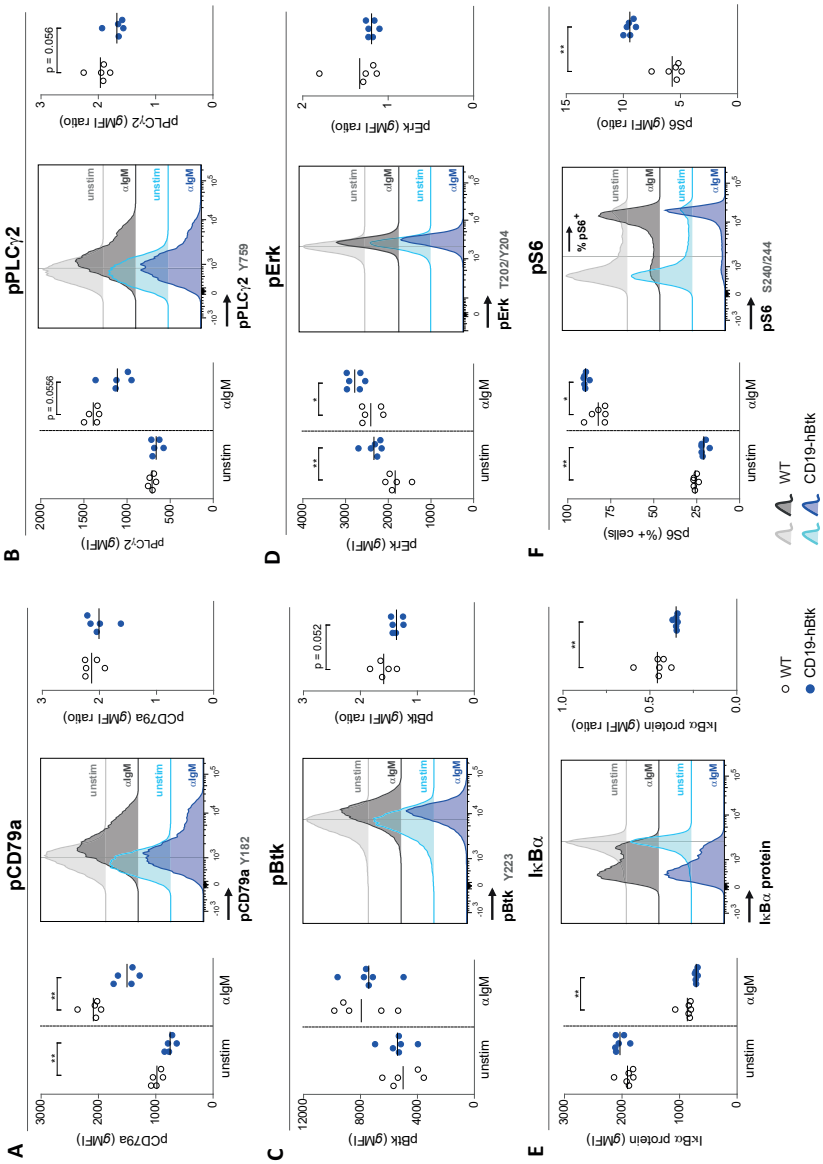


Figure 5. Decreased proximal signaling in CD19-hBtk follicular B cells.

(A-F) Splenic cells of CD19-hBtk Btk-overexpressing mice and wild-type controls were stimulated for either 5 min for pCD79a (A), pPLCγ2 (B), pBtk (C) and pErk (D), 1 h for IκBα (E) or 3 h for pS6 (F) and gated for follicular B cells after the indicated *in vitro* stimulation with anti-IgM. All panels show quantification by geometric mean fluorescence intensity (*g*MFI)(A-E) or by percentage of positive cells (pS6; F)(*left*), a histogram overlay of representative samples (*middle*) and stimulation ratio calculated by fold increase in anti-IgM stimulated *g*MFI compared to unstimulated counterparts (*right*). Symbols represent individual mice and lines indicate mean values. Graphs represent two to three individual experiments, each with five mice per group; CD19-hBtk and WT mice were 8-12 weeks old; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

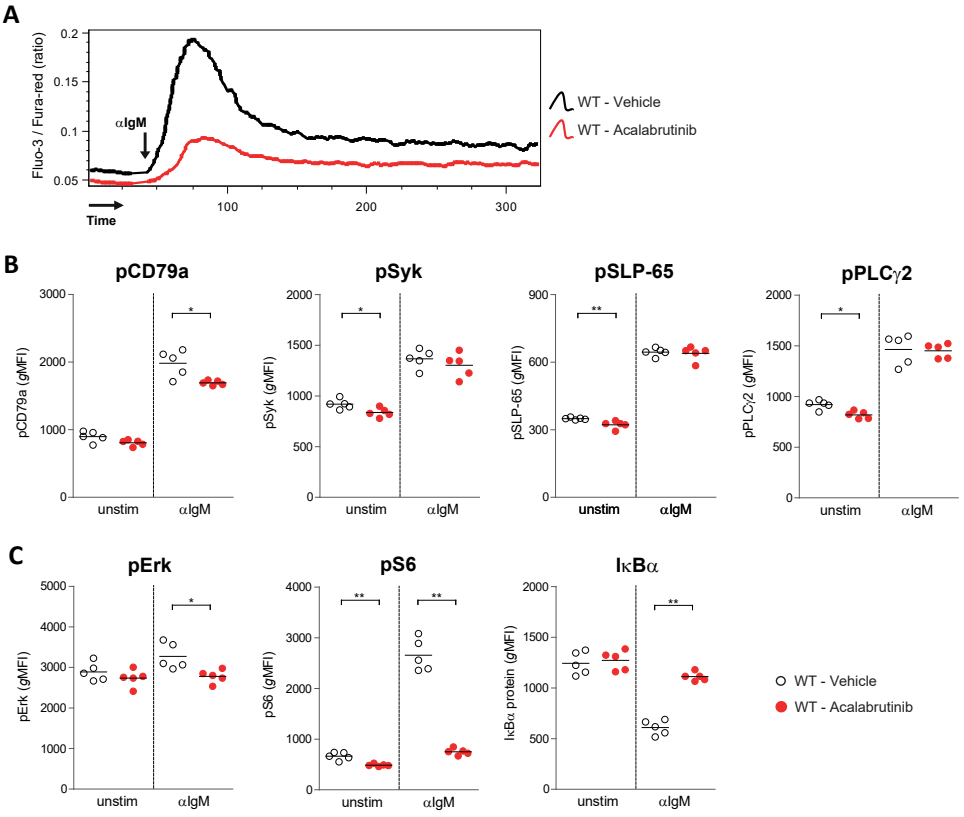


Figure 6. Single-dose in vivo Btk-kinase inhibition affects basal phosphorylation of proximal BCR molecules. CBA/J mice were treated *in vivo* by administration of one single dose of acalabrutinib or vehicle control by oral gavage. (A) Ca^{2+} influx measurement in $B220^{+}$ B cells upon stimulation with 25 µg anti-IgM in vehicle or acalabrutinib-treated mice, representative for five mice analyzed. (B) Quantification by geometric mean fluorescence intensity (*g*MFI) for pCD79a, pSyk, pSLP-65 and pPLCγ2 after after 5 min stimulation of anti-IgM. (C) Quantification by *g*MFI for pErk after 5 min, for IκBα protein after 1 h and pS6 after 180 min of anti-IgM stimulation of follicular B cells. Graphs represent two individual experiments, each with 4-5 mice per group. Symbols represent individual mice and lines indicate mean values; each group contained five 12-week-old CBA/J wild-type (WT) mice; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

Prolonged *in vivo* Btk inhibition mimics rewiring of BCR signaling as observed in Btk-deficient mice

Next, we treated WT mice with acalabrutinib or vehicle in drinking water for 5 days, to investigate rewiring following prolonged kinase inhibition.

Compared to B cells from vehicle-treated mice, calcium influx following anti-IgM stimulation was decreased in B cells from acalabrutinib-treated mice and similar to *Btk*$^{-/-}$ B cells (**Figure 7A**). Follicular B cells from acalabrutinib-treated mice showed increased IgM and similar IgD expression, reminiscent of the IgM/IgD profile of B cells in *Btk*$^{-/-}$ mice, while expression of CD79b was increased and Btk protein was decreased (**Suppl. Fig. 4A**). Phosphorylation of CD79a and PLCγ2 was increased upon stimulation with anti-IgM in acalabrutinib-treated follicular B cells compared to vehicle-treated counterparts (**Figure 7B,C**). We also observed increased BCR responsiveness of acalabrutinib-treated follicular B cells for pSrc-Y424 and pSrc-Y507 (**Figure 5D**), although this was mainly due to the decreased basal signaling (**Suppl. Fig. 4B**).

Prolonged acalabrutinib treatment strongly affected distal BCR signaling: IκBα degradation and phosphorylation of Akt and S6 were significantly reduced (**Suppl. Fig. 4C, 4D**). Acalabrutinib treatment reduced basal Erk signaling, but the anti-IgM-induced phosphorylation was similar, resulting in increased BCR responsiveness for pErk (**Suppl. Fig. 4E**).

Finally, we investigated whether BCR rewiring is also induced upon Btk inhibition in malignant B cells, we employed a chronic lymphocyte leukemia (CLL) mouse model. We transferred EMC6 CLL cells, derived from the *IgH.TE*μ mice that spontaneously develop CLL[35, 36] intraperitoneally into *Rag1*$^{-/-}$ mice (**Figure 7E**). After twelve days, we detected circulating IgM^{+}CD5^{+} CLL cells confirming EMC6 engraftment in these *Rag1*$^{-/-}$ mice, in line with published findings[36]. To study the effect of *in vivo* Btk inhibition in CLL cells, we treated the mice with the Btk-inhibitor ibrutinib, or vehicle as a control, for a maximum of 14 days (**Figure 7E**). Treatment with ibrutinib significantly enhanced the survival as compared to vehicle-treated mice (**Figure 7F**), with two ibrutinib-treated mice reaching the experimental end-point of 20 days after start of treatment. We analyzed spleens of end-stage CLL mice, implying that for ibrutinib-treated mice the analysis was performed ~3-5 days after the end of 14-day treatment. In line with findings in acalabrutinib-treated WT B cells, the engrafted EMC6 CLL cells showed significantly enhanced upstream BCR signaling compared to WT controls, as evidenced by increased phosphorylation of CD79a, Syk, SLP-65 and PLCγ2 (**Figure 7G**). The increased phosphorylation could not be explained by increased total protein levels of these signaling molecules **(Suppl. Fig. 5A,B**).

Taken together, these data show that prolonged *in vivo* Btk inhibition results in rewiring of BCR signaling pathways, both upstream and downstream of Btk, in follicular B cells, paralleling our findings in Btk-deficient mice. Moreover, *in vivo* Btk inhibition was also associated with a similar rewiring of BCR signaling in leukemic B cells.

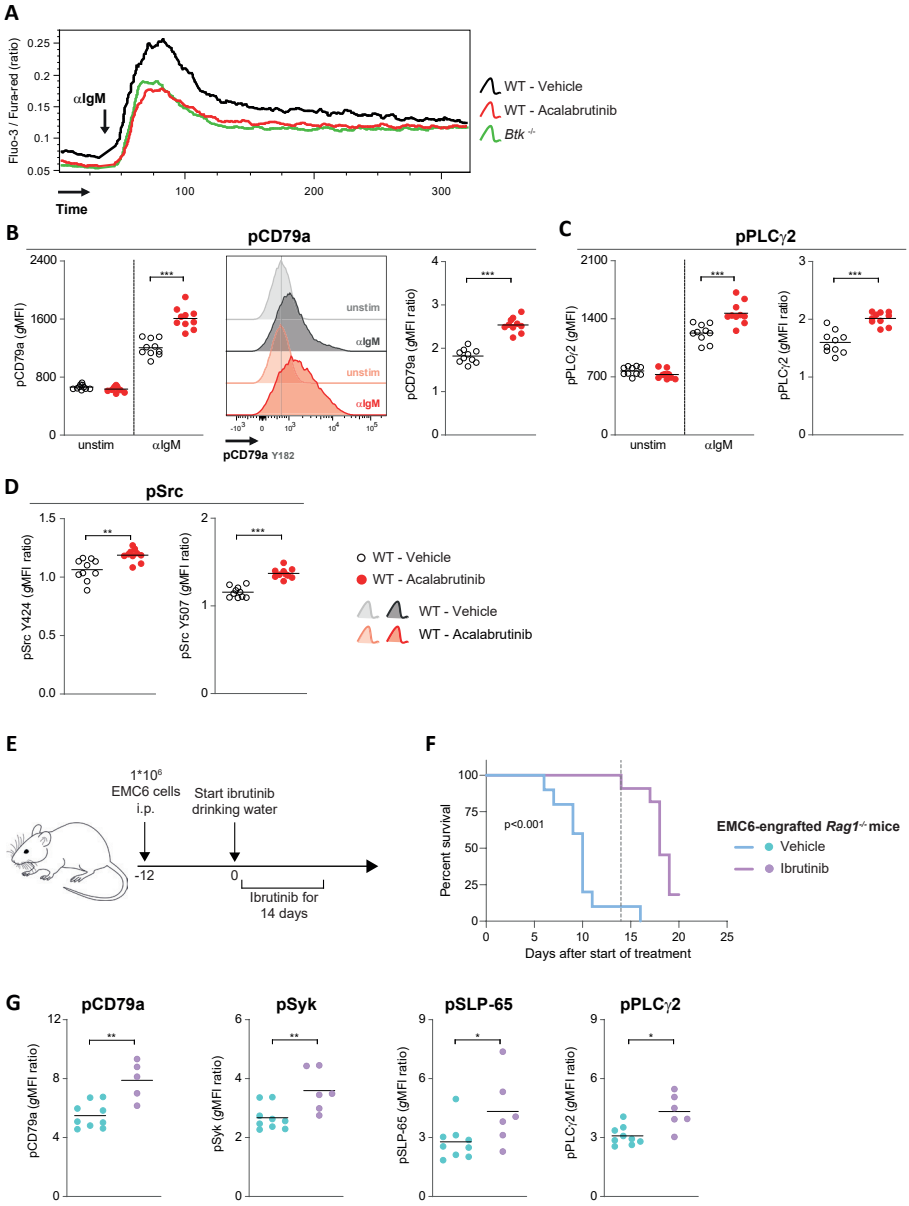


Figure 7. Prolonged Btk-kinase inhibition in vivo leads to enhanced proximal signaling.
CBA/J mice were treated *in vivo* with drinking water containing acalabrutinib or vehicle control for five days. (A) Ca^{2+} influx measurement of $B220^{+}$ B cells upon stimulation with 25 µg anti-IgM in vehicle or acalabrutinib-treated CBA/J mice compared to untreated Btk-deficient (*Btk*$^{-/-}$) mice. (B) Quantification by geometric mean fluorescence intensity (*g*MFI) in follicular B cells after 5 min for pCD79a (*left*), representative histogram overlay (*middle*) and stimulation ratio calculated by fold increase in anti-IgM stimulated *g*MFI compared to unstimulated counterparts (*right*). (C) Quantification by *g*MFI (*left*) and stimulation ratio (calculated by fold increase in anti-IgM stimulated *g*MFI compared to unstimulated counterparts; *right*) of pPLCγ2 after 5 min of anti-IgM stimulation.

(D) Stimulation ratios for pSrc-Y424 and pSrc-Y507. (E) Experimental design for EMC6-engraftment and ibrutinib treatment of *Rag1*$^{-/-}$ mice (F) Retrospective Kaplan-Meier incidence curve depicting survival of EMC6-engrafted *Rag1*$^{-/-}$ mice treated with ibrutinib or vehicle drinking water, n=11 mice per group. Dotted grey line indicates stop of ibrutinib treatment at day 14 (G) Quantification of the stimulation ratio for pCD79a, pSyk, pSLP-65 and pPLCγ2 after 5 min of anti-IgM stimulation in EMC6-engrafted *Rag1*$^{-/-}$ mice. Graphs represent two individual experiments, each with 4-11 mice per group; CBA/J wild-type (WT), Btk$^{-/-}$ mice and *Rag1*$^{-/-}$ mice were 8-12 weeks old; Symbols represent individual mice and lines indicate mean values; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney *U* test.

DISCUSSION

Btk protein is a crucial BCR signaling protein and tight regulation of expression levels and kinase activity are important for normal B cell development and function. Btk inhibition is a successful treatment for several B cell malignancies, but its (long term) effects on signaling have not been assessed. Our findings implicate that Btk inhibition may have more far reaching effects than previously anticipated. In addition to downstream signaling routes, Btk inhibition also affects proximal upstream BCR signaling and rewires the entire BCR signaling cascade.

Downstream signaling by NF-κB and Akt was shown to be critically dependent on Btk protein[23, 24, 37]. Conversely, we previously showed that Akt signaling is enhanced in Btk-overexpressing B cells upon BCR stimulation[34]. *In vitro* blockade of Btk-kinase activity leads to decreased basal signaling of Akt, NF-κB and ERK in B cells from CLL patients and mouse models[36, 38, 39]. Decreased basal downstream signaling was also observed after 1-4 weeks *in vivo* acalabrutinib treatment in a TCL-1 adoptive transfer CLL model[39], however, BCR responsiveness or proximal signaling were not investigated. Here, we also observed effects on basal signaling in non-transformed B cells, however, the most apparent difference was the strongly decreased response to BCR stimulation upon *in vivo* acalabrutinib treatment.

Btk-deficient and X-chromosome-linked immunodeficiency mice show decreased calcium signaling upon BCR stimulation[40, 41]. Whilst basal calcium is unchanged in CD19-hBtk B cells, anti-IgM-induced calcium influx is enhanced in CD19-hBtk B cells[42]. A very recent study showed that B cells with strongly impaired calcium influx capacity show altered pS6 kinetics, resulting in an earlier pS6 peak and a decreased capacity to induce other downstream mediators including Myc and IRF4[43]. This is similar to our findings in *Btk*$^{-/-}$ follicular B cells, which showed increased pS6 at 30 min but decreased pS6 at 180 min of anti-IgM stimulation compared to WT controls. It is therefore likely that Btk-dependent calcium signaling alters pS6 signaling kinetics, and may thereby impact B cell survival.

Interestingly, anti-IgM-induced pErk was markedly increased specifically in follicular B cells from *Btk*$^{-/-}$ and acalabrutinib-treated mice, compared to untreated WT mice. Because Erk was shown to be crucial for survival and differentiation of immature B cells in a mouse strain with low surface BCR levels[44], it is conceivable that enhanced Erk signaling is advantageous for Btk$^{-/-}$ follicular B cells. Further experiments are required to investigate why this is not the case for T1 or MZ B cells in *Btk*$^{-/-}$ mice, in which Erk signaling is comparable to WT mice.

Very surprisingly, we found that Btk activity dictated the signaling of proximal BCR signaling molecules in follicular B cells. While Btk inhibition significantly reduced anti-IgM-mediated CD79a phosphorylation within 3 hours of *in vivo* acalabrutinib treatment, genetic Btk-deficiency or prolonged acalabrutinib treatment increased BCR responsiveness for the entire proximal signaling hub. Our findings are in concordance with the reported increased BCR-induced pCD79a and pSyk in *Btk*$^{-/-}$ mice[45]. In this study, *in vitro* inhibition by ibrutinib resulted in reduced pSyk, even in *Btk*$^{-/-}$ B cells[45], indicating an off-target effect of ibrutinib. Very recently, BCR rewiring was also reported in a mouse model with B-cell specific deletion of TNF receptor associated factor 3 (TRAF3); TRAF3-deficient B cells showed increased BCR responsiveness in upstream signaling molecules, enhanced calcium influx and pErk signaling upon BCR stimulation[46]. This is similar to our findings, although we did not observe increased calcium signaling. This could either relate to calcium being a quite direct downstream of target of Btk, or that increased phosphatase signaling downregulates the calcium response. Likewise, it cannot formally be excluded that off-target effects contributed to BCR-induced kinome fingerprint changes in samples from CLL patients before and after ibrutinib treatment[47]. The observed marked increase in the BCR-induced kinome signature might be related to increased surface IgM expression in patients receiving therapy. However, our analyses in Btk-deficient mice (**Figure 4**) indicated that enhanced signaling was observed irrespectively of the differences in IgM/IgD profile – which controls B cell responsiveness[48, 49] – between *Btk*$^{-/-}$ and WT B cells.

The mechanisms by which Btk controls upstream signaling remain elusive, particularly because Btk differentially affects proximal signaling in follicular, T1 and MZ B cells. Several findings, e.g. for CD79a, indicate that the mechanisms involved are even remarkably complex. First, acalabrutinib can both reduce and increase anti-IgM induced pCD79a (after 3 hours and after 5 days of *in vivo* treatment, respectively). Second, both Btk-deficiency and Btk overexpression reduce CD79a protein levels in B cells. Third, signaling in *Btk*$^{-/-}$ follicular B cells could be enhanced due to increased availability of CD79b molecules for IgM. However, the interpretation of these findings is complicated, because of the concomitant decrease in CD79a, which (i) is the limiting component during BCR assembly in the ER[50] and (ii) involved as homodimer in CD19 signaling B cells[51]. In addition, while our approach provides evidence for extensive rewiring, caution should be taken when interpreting data, because of the presence of multiple – activating or inhibiting – phosphorylation sites on signaling molecules, only some of which can be analyzed by available phospho-specific antibodies. Given that Btk activity regulates many crucial transcription regulators in B cells, including NF-κB, NFAT, Fos/Jun[52, 53], it is likely that Btk inhibition does not only rewire BCR signaling by direct phosphorylation of signaling molecules but also by regulating their transcription. Since we found that rewiring events in vivo takes a couple of days, it is to be expected that BTK does not only act directly as a kinase (and may e.g. activate phosphatases), but also indirectly regulates transcription of signaling molecules or their regulators. This would be supported by the recently reported epigenetic changes induced during ibrutinib therapy[22], as well as our transcriptome comparison of Btk-overexpressing and WT B cells, showing differential expression of many kinases, phosphatases as well as transcription regulators [J.R., R.W.H., unpublished findings].

Our findings may have implications for the treatment of autoimmune and malignant diseases, although it is currently unknown whether BTK inhibition in human B cells is also associated with BCR rewiring. In B cell malignancies potential rewiring may be dampened by sequential or combination therapies of BTK inhibitors with e.g. the Bcl-2 inhibitor venetoclax [3, 54, 55] or potentially PI3K inhibitor idelalisib. However, BCR signaling modulation by Btk inhibition is also relevant for other B-cell-mediated diseases, because Btk inhibitors were effective in numerous autoimmune mouse models[56, 57] and multiple sclerosis patients[7] and are currently tested in clinical trials for autoimmune disease and coronavirus disease 2019 (COVID-19) inflammation[2, 58]. In-depth analysis of BCR responsiveness and activation of B cells from autoimmune or COVID-19 patients following Btk inhibition should show how non-malignant B cells respond to ibrutinib therapy.

For inflammatory diseases the consequences of BCR rewiring will likely be very different, because therapeutic intervention is not aimed at depletion of malignant B cells, but rather at regulation of B cell function. Nevertheless, inferred from our findings in mice that BTK inhibition induces rewiring of both distal and proximal BCR signaling, in leukemic patients also signaling responsiveness of healthy B cells will be substantially altered. Therefore, it will be valuable to include a detailed analysis of BCR signaling, in future clinical trials of Btk inhibitors both in B cell malignancies and in autoimmune disease.

MATERIALS AND METHODS

Mice and genotyping

Btk-deficient (*Btk*$^{-/-}$)[33], CD19-hBtk[59] and Rag1-deficient (*Rag1*$^{-/-}$) were on the C57BL/6 background [60] mice were genotyped by PCR. Non-transgenic littermates or C57/BL6 mice (Charles River, Wilmington, MA, USA) were used as wild-type (WT) controls. For *in vivo* Btk-kinase inhibition experiments, we used WT CBA/J mice (Janvier, Le Genest-Saint-Isle, France). Mice were bred and kept under specified pathogen-free conditions in the Erasmus MC experimental animal facility. All experimental protocols were reviewed and approved by the Erasmus MC Committee for animal experiments (DEC).

Flow cytometry and calcium influx

Splenic cell suspensions were prepared in RPMI 1640 supplemented with 2% FCS (RPMI-2% FCS) by mechanical disruption using 100 μm strainers (Corning, Corning, NY, USA). To stain for CD79a and CD79b protein, 2x10^6 cells were incubated in magnetic-activated cell sorting (MACS) buffer (PBS/0.5% BSA/2mM EDTA) with combinations of monoclonal antibodies (**Suppl. Table 1**) and stained according to previously described procedures[42]. To detect CD79a protein intracellularly, cells were fixed using 2% paraformaldehyde (PFA) in PBS and permeabilized using 0.5% Saponin (Sigma Aldrich, St. Louis, MO, USA) in MACS buffer.

To measure activation of anti-apoptotic and survival pathways, $0.5x10^6$ splenic cells were cultured in RPMI-5% FCS at 37°C for 22 h for Bcl-XL (Cell Signaling Technologies, Danvers, MA, USA), Mcl-1 (Abcam, Cambridge, UK) and c-Rel (Miltenyi Biotec, Bergisch Gladbach, Germany), or 1 h for IκBα (Invitrogen, Waltham, MA, USA) expression. Splenic cells were stimulated with 25 μg/mL anti-mouse antigen-binding $F(ab')_2$-IgM fragments (anti-IgM; Jackson Immunoresearch, West Grove, PA, USA) or left unstimulated. Subsequently, cells were stained for cell surface markers and viability on ice in the dark. To detect intracellular proteins, we fixed the cells with 2% PFA in PBS for 10 min followed by permeabilization using 0.5% Saponin in MACS buffer.

To measure intracellular calcium mobilization, $5x10^6$ splenocytes were incubated with Fluo3-AM and Fura Red-AM fluorogenic probes (Life Technologies, Carlsbad, CA, USA), as previously described[34, 36]. During measurements, cells were stimulated with 25 **μg/mL** anti-IgM after running a 30s baseline.

Upon completion of the staining procedure, samples were measured in MACS buffer on an LSR-II (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo v10 (BD Biosciences).

Phosphoflow cytometry

For phosphoflow experiments, $0.5x10^6$ cells were cultured in RPMI-2% FCS at 37°C for 1 min for pPI3K p85 (Cell Signaling Technologies), 3 min for pSHP-1 (Cell Signaling Technologies), 5 min for pSrc (Abcam), pLyn (Abwiz Bio, San Diego, CA, USA), pCD79a (Cell Signaling Technologies), pSyk, pSLP-65, pPLCγ2 and pErk (all BD Biosciences) or for 3 h for pS6 and pAkt (both Cell Signaling Technologies) with 25 μg/mL anti-IgM or antigen-binding $F(ab')_2$-Igκ fragments (anti-IgK; Jackson Immunoresearch). After fixation with the eBioscience FoxP3 staining kit Fix/Perm solution, cells were washed twice with the accompanying Perm/Wash solution (Invitrogen). Next, cells were incubated with varying combinations of monoclonal antibodies (**Suppl. Table 1**) and stained according to previously described procedures[31]. After staining for the phosphoproteins, samples were measured in MACS buffer on an LSR-II (BD Biosciences) and analyzed using FlowJo v10 (BD Biosciences).

In vitro and *in vivo* treatment with acalabrutinib

In vitro cultures were performed by pre-treating $0.5x10^6$ splenic cells with 1μM acalabrutinib (ACP-196; MedChemExpress, Monmouth Junction, NJ, USA) or 1μM DMSO in RPMI-2% FCS for 3 h at 37°C. Cells were subsequently stimulated with 25 μg/mL anti-IgM or alternatively RPMI-2% FCS was added as unstimulated control.

For single-dose experiments for *in vivo* Btk-kinase inhibition, CBA/J WT mice were treated with one dose of 25 mg/kg acalabrutinib in vehicle solution (Trappsol®Cyclo (2-Hydroxypropyl-beta-cyclodextrin (HPβCD)); CTD inc, Gainesville, FL, USA) or vehicle alone by oral gavage. Mice were sacrificed 3 h after acalabrutinib administration.

For prolonged *in vivo* treatment, CBA/J WT mice received 0.16 mg/mL acalabrutinib diluted in vehicle or vehicle alone formulated in drinking water[39] for five days, which corresponds with administration of a daily dose of 25 mg/kg.

Adoptive transfer EMC6 in *Rag1*$^{-/-}$ mice and ibrutinib treatment

To study the effects of Btk inhibitors on BCR signaling in leukemia, 1x10^6 cells EMC6 CLL cells – a cell line derived from the *IgH.TE*μ CLL mouse model[35] – were intraperitoneally injected into *Rag1*$^{-/-}$ mice[36]. Mice were monitored for the onset of leukemia by blood screening and randomized into different groups based on the percentage of leukemic cells in circulation prior to treatment, as previously described [38]. After twelve days, EMC6-engrafted *Rag1*$^{-/-}$ mice received 0.16 mg/mL ibrutinib [61] diluted in vehicle or vehicle alone formulated in drinking water for 14 days. After 14 days of treatment, mice again received regular drinking water. Mice were euthanized upon development of severe signs of disease, e.g. difficulty to breath and aversion to activity, or at the experimental end-point of 20 days after start of ibrutinib treatment.

Statistical analysis

Statistical differences were calculated in GraphPad Prism 5 software (GraphPad Software Inc; San Diego, CA, USA) by Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparison posthoc test. A Log Rank test was performed to calculate differences in survival for CLL-engrafted *Rag1*$^{-/-}$ mice. P values <0.05 were considered significant.

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AUTHOR CONTRIBUTIONS

JR designed the research, performed experiments, analyzed the data, and wrote the manuscript. MdB, SN, SPS and JvH performed experiments and analyzed the data. OC and RH contributed to the research design and the writing of the manuscript and supervised the study. All co-authors approved the final manuscript.

COMPETING INTRESTS

The authors declare to have no financial conflicts of interest.

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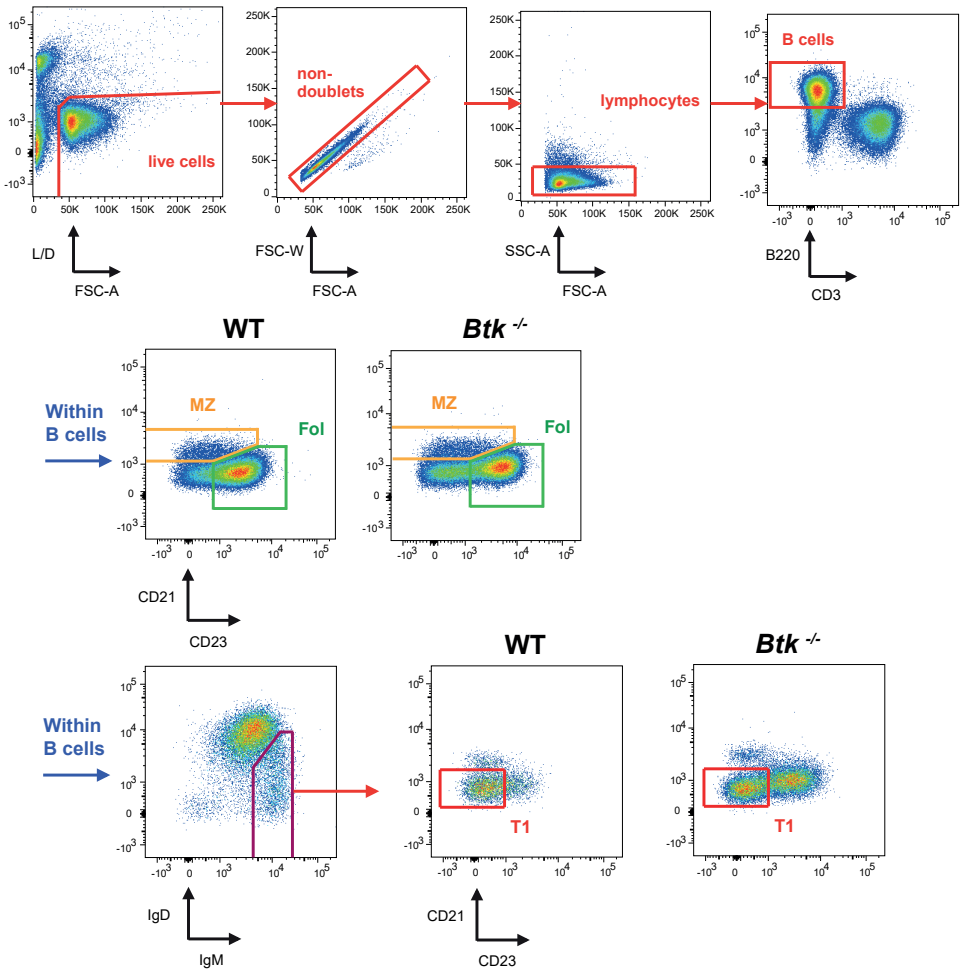
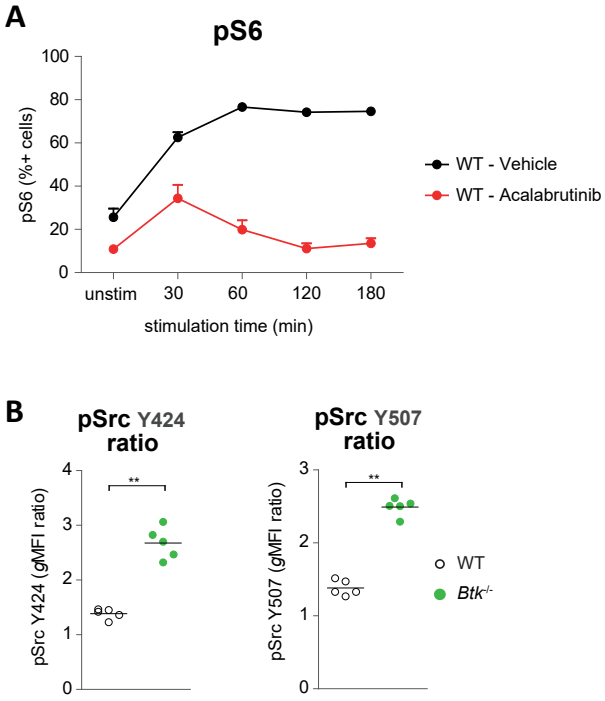
SUPPLEMENTAL MATERIAL

Supplemental Table 1. Antibodies used for (phospho)flow cytometry.

Antibody	Conjugate	Clone	Manufacturer
Bcl-XL	-	54H6	Cell Signaling Technologies
B220	AF700	RA3-6B2	Invitrogen
CD21 / CD35	FITC	eBio4E3	Invitrogen
	BV605	7G6	BD Biosciences
CD23	Biotin	B3B4	BD Biosciences
	PE-Cy7	B3B4	Invitrogen
CD3	APC-eFluor 780	17A2	Invitrogen
	BV421	145-2C11	BD Biosciences
CD5	APC	53-7.3	Invitrogen
CD79a	PE	HM47	Invitrogen
CD79b	FITC	HM79b	BD Biosciences
CD93	APC	AA4.1	Invitrogen
c-Rel	PE	REA397	Miltenyi Biotec
Fixable Viability Dye	eFluor 506	n.a.	Invitrogen
IgD	BV711	11-26c.2a	BD Biosciences
Igκ	Biotin	187.1	BD Biosciences
Igλ	Biotin	R26-46	BD Biosciences
IgM	PE-Cy7	II/41	BD Biosciences
IκBα	PE	MFRDTRK	Invitrogen
Mcl-1	-	Y37	Abcam
pAkt (S473)	-	D9E	Cell Signaling Technologies
pBtk / pItk (Y223/Y180)	APC	N35-86	BD phosflow
pCD79a (Y182)	Alexa Fluor 647	D1B9	Cell Signaling Technologies
pErk1/2 (T202/pY204)	PE	20A	BD phosflow
pPI3K p85 (Y458)	-	E3UI1H	Cell Signaling Technologies
pPLCγ2 (Y759)	Alexa Fluor 647	K86-689.37	BD phosflow
pSHP-1 (Y564)	-	D11G5	Cell Signaling Technologies
pSLP-65 (Y84)	PE	J117-1278	BD phosflow
pSrc (Y424)	-	EP503Y	Abcam
pSrc (Y507)	-	5B6	Abwiz Bio
pSyk (Y348)	PE	I120-722	BD phosflow
pS6 (S240/244)	-	D68F8	Cell Signaling Technologies
Anti-rabbit	PE	n.a.	Jackson ImmunoResearch
Streptavidin	BV786	n.a.	BD Biosciences

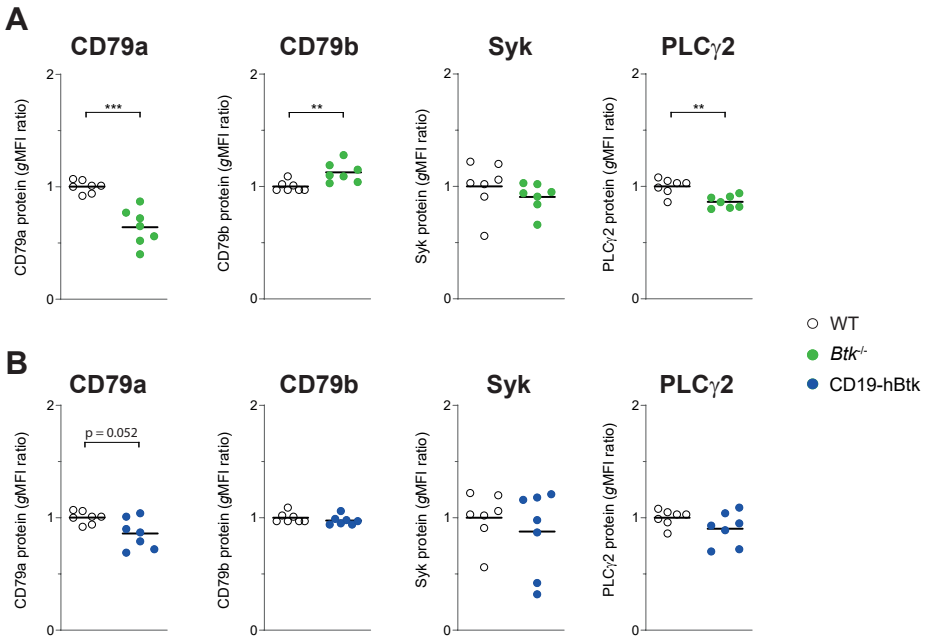


Supplemental Figure 1. Gating strategy phosphoflow. Gating strategy for live, single lymphocytes.
Within lymphocytes, we gated for B cells ($B220^{+}CD3^{-}$). From B cells, we gated for marginal zone (MZ) B cells ($B220^{+}CD21^{+}CD23^{-}$), follicular (Fol) B cells ($B220^{+}CD21\text{-}CD23^{+}$) and transitional type 1 (T1) B cells ($B220^{+}IgD^{-}IgM^{++}CD21^{-}CD23^{-}$). Examples for all subsets are shown for Btk-deficient (*Btk*$^{-/-}$) and wild-type (WT) mice. L/D = live/dead viability stain; FSC-A = Forwards-Scatter Area; FSC-W = Forward-Scatter Width; SSC-A = Side-Scatter Area.



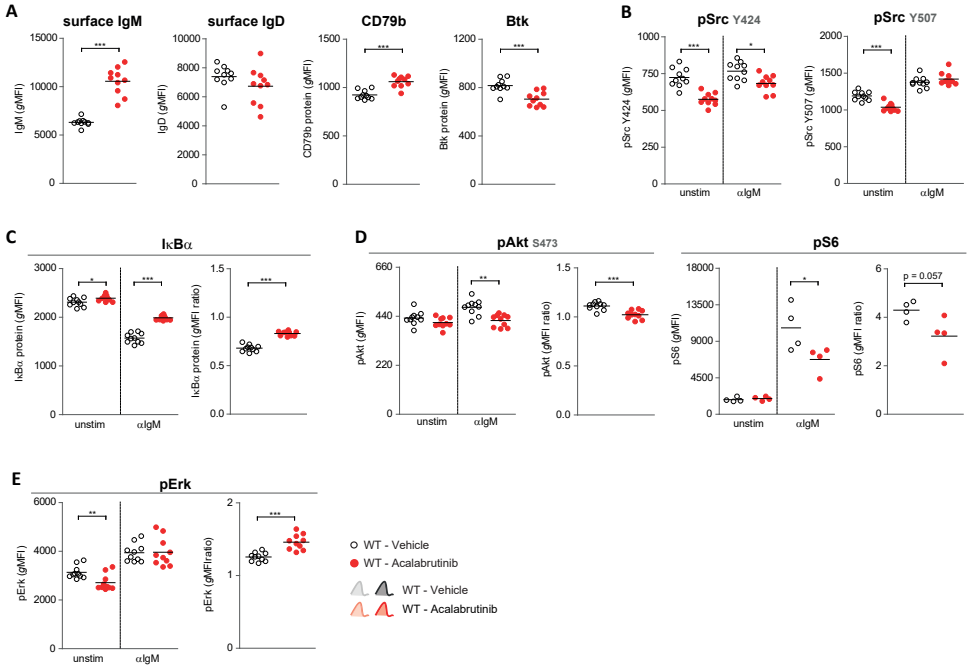
Supplemental Figure 2. Ribosomal protein pS6 kinetics with Btk inhibitor acalabrutinib and Src kinase responsiveness.

(A) S6 signaling kinetics represented by percentage of positive cells in wild-type (WT) follicular splenic B cells after 30, 60, 120, 180 min incubation with anti-IgM stimulation in presence of Btk-inhibitor acalabrutinib or Vehicle-control. (B) Stimulation ratio of pSrc-Y424 and pSrc-Y507 calculated by fold increase in anti-IgM stimulated geometric mean fluorescence intensity (*g*MFI) compared to unstimulated counterparts for follicular B cells of Btk-deficient (*Btk*$^{-/-}$) and WT mice. Symbols represent individual mice and lines indicate mean values. Graphs represent two to three individual experiments, each with 5 mice per group; *Btk*$^{-/-}$ and WT controls were 8-12 weeks old; **p<0.01 by Mann-Whitney *U* test.



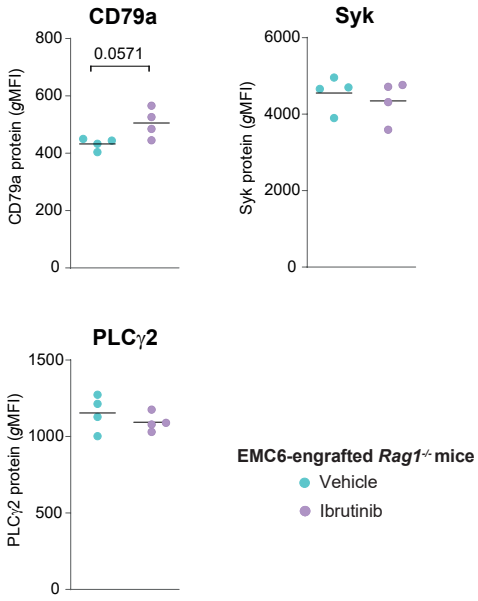
Supplemental Figure 3. Expression levels of BCR signaling molecules in Btk-deficient and CD19-hBtk follicular B cells.

Quantification of CD79a intracellular protein and CD79b, Syk and PLCγ2 extracellular protein expression by geometric mean fluorescence intensity (*g*MFI) ratios in follicular B cells from *Btk*$^{-/-}$ mice (A) and CD19-hBtk mice, compared to wild-type (WT) controls. *g*MFI ratios were used to normalize expression for *Btk*$^{-/-}$ and CD19-hBtk B cells to WT values, which were set to 1, from two individual experiments. Symbols represent individual mice and lines indicate mean values. *Btk*$^{-/-}$, CD19-hBtk and WT mice were 10-30 weeks old; **p<0.01, ***p<0.001 by Mann-Whitney *U* test.



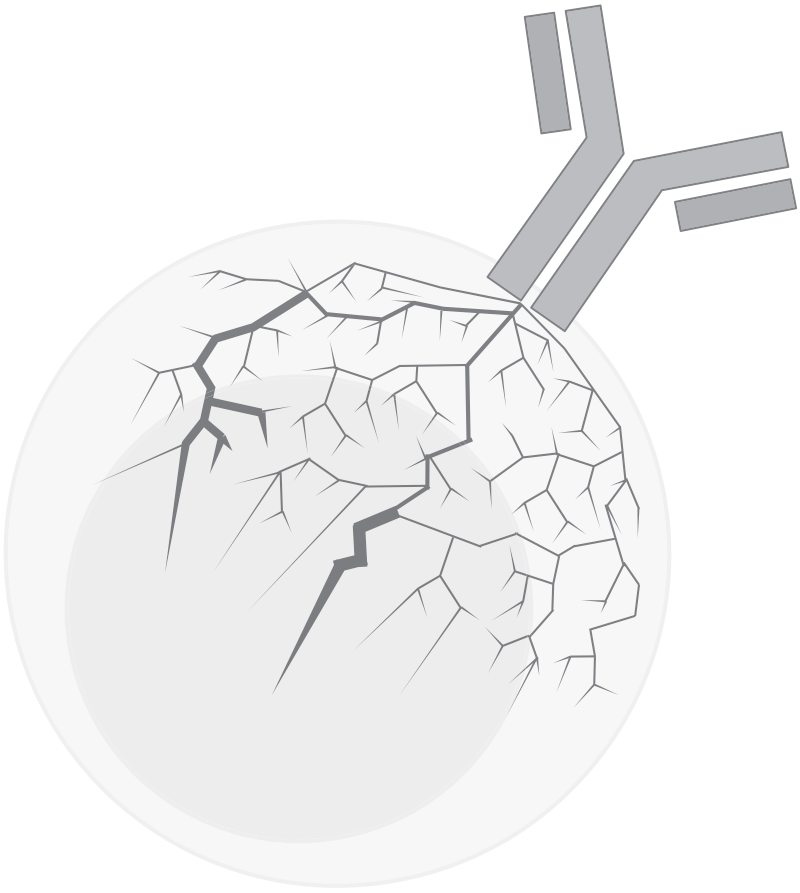
Supplemental Figure 4. Five-day in vivo treatment alters BCR expression, expression of signaling molecules and increases proximal BCR signaling.

(A) Quantification of surface IgM, surface IgD, CD79b protein and Btk protein expression by geometric mean fluorescence intensity (*g*MFI) values in acalabrutinib-treated follicular B cells compared to vehicle-treated controls. (B) Stimulation ratios calculated by fold increase in anti-IgM stimulated *g*MFI in follicular B cells compared to unstimulated counterparts for pSrc-Y424 and pSrc-Y507. (C-E) Quantification by *g*MFI (*left*) and stimulation ratio (calculated by fold increase in anti-IgM stimulated *g*MFI compared to unstimulated counterparts; *right*) of pPLCγ2 after 5 min (C), IκBα protein after 1 h (D), pAkt and pS6 after 180 min (E) and pErk after 5 min of anti-IgM stimulation. Symbols represent individual mice and lines indicate mean values. Graphs represent two individual experiments, each with 4-10 mice per group; treated wild-type (WT) CBA/J mice were 8-12 weeks old; *p<0.05, *** p<0.001 by Mann-Whitney *U* test.



Supplemental Figure 5. Protein expression levels of BCR signaling molecules after ibrutinib treatment of EMC6-engrafted Rag1-deficient (Rag1$^{-/-}$) mice.

Quantification of expression of the indicated signaling proteins using intracellular flow cytometry, shown as geometric mean fluorescence intensity (gMFI) values in EMC-6 CLL cells transferred into into *Rag1*$^{-/-}$ mice that received ibrutinib or vehicle. Symbols represent individual mice and lines indicate mean values. Graphs represent two individual experiments, each with 3-5 mice per group; mice were 8-15 weeks old.



CHAPTER 6

Enhanced expression of Bruton's tyrosine kinase in B cells drives systemic autoimmunity by disrupting T cell homeostasis

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ABSTRACT

Upon B cell receptor (BCR) stimulation, naïve B cells increase protein levels of the key downstream signaling molecule Bruton's tyrosine kinase (BTK). Transgenic CD19-hBtk mice with B cell-specific BTK overexpression show spontaneous germinal center formation, anti-nuclear autoantibodies and systemic autoimmunity resembling lupus and Sjögren's syndrome. However, it remains unknown how T cells are engaged in this pathology. Here, we found that CD19-hBtk B cells were high in IL-6 and IL-10, and disrupted T cell homeostasis in vivo. CD19-hBtk B cells promoted IFNγ production by T cells and expression of the immune-checkpoint protein ICOS on T cells, and induced follicular T helper cell differentiation. Crosses with CD40L-deficient mice revealed that increased IL-6 production and autoimmune pathology in CD19-hBtk mice was dependent on B-T cell interaction, whereas IL-10 production and IgM autoantibody formation were CD40L-independent. Surprisingly, in Btk overexpressing mice naïve B cells manifested increased CD86 expression, which was dependent on CD40L, suggesting that T cells interact with B cells in a very early stage of immune pathology. These findings indicate that increased BTK-mediated signaling in B cells involves a positive feedback loop that establishes T cell-propagated autoimmune pathology, making BTK an attractive therapeutic target in autoimmune disease.

INTRODUCTION

A breakdown of B cell tolerance is thought to be a major pathogenic event in systemic autoimmune disorders, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome (1, 2). Autoantibodies directed against various nuclear self-antigens often appear in patient serum before the onset of clinical symptoms (3) and - particularly in SLE - form circulating immune complexes that elicit inflammation upon deposition in joints, eyes, skin, lungs or kidneys (1, 4). Genetic studies indicate that B cell-intrinsic defects are central to autoimmune disease development (5-7). Susceptibility loci include genes implicated in B cell receptor (BCR) signaling (*LYN, BLK, BANK1, PTPN22,* and *PXK*) and NF-κB signaling. *Furthermore, m*ouse models have shown that single-gene defects in BCR signaling molecules or inhibitory co-receptors can induce autoimmunity (reviewed in ref. (8)). Aberrant emergence, activation and persistence of autoreactive B cells are thought to be dependent on other immune cells (9, 10). Whether and how autoreactive B cells propagate disease and to which extent they act through other cell types is largely unknown.

Conversely, follicular T helper (Tfh) cells contribute to autoimmune disease development through activation of autoreactive B cells in germinal centers (GCs), resulting in the formation of plasma cells producing high-affinity autoantibodies (11-13). B cells are thought not to prime but to consolidate Tfh differentiation and to support their function and expansion (14). For example, B cells guide Tfh responses through the production of IL-6 (15) or ICOS ligand (ICOSL) expression (16) and provide antigenic stimulation to Tfh cells when antigen availability in GCs is waning (17, 18). On the other hand, Tfh cells are important producers of IL-21, a key cytokine involved in B cell activation. Dysregulated Tfh cell formation is found in various lupus-prone mouse models, including *Sanroque* mice harboring a mutation in the RNA-binding protein Roquin-1 (19) or BXSB-Yaa mice in which development of glomerulonephritis is critically dependent on IL-21R signals (20).

We recently observed that overexpression of the BCR signaling molecule Bruton's tyrosine kinase (BTK) restricted to B cells in CD19-hBtk transgenic mice is sufficient to induce an SLE/Sjögren-like disease phenotype (21). Btk protein expression in mouse B cells is normally tightly controlled and upregulated upon BCR stimulation by complex regulatory mechanisms involving microRNAs and feed-forward NF-κB activation (22-24). B cells overexpressing BTK are selectively hyperresponsive to BCR stimulation, showing enhanced Ca^{2+} influx and NF-κB activation and resistance to Fas-mediated apoptosis *in vitro,* and are not effectively eliminated *in vivo* when autoreactive (21). A pathogenic role for BTK in rheumatic diseases is further emerging from mouse studies demonstrating that small molecule BTK inhibitors prevent or ameliorate lupus nephritis and experimental arthritis (25-31). However, BTK inhibitors are likely to have effects beyond BCR signaling, because BTK functions downstream of many receptors, including chemokine and toll-like receptors (TLR) and is also expressed in myeloid cells (32). Apart from direct effects on BTK-expressing cells, BTK inhibition was associated with diminished activation of both helper and cytotoxic T cells (25), raising the question how these T cells are indirectly activated in a BTK-dependent manner.

To elucidate how BTK signaling may engage pathogenic T cells in autoimmunity, here we investigated T cell activation, differentiation and B-T cell interaction in aging CD19-hBtk mice. We found that increased BTK expression in mice supported spontaneous autoimmunity *in vivo*, both independently of T cells and by promoting the induction of Tfh cells. Tfh induction was also increased in Btk transgenic mice upon TNP-KLH immunization and collagen induced arthritis induction. However, affinity maturation was not affected. Interestingly, increased IL-6 production by B cells and IgG autoantibody formation was T cell dependent, as was increased CD86 expression by naïve B cells. Taken together, these data demonstrate that enhanced BTK signaling in B cells can establish a T cell-driven pro-inflammatory loop resulting in autoimmune pathology, making BTK inhibition an attractive therapeutic strategy.

MATERIALS AND METHODS

Mice and genotyping

CD19-hBtk (33) and *Cd40l*$^{-/-}$ (34) mice have been described. These mouse strains were backcrossed to the c57bl/6 genetic background for >10 generations. Mice were genotyped by PCR. Wild type littermates were used as controls. Mice were kept at specified pathogen free conditions at the Erasmus MC experimental animal facility, and experimental procedures were approved by the Erasmus MC committee for animal experiments.

Flow cytometry procedures

Single-cell suspensions were prepared from mouse spleens, bone marrow and lymph nodes using 100 μm cell strainers (BD Falcon™) in magnetic activated cell sorting (MACS) buffer (PBS/0.5% bovine serum albumin (BSA)/2mM EDTA). Fluorescent labeling of cell membrane molecules was performed as described previously (21). For isotype-specific staining of intracellular Ig in plasma cells, cells were fixed and permeabilized using Cytofix/Cytoperm™ Buffer and Perm/Wash™ Buffer respectively (BD Biosciences), according to manufacturer's instructions. For the measurement of FoxP3 expression by CD4 T cells, cells were fixed and permeabilized with eBioscience FoxP3 staining kit. For the measurement of intracellular cytokines, cells were fixed in PBS/2% paraformaldehyde (PFA), permeabilized and stained in MACS buffer containing 0.5% saponin (Sigma-Aldrich). Gal-β(1-3)-GalNAc carbohydrates were stained with biotin-conjugated PNA (peanut agglutinin, Sigma-Aldrich), and indirect staining of biotin-conjugated antibodies and reagents was performed with fluorochrome-coupled streptavidin (eBioscience). For antibodies used, see supplementary Table I.

Quantitiative PCR experiments

Naïve ($CD3^+CD4^+CD25^-CD62L^+$) T cells and total memory ($CD3^+CD4^+CD25^-CD62L^-$) T cells were sorted from spleens of 32 week old WT and CD19-hBtk mice, using a FACS Aria equipped with BD FACS Diva software (BD Biosciences, San Jose, CA, USA). Purity of the obtained populations was >96%. RNA was isolated from the cells using Qiagen RNeasy microkit according to manufacturers' instructions. IL-21-specific primers were designed using the ProbeFinder software (Roche): CCATCAAACCCTGGAAACAA and TCACAGGAAGGGCATTTAGC. Quantitative RT-PCR was performed using standard precedures and the ABI Prism 7300 set-up and IL-21 expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

Collagen-induced arthritis (CIA) experiments

Mice were intradermally immunized with 100 µg of chicken collagen type 2 (CII; Chondrex) emulsified in complete Freund's adjuvant (CFA). At day 21 a secondary intradermal immunization was performed by injecting 100 µg of chicken CII emulsified in CFA. The evaluation of arthritis symptoms and severity was performed as described (35). Measurement of serum anti-chicken and anti-mouse CII IgG2c antibodies by ELISA was performed as previously described (35).

TNP-KLH immunizations and anti-TNP-KLH antibody ELISAs

Mouse serum and lymphoid organs were analyzed 7 days after intraperitoneal injection of 0.1 mg TNP-KLH (Biosearch Technologies) and 0.8 mg Imject alum adjuvant (Thermo Scientific) in PBS or 7 days after booster injection of 0.1 mg TNP-KLH in PBS given 6 weeks after primary immunization. Serum IgG1-specific anti-TNP-KLH antibody levels were determined using a sandwich enzyme-linked immunosorbent assay (ELISA). To discriminate high versus total affinity anti-TNP-KLH IgG1 antibodies, plates were coated with TNP(5)-KLH and TNP(16)-KLH respectively. After incubation of TNP-KLH coated plates with diluted serum, bound TNP-KLH-specific IgG1 antibodies were detected using goat-anti mouse IgG1 secondary antibodies (Southern Biotech).

Immunohistochemistry and Hep2 staining

Immunohistochemical stainings were performed as published (21). Sections were incubated with anti-CD3 (eBioscience) and anti-IgM antibodies (BD Bioscience). Hep2 reactivity of IgM antibodies was performed as described (21). For combination staining of IgM and IgG antibodies, fluorescently labelled anti-IgM and anti-IgG $F(ab)_2$ fragments were used (Jackson Immunoresearch).

Anti-DNA ELISA

Plates were coated with 'Deoxyribonucleic acid–cellulose, double-stranded from calf thymus DNA' (Sigma). Serum samples were incubated in serial dilutions and goat-anti-mouse biotinylated total IgG antibody (Southern Biotec) was used to detect DNA-specific IgG antibodies.

Statistics

Significance of continuous data was calculated using the non-parametric Mann Whitney U test. P-values <0.05 were considered significant.

RESULTS

Btk overexpression in B cells increases their capacity to interact with T cells

We previously reported that 12-week-old CD19-hBtk mice show spontaneous GC and increased plasma cell formation in the spleen and that aging CD19-hBtk mice develop anti-nuclear Abs (ANA) and autoimmune pathology in kidney, lung and salivary glands (21). To study the impact of BTK overexpression on the capacity of B cells to interact with T cells in aging mice, we first characterized the B cell compartment of CD19-hBtk mice and wild type littermate controls (WT) at ~30-33 weeks of age. Total splenic B cell numbers were comparable between CD19-hBtk and WT mice (**Figure 1A**). In line with our previous findings, proportions of $CD95^{+}IgD^{-}$ GC and programmed death-ligand 2 $(PD\text{-}L2)^{+}CD80^{+}$ memory B cells were significantly increased in the spleens of CD19-hBtk mice (**Figure 1B**). Accordingly, $CD138^{+}$ IgM^{+} and IgG^{+} plasma cells were also increased in spleen and bone marrow (**Figure 1C and D**, respectively). (See for gating strategy of B cell subsets and plasma cells: Supplementary **Figure 1A**) In addition, the proportions of IL-6 and IL-10-producing B cells were significantly increased in spleens of CD19-hBtk mice (**Figure 1E**). Interestingly, IL-10 producing B cells expressed higher levels of CD21 or CD5, indicating that many of these cells have a marginal zone or B1 cell phenotype, whereas IL-6 producing B cells did not express these markers (**Suppl. Figure 1B**). Similar to our previous findings in 12-week-old mice (21), we noticed that surface expression of the co-stimulatory molecules CD80 and CD86 was increased on CD19-hBtk B cells compared with WT B cells (data not shown). Expression of ICOS-L and the inhibitory molecule programmed death-ligand 1 (PD-L1) on GC B cells was comparable in CD19-hBtk and WT mice (**Figure 1F**). Interestingly, ICOS-L was significantly decreased on CD19-hBtk memory B cells (**Figure 1G**), suggesting its downregulation as a result of recent T cell engagement, as described for memory B cells in SLE patients (36).

Taken together, we observed (i) increased formation of GC and memory B cells, (ii) enhanced cytokine production by B cells and (iii) decreased ICOSL expression on memory B cells, all of which suggest that Btk overexpression stimulates B-T cell interaction.

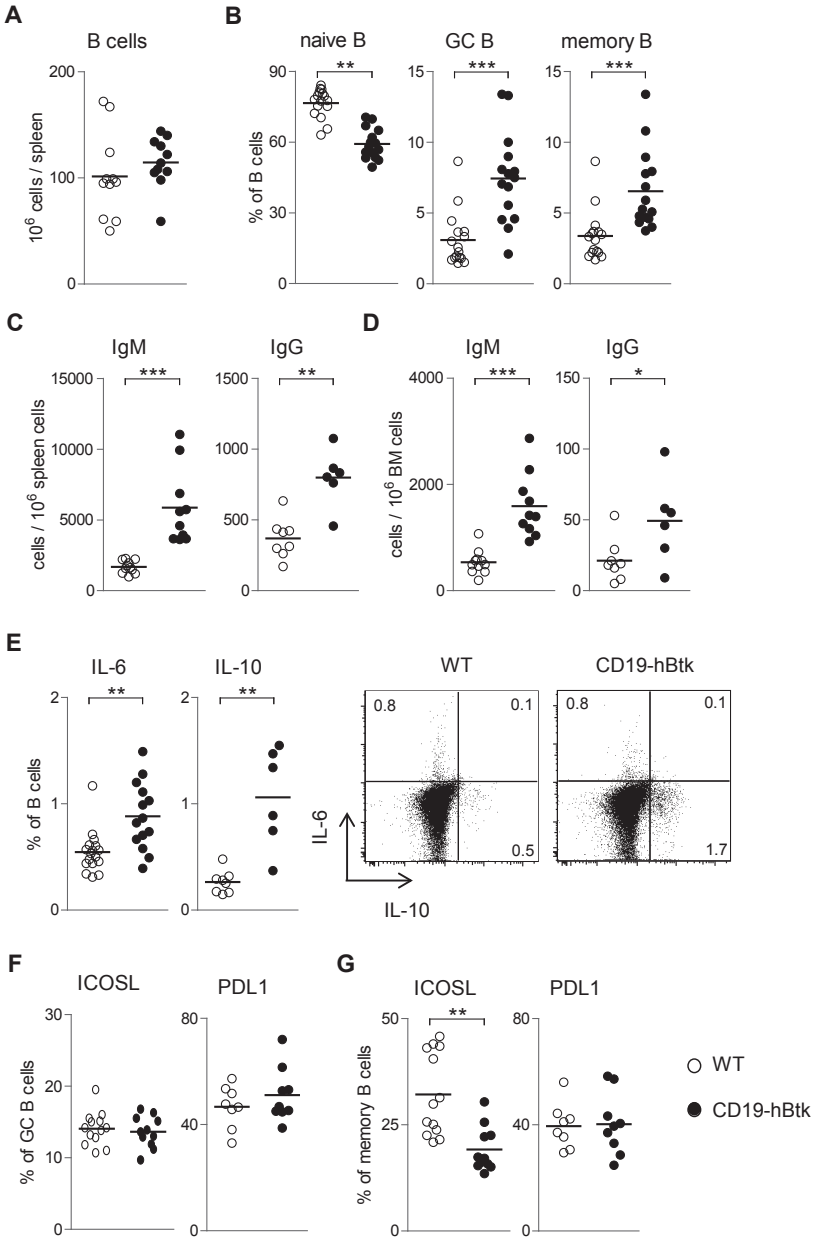

Figure 1. Increased B cell activation in aging CD19-hBtk mice versus WT littermate controls.
[A] Total splenic B cell numbers. [B] Proportions of naïve (CD19+IgD+CD95-), GC (CD19+IgD-CD95+) and memory (CD19+PDL2+CD80+) B cells of total aging splenic B cells. [C-D] Splenic [C] and bone marrow [D] IgM and IgG plasma cells. [E] Proportions of IL-6 and IL-10 producing B cells in spleen. Representative flow cytometry graphs are shown. [F-G] ICOSL and PDL1 expression by GC [F] and memory [G] B cells in spleen. Graphs represent 1-4 individual experiments; mice were ~30-33 weeks old; lines indicate mean values; *p<0.05; **p<0.01; ***p<0.001.

Btk overexpression in B cells is associated with enhanced T cell activation and Tfh differentiation in aging mice

Next we studied the splenic T cell compartment in CD19-hBtk mice. Whereas at ~8 weeks of age, proportions of T cell subsets and cytokine production were comparable between CD19-hBtk and WT mice (data not shown), total numbers of $CD4^+$ T cells were significantly increased in ~30-33-week-old CD19-hBtk mice (**Figure 2A**). Moreover, CD19-hBtk mice showed significantly increased proportions of follicular T cells and $FoxP3^+$ regulatory T cells (Tregs), both non-follicular Tregs and follicular Tregs (Tfr; **Figure 2B**). (Gating strategy **Suppl. Figure 2**) Both PD1-high and PD1-intermediate Tfh and Tfr cells were increased in CD19-hBtk mice. Proportions of residual $CD4^+$ T cells, including Th1, Th2 and Th17 cells, were reduced. Expression of the cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which transmits an inhibitory signal to T cells, was selectively increased on Tfh. Most interestingly, expression of ICOS, which is essential for Tfh survival and effective T helper responses (37-40) was increased on all $CD4^+$ T cell subsets in CD19-hBtk mice (**Figure 2C**). Analysis of $CD4^+$ T cells for intracellular cytokines after 4hrs of PMA/ionomycin stimulation revealed that IFNγ and IL-10 production were significantly increased in $CD4^+$ T cells from CD19-hBtk mice (**Figure 2D**). Also $CD8^+$ T cells manifested increased IFNγ production (data not shown). IL-21 mRNA expression, which was significantly higher in sorted memory $CD4^+CD25^-CD62L^-$ T cells than in naive $CD4^+CD25^-CD62L^+$ T cells, was increased in T cells from CD19-hBtk mice compared to non-transgenic littermates, reflecting the increased proportion of Tfh cells in these mice (**Figure 2E**).

Both excessive IFNγ production and enhanced ICOS signaling have been shown to contribute to Tfh accumulation in lupus-prone *Sanroque* mice (19, 41). It is therefore conceivable that the capacity of Btk-overexpressing B cells to drive IFNγ production by T cells and to promote expression of ICOS on the T cell surface supports their engagement in Tfh differentiation *in vivo* in aging CD19-hBtk mice.

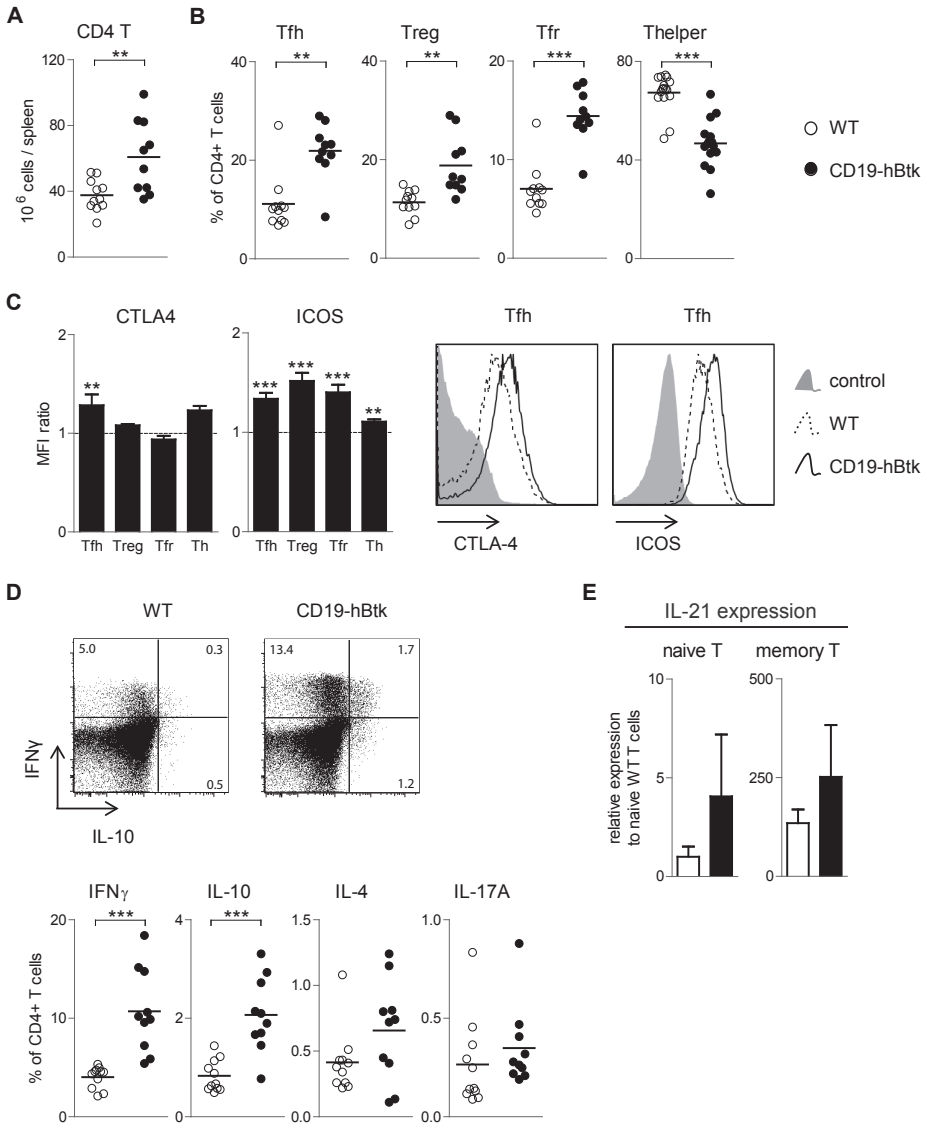

Figure 2. Increased T cell activation in aging CD19-hBtk mice versus WT littermate controls.
[A] Total splenic CD4+ T cells. [B] Proportions of splenic follicular T helper (CD4+CXCR5+PD1+FoxP3-), regulatory T (CD4+CXCR5-PD1-FoxP3+), follicular regulatory T (CD4+CXCR5+PD1+FoxP3+) and residual T helper cells (CD4+CXCR5-PD1-FoxP3-). [C] Relative CTLA4 (n=6) and ICOS (n=15) expression by T cell populations as shown in [B] in CD19-hBtk mice. Line indicates average expression in WT mice (set to 1). Representative flow cytometry graphs are shown as histogram overlaps for Tfh cells of the indicated mice. Control is CD19+ cells in WT mice. [D] Proportions of IFNγ, IL-10, IL-4 and IL-17A producing splenic CD4+ T cells. Representative flow cytometry graphs are shown for IFNγ and IL-10 as dot plots from gated CD4+ T cells. [E] mRNA expression analysis of IL-21 in naïve $CD3^+CD4^+CD25^-CD62L^+$ and memory $CD3^+CD4^+CD25^-CD62L^-$ T cells. Average expression in WT naïve T cells is set to 1. Graphs represent 1-4 individual experiments; lines indicate mean values; **p<0.01; ***p<0.001.

Btk overexpression in B cells promotes the induction of Tfh differentiation in vivo

Next, we aimed to directly investigate whether Btk-overexpressing B cells promote Tfh differentiation in young mice, both in the context of an *in vivo* immunization to a T-cell dependent antigen and in an autoimmune reaction based on molecular mimicry.

We studied Tfh cell induction and antibody affinity maturation in mice upon immunization with the T cell-dependent model antigen trinitrophenol keyhole limpet hemocyanin (TNP-KLH). Mice were immunized with TNP-KLH in alum and boosted six weeks later with TNP-KLH in saline. At day 7 after the primary or secondary injection, we found higher numbers of GC B cells and Tfh cells in the spleens of CD19-hBtk mice, compared with WT controls (**Figure 3A and 3B** for primary and secondary response, respectively). Interestingly, after the primary immunization, the sera contained increased levels of total anti-TNP IgG1 but comparable levels of high-affinity anti-TNP IgG1 antibodies (**Figure 3C**), indicating that low-affinity antibodies were increased in CD19-hBtk mice. This would be consistent with the observed increased survival of Btk overexpressing B cells upon BCR stimulation *in vitro* (21). Despite this increase of low-affinity IgG1 antibody formation in the primary response in CD19-hBtk mice, we found that after the boost serum levels of both total and high-affinity anti-TNP IgG1 and IgM antibodies were comparable between the two groups of mice (**Figure 3D** and **Suppl. Figure 3**). Thus, an aberrant primary and secondary response in CD19-hBtk mice, characterized by increased formation of GC B cells and Tfh cells, nevertheless allowed apparently normal affinity maturation and plasma cell differentiation after secondary immunization.

To investigate the effects of Btk overexpression on local Tfh differentiation in draining lymph nodes in an autoimmune model, we performed collagen induced arthritis (CIA) experiments. Eight-week-old CD19-hBtk and WT mice were immunized with emulsified chicken collagen type II (CII) in complete Freund's adjuvant and boosted at day 21. At day 38, the peak of disease severity, total splenic B cell numbers were comparable in CD19-hBtk and WT mice, but proportions of GC B cells, and IgM^+ or IgG^+ plasma cells were increased in CD19-hBtk mice (**Figure 4A, 4B**). Moreover, in CD19-hBTK mice the proportions of Tfh cells were increased in spleens, although not significantly, and in popliteal lymph nodes ($p<0.05$), compared with WT mice (**Figure 4C**). Also, the expression of IFNγ and IL-17A in $CD4^+$ T cells was significantly increased in lymph nodes (**Figure 4D**). Despite the enhanced T cell activation and Tfh differentiation, CD19-hBtk mice exhibited no detectable increase in disease severity and only a slight increase in disease incidence (**Figure 4E**). Accordingly, serum levels of both anti-chicken CII IgG2c and autoreactive anti-mouse CII IgG2c antibodies were comparable between CD19-hBtk and wild type mice (**Figure 4F**).

From these findings, we conclude that Btk overexpression in B cells induces enhanced Tfh differentiation *in vivo*, but does not substantially hamper affinity maturation nor augment autoantibody responses to collagen.

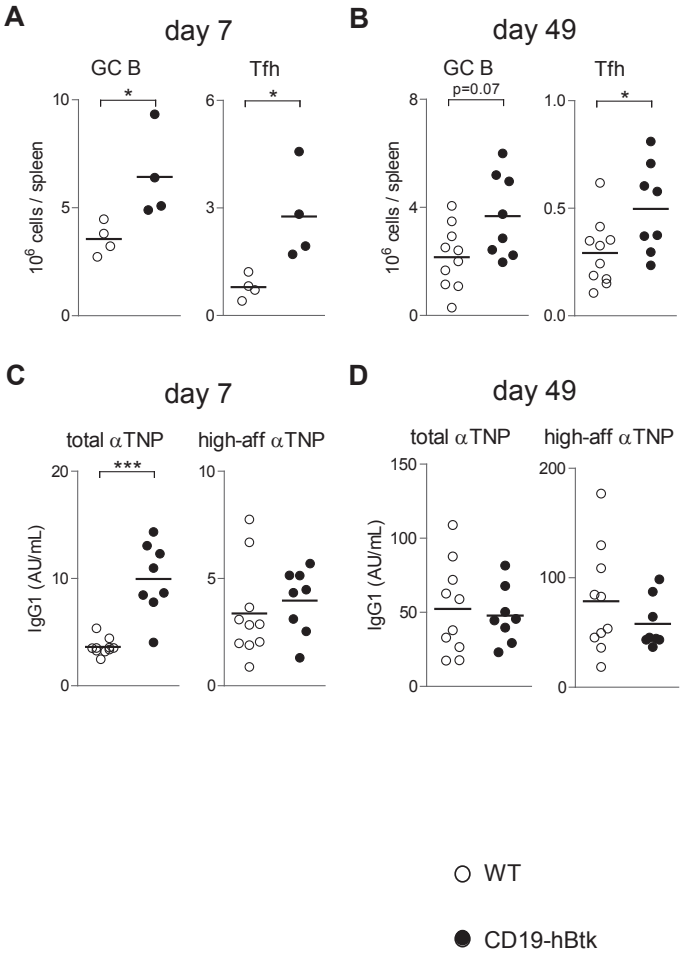

Figure 3. Affinity maturation is unaffected in CD19-hBtk mice upon TNP-KLH immunization.
[A-B] Splenic GC B and follicular T helper cells 7 days after primary immunization [A] and 7 days after secondary immunization (day 49) [B] with TNP-KLH. [C-D] Total and high affinity anti-TNP IgG1 antibodies in serum 7 days after primary immunization [C] and 7 days after secondary immunization (day 49) with TNP-KLH [D]. Lines indicate mean values; *p<0.05; ***p<0.001.

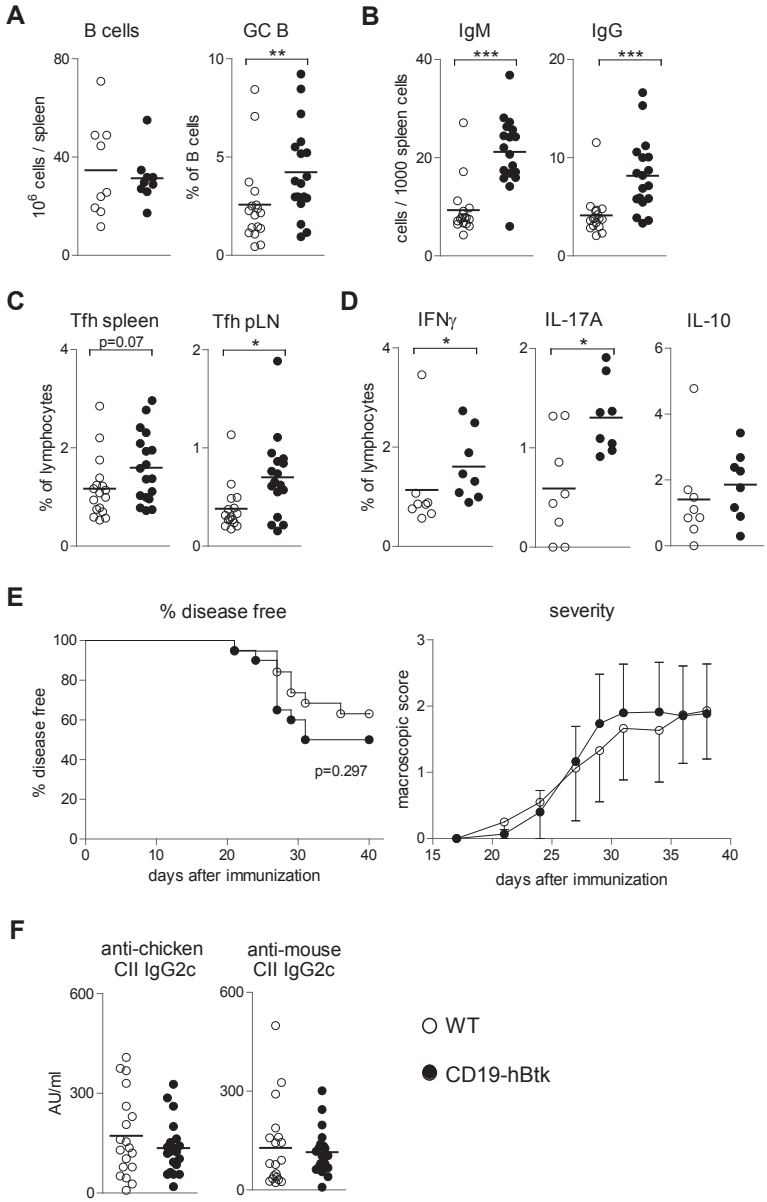

Figure 4. Enhanced Tfh formation and cytokine production in CIA in CD19-hBtk mice.

[A] Total splenic B cells and proportions of GC B cells in CIA. [B] Splenic IgM and IgG plasma cells in CIA. [C] Proportions of follicular T helper cells in spleen and popliteal lymph node in CIA. [D] Proportions of IFNγ, IL-17A and IL-10 producing CD4+ T cells in popliteal lymph nodes in CIA. [E] Proportion of disease free animals over time (n=20 CD19-hBtk and n=19 WT mice) and macroscopic arthritis score in diseased animals (redness and swelling of all paws scored, total maximum score is 8, animals were euthanized for ethical reasons at score ≥6). [F] Serum anti-chicken and anti-mouse CII-specific IgG2c antibodies 38 days after immunization. Lines indicate mean values; *p<0.05; **p<0.01; ***p<0.001.

Defects in the B cell compartment in CD19-hBtk mice are partially T-cell dependent

To determine the contribution of T cells to the autoimmune pathology in CD19-hBtk mice, we crossed these mice on a CD40L-deficient background. CD40L is expressed on activated T cells and engages CD40 on B cells, thereby facilitating T-B cell interaction, and is essential for the formation of GCs (42). Total B cell numbers in the spleens of ~30-33-week old *Cd40l*$^{-/-}$ CD19-hBtk mice were slightly decreased compared to WT, CD19-hBtk or *Cd40l*$^{-/-}$ mice (**Figure 5A**). Proportions of immature, follicular and marginal zone B cells were comparable in the four mouse groups (data not shown; **Suppl. Figure 4A**). Interestingly, IL-6 expressing and IFN γ expressing B cells, but not IL-10 expressing B cells, were restored to normal proportions in *Cd40l*$^{-/-}$ CD19-hBtk mice (**Figure 5B**). This would be consistent with the finding that many of the IL-10 expressing B cells have a MZ or B-1 phenotype (**Suppl. Figure 1B**), which are B cells involved in T-cell independent responses (43). Also CD86 expression on IgD^{+}CD95^{-} naïve B cells was increased in CD19-hBtk mice compared to WT, but not in *Cd40l*$^{-/-}$ CD19-hBtk mice (**Figure 5C**). This finding suggests an interaction of T cells with naïve B cells. Indeed, enhanced cytokine production by T cells or differentiation into both PD1-high and PD1-intermediate follicular T cells was not observed in aging *Cd40l*$^{-/-}$CD19-hBtk mice (**Figure 5D and 5E**).

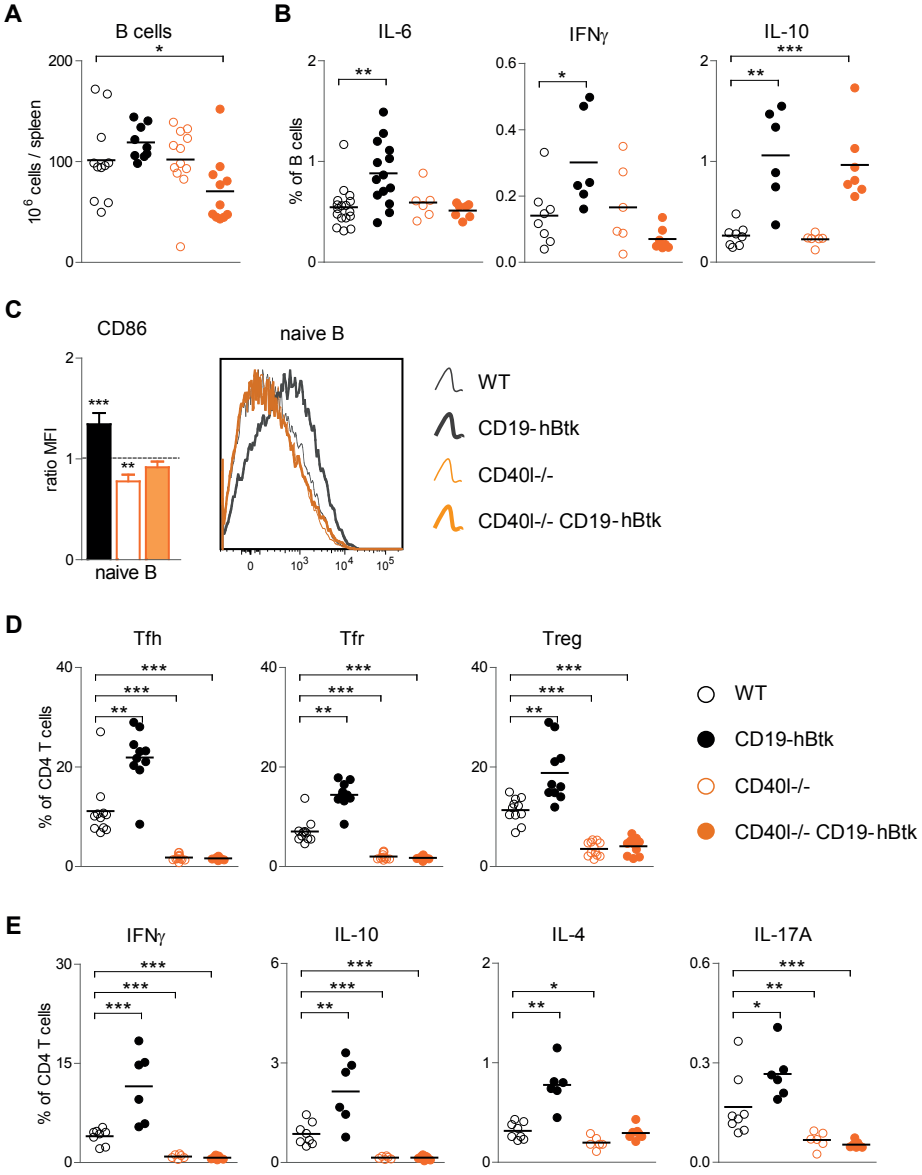

Figure 5. B-T cell interaction through CD40-CD40L is required for increased B and T cell activation in CD19-hBtk mice.

[A] Total splenic B cell numbers in WT littermate controls, CD19-hBtk, *CD40l-/-* and *CD40l-/-* CD19-hBtk mice. [B] Proportions of splenic IL-6 and IL-10 producing B cells. [C] CD86 expression in naïve B cells. Bar graph depicts relative MFI (MFI/average MFI of WT mice). [D] Proportions of splenic follicular T helper (CD4+CXCR5+PD1+FoxP3-), regulatory T (CD4+CXCR5-PD1-FoxP3+), follicular regulatory T (CD4+CXCR5+PD1+FoxP3+) and residual T helper cells (CD4+CXCR5-PD1-FoxP3-). [E] Proportions of cytokine producing CD4+ T cells. Lines indicate mean values; *p<0.05; **p<0.01; ***p<0.001.

As expected, GC B cells were lacking in *Cd40l*$^{-/-}$ and *Cd40l*$^{-/-}$ CD19-hBtk mice (**Figure 6A**). The increase in the proportions of memory B cells and IgG^{+} plasma cells, as seen in CD19-hBtk mice, was absent in *Cd40l*$^{-/-}$ CD19-hBtk mice (**Figure 6A, 6B**). However, the numbers of IgM^{+} plasma cells in the spleens of *Cd40l*$^{-/-}$ CD19-hBtk mice were comparable to those in CD40L-expressing CD19-hBtk mice and were significantly increased compared to WT or *Cd40l*$^{-/-}$ control mice (**Figure 6B**; **Suppl. Figure 4B**). The autoimmune phenotype of CD19-hBtk mice featured IgM and IgG antibody deposition in the kidneys (21). In contrast, histological analysis of the kidneys from *Cd40l*$^{-/-}$ CD19-hBtk mice revealed significant IgM, but no IgG deposition, nor thickening of glomerular membranes (**Figure 6C** and data not shown). Accordingly, serum IgM from these mice clearly displayed more autoreactivity in a Hep2 assay than did IgM from WT or *Cd40l*$^{-/-}$ mice, revealing a cytoplasmic staining pattern comparable to IgM from CD19-hBtk mice (**Figure 6E**). In contrast to our previous findings in CD19-hBtk mice (21), no autoreactive IgG antibodies were found in serum of *Cd40l*$^{-/-}$ CD19-hBtk mice by Hep2 staining, and anti-DNA IgG antibodies in serum were absent in *Cd40l*$^{-/-}$ CD19-hBtk mice (**Figure 6F, 6G**). Total serum IgM in CD19-hBtk and *Cd40l*$^{-/-}$ CD19-hBtk mice was not increased compared with WT mice (data not shown). Infiltration of various organs, including lung, kidney and salivary gland by immune cells was found in CD19-hBtk (21) but not in *Cd40l*$^{-/-}$ CD19-hBtk mice, indicating that the autoimmune pathology induced by BTK overexpression in B cells fully dependent on B-T cell interaction (shown for salivary glands in **Figure 6D**).

Taken together, these data revealed that overexpression of Btk in B cells resulted in defects that were partly T cell-independent, such as increased IL-10 expression by B cells and elevated levels of circulating autoreactive IgM antibodies, and partly T cell-dependent, including increased CD86 expression on naïve B cells, increased GC and memory B cell formation, enhanced IL-6 production, autoreactive IgG antibodies in serum, IgG antibody deposition in kidneys and immune infiltration of target organs.

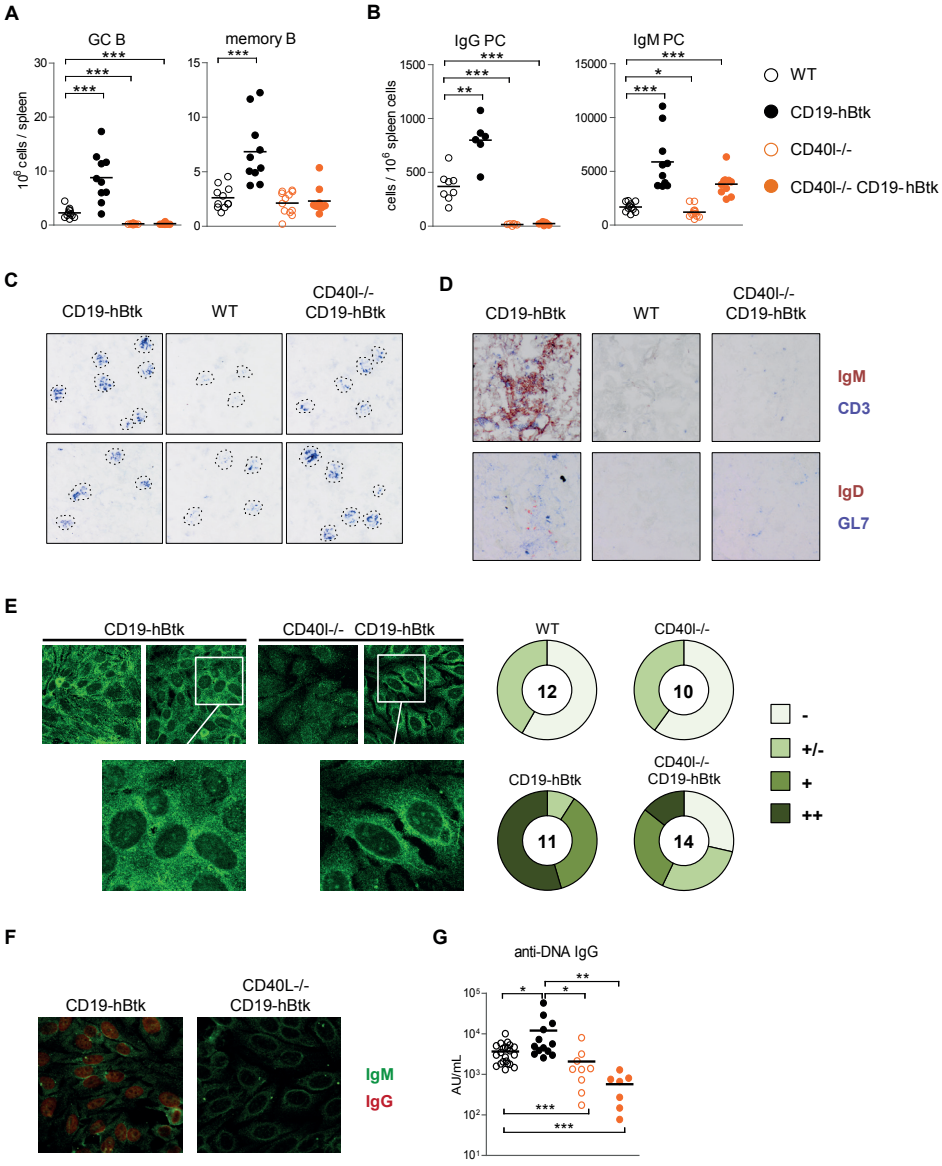

Figure 6. IgM auto-antibody formation in aged CD19-hBtk mice is T cell independent.
[A] Total numbers of splenic GC and memory B cells. [B] Splenic IgG+ and IgM+ plasma cells. [C] Histological analysis of kidney sections for glomerular IgM deposition (blue). [D] Histological analysis of salivary gland sections for CD3+ T cells (blue) and IgM+ B cells (red) (top panel) and GL7+ GC B cells (blue) and IgD+ B cells (red) (lower panel). [E] Serum IgM antibody reactivity with Hep2 cells. Representative pictures are shown for two CD19-hBtk and *CD40l-/-* CD19-hBtk mice. Numbers in pie-charts indicate numbers of animals analyzed; – no staining, +/- mild staining, + moderate staining, ++ strong staining. [F] Representative pictures of serum IgM (green) and IgG (red) reactivity with Hep2 cells. [G] Anti-DNA IgG antibody levels in serum. Graphs represent 1-4 individual experiments; lines indicate mean values; *p<0.05; **p<0.01; ***p<0.001.

DISCUSSION

Various mouse models and patients with primary immunodeficiencies, including X-linked agammaglobulinemia resulting from Btk deficiency, show that B cells play a crucial role in the generation of Tfh cells (13, 44). Conversely, we now provide evidence that B cells with elevated Btk protein expression have the capacity to disrupt T cell homeostasis and to induce excessive Tfh differentiation associated with spontaneous GC formation and autoimmunity. In aging mice, Btk overexpressing B cells engage T cells in a positive feedback loop that establishes T-cell propagated systemic autoimmune pathology. Btk overexpression induces IL-6 production in B cells *in vivo* (through a CD40L-dependent pathway) and promotes IFNγ production and surface ICOS expression by T cells, as well as the formation of Tfh cells.

Mouse crosses on the *Cd40l*$^{-/-}$ background showed that the anti-nuclear IgG associated autoimmune pathology in aging CD19h-hBtk mice required active involvement of T cells. Autoimmunity develops despite enhanced Treg and Tfr cell differentiation and increased IL-10 production by both B and T cells. Apparently, these Tfr cells, which are thought to be responsible for limiting the GC response (45, 46), fail to inhibit reciprocal activation of B and Tfh cells in aging CD19-hBtk mice. The inhibitory function of Treg and Tfr cells requires surface CTLA4 expression (47, 48), which in CD19h-hBtk mice was comparable to WT mice. Also CTLA4 on the surface of Tfh cells has the capacity to inhibit B cell function (47, 48). Because CTLA4 expression was even increased in CD19-hBtk mice (Fig. 2C), we conclude that Btk overexpression in B cells reduces their susceptibility to inhibitory signals from T cells. This could be explained by high expression of CD80 and CD86 on B cells induced by Btk overexpression (21), since it has been shown that transfer of CD80- and CD86-expressing B cells alone was sufficient to induce Tfh formation (47).

Induction of Tfh by CD19-hBtk B cells in the context of spontaneous arising GCs, as seen in aging mice, could be enhanced in young animals, either by immunization with a model antigen or in a CIA autoimmune model. Primary immunization with the model antigen TNP-KLH resulted in higher serum levels of low-affinity anti-TNP antibodies in CD19-hBtk mice than in wild-type mice. Because CD19-hBtk B cells are more resistant to FAS-mediated apoptosis (21), it is likely that B cells harboring low affinity BCRs that would normally be counter selected in a GC response can survive. However, this did not lead to enhanced formation or maintenance of low-affinity memory B cells, as levels of both total and high-affinity anti-TNP IgG1 antibodies were comparable after secondary immunization. Thus, repertoire selection after secondary immunization did not appear to be affected by the relative resistance to FAS-mediated apoptosis of CD19-hBtk transgenic GC B cells, showing that low-affinity CD19-hBtk transgenic B cells still had a selective disadvantage when competing with high-affinity antigen-binding CD19-hBtk transgenic B cells. Likewise, in the CIA experiments, the Tfh numbers in draining lymph nodes were increased in CD19-hBtk mice, but this was not associated with higher disease scores or enhanced anti-mouse CII auto-antibody formation. From these findings, we conclude that affinity maturation in CD19-hBtk mice is unperturbed and that the induced Tfh cells are functional in BCR repertoire selection. We hypothesize that Tfh cells in CD19-hBtk mice may recognize peptides from nucleic acid-

associated proteins, whereby the formation of pathogenic anti-nuclear antibodies depends on TLR signaling in B cells, as was previously shown for the extrafollicular plasmablast response in MRL.fs (lpr) mice (49). In addition, the increased GC B cell and Tfh development in CD19-hBtk mice depend on TLR signaling, as numbers were restored to wild type levels after crosses with MyD88-deficient mice (OBJC and RWH, unpublished). An efficient GC-dependent autoreactive BCR repertoire selection mechanism in aging mice would convert specificities of circulating antibodies from quite weak anti-cytoplasmic IgM (as also seen in *Cd40l*$^{-/-}$ mice) into strong anti-nuclear IgG autoantibodies. The patterns of Hep2 reactivity that we found for IgM antibodies suggest that they bind to ribosomes, similar to patterns found in SLE patients.

Although CD40L is primarily expressed on activated CD4+ T cells and mostly studied because of its prominent role B-T cell interaction, CD40L is also present on several other cell types, including activated B cells, platelets, DCs and smooth muscle cells (50). It is therefore conceivable that - next to B-T cell interaction - additional immunological pathways involved in autoimmunity may be hampered in *Cd40l*$^{-/-}$ mice. In this context, it is relevant that it has been shown that DCs can express both CD40 and CD40L and have the capacity to directly influence various B cell processes, including proliferation, differentiation and Ig class-switch recombination (50-52). However, although marginal zone DCs were found to induce robust T-cell dependent antibody responses, they did not induce GCs or long-lived B cell responses but rather invoke T-cell dependent extra-follicular antibody responses after antigenic stimulation (52). Therefore, direct DC-B cell interactions do not likely explain the phenotype of CD19-hBTK Tg mice that is characterized by spontaneous GC formation.

Taken together, our findings support a model in which Btk overexpression induces spontaneous GCs and autoimmune pathology by upregulation of CD80 and CD86, which promotes Tfh cell formation and at the same time obstructs CTLA4-dependent regulation by Tregs and Tfr cells. As a result of ongoing B-Tfh cell interaction, B cells increase IL-6 production and T cells exhibit excessive IFNγ production and surface expression of ICOS, which lead to pathogenic accumulation of GCs and plasmablasts that produce anti-nuclear autoantibodies.

To date, several studies investigating the efficacy of BTK inhibitors in rodent models for autoimmune diseases, including SLE and RA, have shown a beneficial effect on disease progression (25-29). Although effects of BTK can partly be explained by direct effects on B cells and autoantibody production, BTK inhibition clearly affects other cell types, including monocytes, macrophages and mast cells, for example through disruption of FcR signaling in myeloid cells. However, it is conceivable that interference with B-T cell interaction may also significantly contribute to therapeutic effect of BTK inhibition. The observed response in SLE and pSS patients to BAFF/BLyS-neutralizing antibody Belumimab further implies that targeting B cell survival and activation is an effective treatment strategy (53, 54). Interestingly, in human B cells, BTK protein levels increase upon activation *in vitro* and BTK levels are increased in human primary Sjögren's syndrome patients *in vivo* (OBJC and RWH, unpublished). Because the BTK inhibitors ibrutinib and acalabrutinib, which show high

response rates as a monotherapy in patients with various B cell malignancies, is generally w*ell tolerated (55, 56),* targeting BTK could be a promising new therapeutic strategy in the treatment of systemic autoimmune diseases.

In summary, we have established that increased expression of Btk is sufficient to induce excessive Tfh differentiation and spontaneous GC formation associated with the production of pathogenic anti-nuclear autoantibodies. We conclude that in autoimmune disease BTK-mediated signaling in B cells establishes or maintains T cell-propagated pathology, making BTK an attractive therapeutic target.

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SUPPLEMENTAL MATERIAL

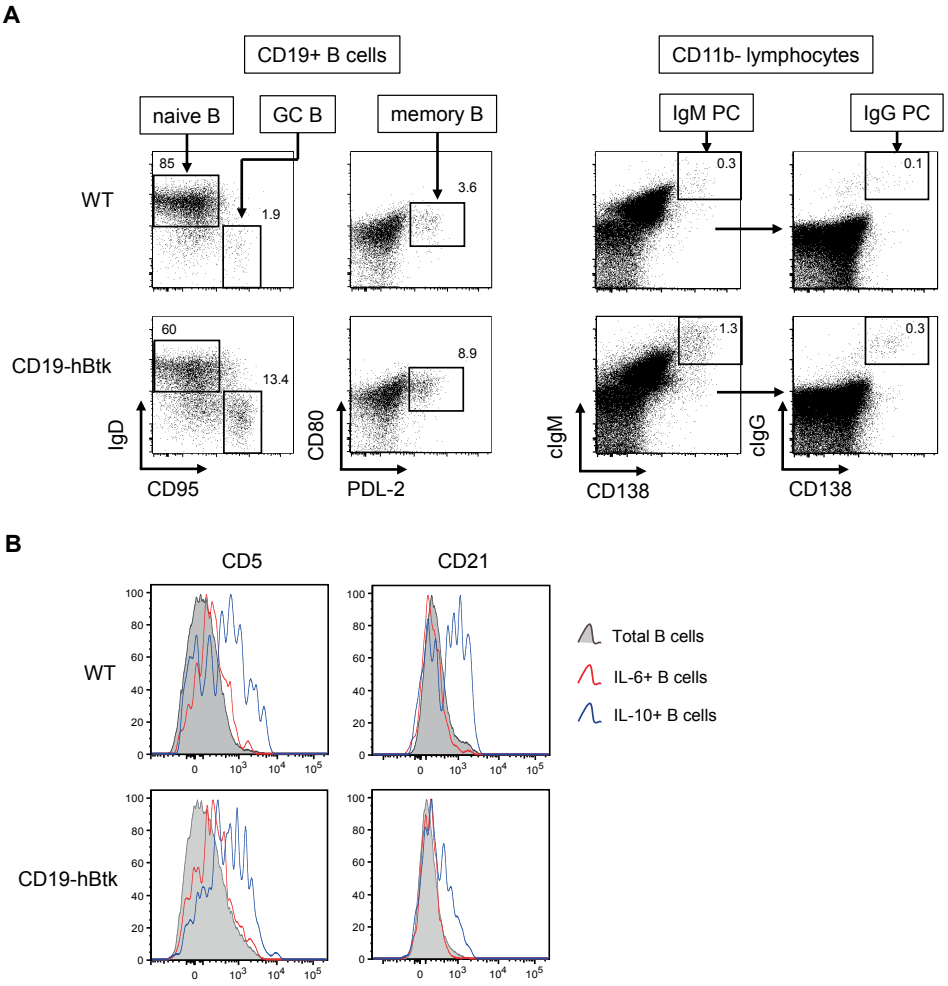


Figure S1. [A] Gating strategy and representative flow plots of different B cell subsets (left panel): naïve (CD19+IgD+CD95-), GC (CD19+IgD-CD95+) and memory (CD19+PDL2+CD80+) B cells, and of plasma cells (right panel): IgM plasma cells (CD11b-CD138+cIgM+) and IgG plasma cells (CD11b-CD138+cIgM-cIgG+). [B] CD5 and CD21 expression in IL-6 expressing (red), IL-10 expressing (blue) and total splenic B cells as control (gray) in ~30-33 week old WT and CD19-hBtk mice. [B] is representative for n=3 per group.

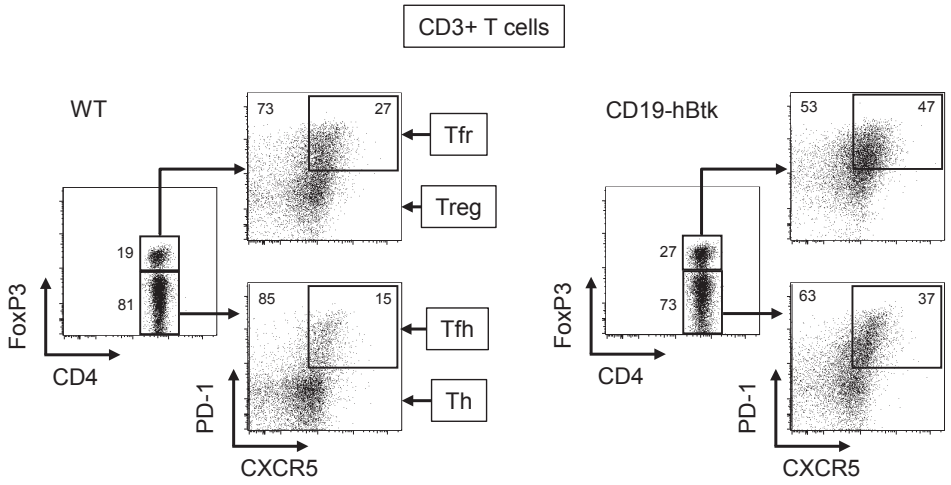


Figure S2. Gating strategy and representative flow plots of different T cell subset: splenic follicular T helper (CD4+CXCR5+PD1+FoxP3-), regulatory T (CD4+CXCR5-PD1-FoxP3+), follicular regulatory T (CD4+CXCR5+PD1+FoxP3+) and residual T helper cells (CD4+CXCR5-PD1-FoxP3-).

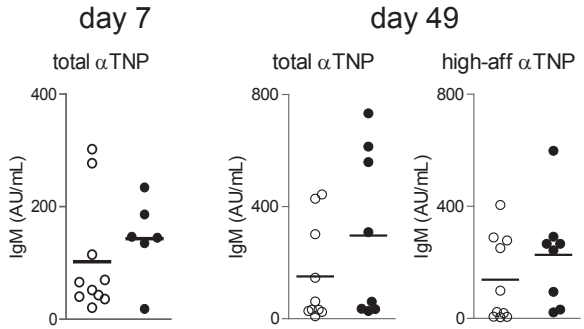


Figure S3. Total anti-TNP IgM antibodies in serum 7 days after primary immunization (day 7) and total and high-affinity anti-TNP IgM antibodies 7 days after secondary immunization (day 49) with TNP-KLH. Lines indicate mean values.

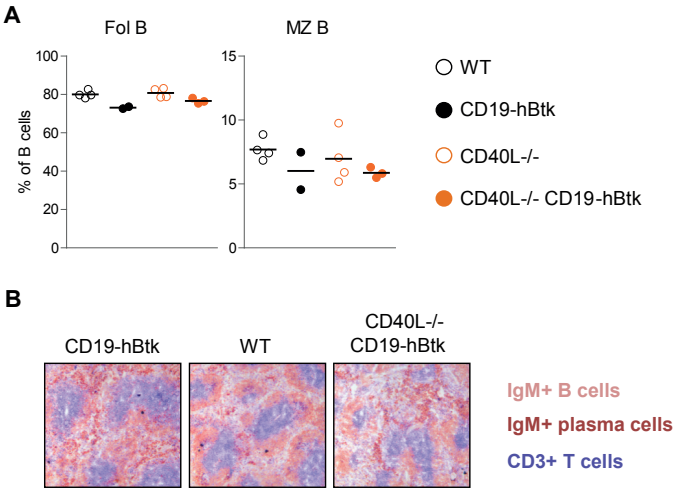
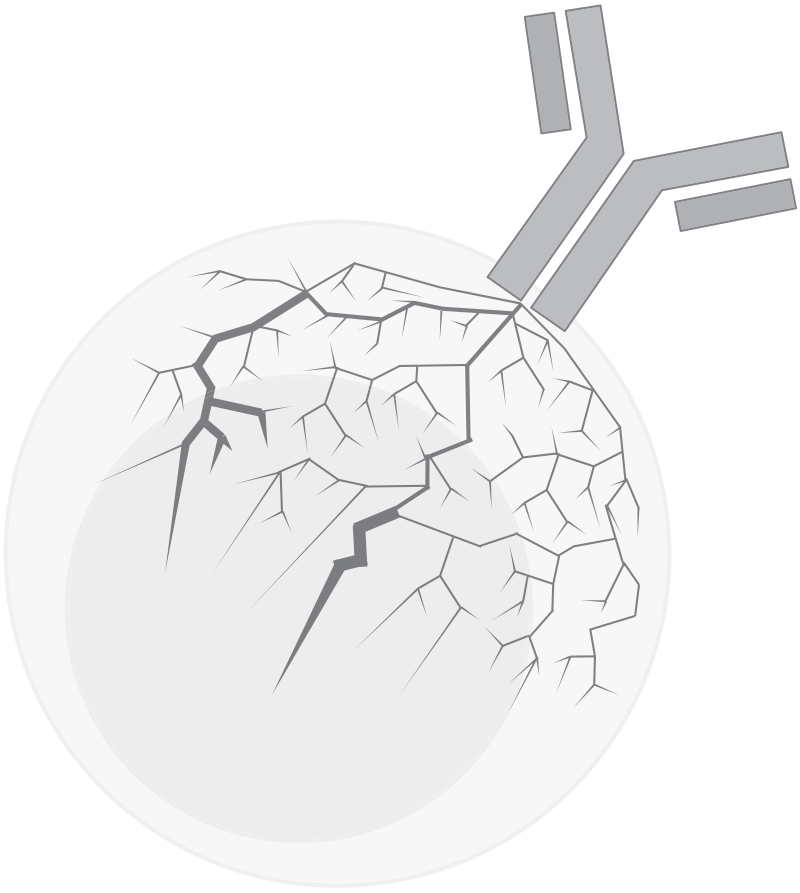


Figure S4. [A] Proportions of splenic follicular (CD21+/-CD23+) and marginal zone (CD21hi CD23-) B cells in WT littermate controls, CD19-hBtk, CD40l-/- and CD40l-/- CD19-hBtk mice. [B] Histological analysis of IgM+ plasma cells in the spleen; CD3+ T cells (blue), IgM+ B cells (light red), IgM+ plasma cells (dark red).



CHAPTER 7

Toll-Like Receptor signaling drives Btk-mediated autoimmune disease

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ABSTRACT

Bruton′s tyrosine kinase (Btk) is a signaling molecule involved in development and activation of B-cells through B-cell receptor (BCR) and Toll-like receptor (TLR) signaling. We have previously shown that transgenic mice that overexpress human Btk under the control of the CD19 promoter (CD19-hBtk) display spontaneous germinal center formation, increased cytokine production, anti-nuclear autoantibodies and systemic autoimmune disease upon aging. As TLR and BCR signaling are both implicated in autoimmunity, we studied their impact on splenic B-cells. Using phosphoflow cytometry, we observed that phosphorylation of ribosomal protein S6, a downstream Akt target, was increased in CD19-hBtk B cells following BCR stimulation or combined BCR/TLR stimulation, when compared with wild-type B cells. The CD19-hBtk transgene enhanced BCR-induced B cell survival and proliferation, but had an opposite effect following TLR9 or combined BCR/TLR9 stimulation. Although the expression of TLR9 was reduced in CD19-hBtk B cells compared to wild-type B cells, a synergistic effect of TLR9 and BCR stimulation on the induction of CD25 and CD80 was observed in CD19-hBtk B cells. In splenic follicular and marginal zone B cells from aging CD19-hBtk mice BCR signaling stimulated *in vitro* IL-10 production in synergy with TLR4 and particularly TLR9 stimulation, but not with TLR3 and TLR7. The enhanced capacity of CD19-hBtk follicular B cells to produce the pro-inflammatory cytokines IFNγ and IL-6 compared with wild-type B cells was however not further increased following *in vitro* BCR or TLR9 stimulation. Finally, we used crosses with mice deficient for the TLR-associated molecule myeloid differentiation primary response 88 (MyD88) to show that TLR signaling was crucial for spontaneous formation of germinal centers, increased IFNγ and IL-6 production by B cells and anti-nuclear autoantibody induction in CD19-hBtk mice. Taken together, we conclude that high Btk expression does not only increase B cell survival following BCR stimulation, but also renders B cells more sensitive to TLR stimulation, resulting in increased expression of CD80 and IL-10 in activated B cells. Although BCR-TLR interplay is complex, our findings show that both signaling pathways are crucial for the development of pathology in a Btk-dependent model for systemic autoimmune disease.

INTRODUCTION

B cells are crucial players in autoimmunity, as B cell depletion therapy was proven effective in patients with several systemic autoimmune diseases including Sjögren's syndrome (SjS) and rheumatoid arthritis (RA) (1, 2). These diseases are marked by altered B cell selection leading to the production of autoreactive antibodies, an important hallmark in the pathology of systemic autoimmune diseases.

Signaling via the B cell receptor (BCR) is essential for B cell survival (3). B cells are selected in the bone marrow (BM) at the large pre-B cell and the immature B cell stage for functional rearrangements of the immunoglobulin (Ig) heavy and light chain genes, respectively. Subsequently, checkpoints follow to select non-self-reactive B cells, both in the BM and in the periphery where maturing B cells, referred to as transitional B cells, undergo stringent selection. BCR signaling is crucial for the selection of B cells. However, evidence is accumulating that additional signals derived from CD40, Toll-like receptors (TLRs) and BAFFR also affect selection of B cells (4).

A crucial signaling molecule in the development, survival and activation of B cells is Bruton's tyrosine kinase (Btk), a member of the Tec family of non-receptor kinases. Btk is expressed in almost all cells of the hematopoietic lineage, except T cells and plasma cells (5, 6) and the expression of appropriate levels of Btk is crucial for normal B cell development (7, 8). Functionally, Btk is critically involved in many signaling pathways, such as BCR, TLR and chemokine receptor signaling (9). Patients with loss-of-function mutations in the *BTK* gene present with X-Linked agammaglobulinemia (XLA), an inherited immunodeficiency marked by an almost complete arrest of B cell development at the pre-B cell stage in the BM and a near absence of peripheral B cells and circulating Ig (10, 11). In mice, Btk-deficiency does not result in an arrest in B cell development in the BM, although pre-B cell differentiation is somewhat impaired; due to a defective transitional B cell maturation the numbers of peripheral B cells are decreased (12-14). We have previously shown that BTK protein levels are different across human peripheral blood B cell subsets (15). Moreover, both in human and in mice BTK protein levels are upregulated when mature B cells are activated *in vitro* by various signals including those initiated by BCR, TLR and CD40 stimulation (8). Taken together, these findings demonstrate the importance of Btk and indicate that its expression is tightly regulated.

We have generated transgenic mice that overexpress human Btk (hBtk) under the control of the CD19 promoter region (CD19-hBtk). B cells from these mice show increased survival and cytokine production and have the capacity to engage T cells in spontaneous germinal center (GC) formation (8). CD19-hBtk transgenic mice develop autoimmune pathology, characterized by lymphocyte infiltrates in several tissues including salivary glands and production of anti-nuclear autoantibodies (ANAs), which was observed from the age of 25 weeks onwards (8). This Btk-mediated autoimmunity phenotype is largely dependent on interaction with T cells (16) and resembles human systemic lupus erythematosus (SLE) and SjS. Human autoimmune disease is also associated with increased BTK expression: we recently showed that patients with RA and SjS have increased BTK protein levels in B cells

from peripheral blood, compared with healthy controls (15). It remains unclear, however, whether the hBtk-mediated autoimmune phenotype in the mouse strictly depends on BCR signaling or on additional signaling pathways.

The role of TLR signaling in the development of autoimmune diseases has been widely studied (17-25) and synergistic signaling of the BCR and TLRs has been implicated in systemic autoimmune disease in animal models (21, 26). Several lines of evidence indicate that Btk is critically involved in this BCR-TLR synergy. Btk can directly interact with the myeloid differentiation primary response 88 (MyD88) protein (27), an adaptor molecule downstream of many TLRs. Interestingly, TLR9 stimulation appears to affect B cell differentiation, as it was recently shown that engagement of TLR9, which recognizes dsDNA, can antagonize antigen processing and affinity maturation of antigen-specific B cells (28). The relevance of Btk in TLR-mediated B cell activation is supported by the finding that Btk-deficient B cells produced less IL-10 upon TLR9 stimulation compared with B cells with physiological Btk levels (29). In addition, Btk was shown to mediate synergistic signaling between the BCR and TLR9 (30), which is crucial for activation of autoreactive B cells (26). Therefore it is conceivable that BCR-TLR synergy contributes to the initiation or maintenance of the autoimmune phenotype of BTK overexpressing transgenic mice.

In this report, we aimed to determine the contribution of TLR signaling and BCR-TLR synergistic signaling to B cell activation in our mouse model of Btk-mediated autoimmune disease. Because the anti-dsDNA autoantibodies are prominent in aging CD19-hBtk transgenic mice (8), we focused hereby on TLR9. We first analyzed phosphorylation of various BCR and TLR downstream signaling molecules in splenic B cells. Consistent with increased survival of Btk-overexpressing B cells, when compared with wild-type (WT) B cells, CD19-hBtk transgenic B cells displayed increased phosphorylation of the ribosomal protein S6 upon BCR stimulation. Although the expression of TLR9 was reduced in CD19-hBtk B cells compared to wild-type B cells, a robust synergistic effect of TLR9 and BCR stimulation on S6 phosphorylation, CD25 and CD80 expression, and IL-10 production was observed in CD19-hBtk B cells. Together with the finding that TLR signaling was crucial for CD19-hBtk-mediated autoimmunity *in vivo*, these results point to a role of Btk in BCR-TLR synergy in the context of autoimmune disease development.

METHODS

Mice and genotyping

CD19-hBtk (31), Btk-deficient (12) and *IgH.TE*μ mice (32) were previously described. *Myd88*$^{LSL/LSL}$ (*Myd88*$^{-/-}$) mice (33) were crossed on CD19-hBtk mice to create the *Myd88*$^{-/-}$ CD19-hBtk line. Mice were genotyped by PCR and MyD88-sufficient non-hBtk transgenic littermates or C57/BL6 mice (Charles River) were used as WT controls. *Myd88*$^{-/-}$ mice were crossed on *IgH.TE*μ mice (>F3 sv129xC57BL/6) and onset of leukemia was monitored every 3-6 weeks by peripheral blood screening for monoclonal B cell expansion. Mice were bred and kept under specified pathogen-free conditions in the Erasmus MC experimental animal facility. All experimental protocols were reviewed and approved by the Erasmus MC Committee of animal experiments (DEC).

Flow cytometry procedures and calcium influx

Cell suspensions of spleen and BM were obtained using 100 μm cell strainers in magnetic-activated cell sorting (MACS) buffer (PBS/0.5% BSA/2mM EDTA), as previously described (16). 2x10^6 cells were incubated with varying combinations of monoclonal antibodies (**Table S1A**) and stained according to previously described procedures, whereby isotype and fluorescence minus one (FMO) controls and non-expressing cells were used to set-up and validate the staining procedures (8). To stain for the isotype of intracellular immunoglobulins (Ig) in plasma cells, cells were fixed with BD Cytofix/Perm Buffer (BD Biosciences) and permeabilized with BD Perm/Wash Buffer (BD Biosciences). The eBioscience FoxP3 staining kit (eBioscience) was used to fix and permeabilize cells to stain for FoxP3 expression. For intracellular staining of cytokine-expressing cells, samples were fixed in PBS/2% paraformaldehyde and permeabilized and stained in MACS buffer containing 0.5% saponin (Sigma-Aldrich). For measuring intracellular calcium mobilization, 5x10^6 splenocytes were incubated with fluorogenic probes Fluo3-AM and Fura Red-AM (Life Technologies), essentially as previously described (34), except that we stained for B220$^+$ splenocytes. Cell cycle staining using propidium iodide (PI) was performed as previously described (14). Leukemic B cells (CD19$^+$CD5$^+$) were stained with FITC-labeled phosphatidylcholine (PtC) liposomes (DOPC/CHOL 55:45, Formumax Scientific Inc.) in MACS Buffer. All measurements were performed on an LSRII flow cytometer (BD Biosciences) and results were analyzed using FlowJo Version 9.7.6 software (TreeStar Inc).

Phosphoflow cytometry

To determine phosphorylation of intracellular proteins 0.5-1x10^6 cells were cultured in 2% FCS in RPMI at 37°C for 5 minutes (pCD79a, pSyk and pPLCγ2) or for 3 hours (pS6 and pAkt) with 20 μg/mL anti-mouse antigen-binding F(ab')$_2$ -IgM fragments (αIgM; Jackson

ImmunoResearch), 2 μM (for CD79a, pPLCγ2 and pAkt) or 0.1 μM (pS6) CpG (ODN 1668; Invitrogen) or combinations thereof prior to fixation with the eBioscience FoxP3 staining kit Fix/Perm solution (eBioscience). Cells were centrifuged and washed twice with eBioscience FoxP3 staining kit Perm/Wash solution (eBioscience) and subsequently stained for 30 minutes at 4°C with markers to identify B cells and T cells (**Table S1B**), followed by staining for the appropriate phospho-target (30 minutes at RT). Cells stained for pS6 were subsequently stained with anti-rabbit PE antibody (Jackson ImmunoResearch) 15 minutes at RT. Isotype and FMO controls were included in the set-up of the staining procedure, verifying the signal intensities of the phospho-targets. In addition, non-expressing T cells were used as internal controls for BCR-restricted signaling molecules in all experiments. All measurements were performed on an LSRII flow cytometer (BD Biosciences), and results were analyzed using FlowJo Version 9.7.6 software (TreeStar Inc).

In vitro stimulation for cytokine expression

To measure cytokine-expressing lymphocytes, splenic cell suspensions were stimulated for four hours at 37°C using 50 ng/ml *Phorbol* 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both Sigma-Aldrich), 10 μg/mL αIgM (Jackson ImmunoResearch), 200 μg/mL Poly:IC (Invivogen), 1.6 μg/mL Lipopolysaccharide (LPS; Sigma-Aldrich), 40 μg/mL imidazoquinoline (Imiquimod VacciGrade™; Invivogen) and/or 2 μM CpG (ODN 1668; Invitrogen) in combination with monensin (Golgistop; BD Biosciences). To evaluate the effect of Btk inhibition, 1 μM ibrutinib (PCI-32765; Sigma-Aldrich) was added to the *in vitro* cultures.

MACS purification and *in vitro* B cell cultures

Splenic cell suspensions from CD19-hBtk and WT control mice were prepared in MACS buffer. MACS procedure and culture was performed as previously published (8). Cells were stained with biotinylated antibodies (**Table S1C**) followed by streptavidin-coupled magnetic beads (Miltenyi Biotec) and unlabeled naïve B2 cell fractions were collected by magnetic depletion of labeled cells with a purity of >92%. Purified naïve B cells were stimulated for 48 hours to evaluate cell cycle progression and 48 or 72 hours for activation markers with 10 μg/mL αIgM (Jackson ImmunoResearch), 2 μM CpG (ODN 1668; Invitrogen) or combinations thereof in culture medium (RPMI 1640/ 10% FCS/ 50 μg/mL gentamycin/ 0.05 mM ß-mercaptoethanol). To evaluate the effect of Btk inhibition, 1μM ibrutinib (PCI-32765) was added to *in vitro* culture conditions.

Immunohistochemistry

Myd88$^{-/-}$CD19-hBtk, CD19-hBtk and age-matched WT control mice were sacrificed at 28-33 weeks of age. Salivary glands and kidneys were collected, embedded in O.C.T-compound

(Sakura) and stored at -80°C. Immunohistochemical staining was performed as previously described (35). Slides were washed with PBS and stained for 60 minutes with anti-CD3 (eBioscience), anti-IgM or anti-IgG2c rat anti-mouse antibodies (BD Biosciences), followed by a counterstaining with anti-rat Alkaline Phosphatase (AP)-labeled antibodies (Jackson ImmunoResearch). Anti-CD3-stained slides were stained for 60 minutes with IgM^{FITC} rat anti-mouse antibodies (BD Biosciences), followed by counterstaining with streptavidin, anti-FITC or anti-PE Peroxidase (PO)-labeled antibodies (Rockland). Slides were embedded in Kaiser glycerol gelatin (Merck).

HEp-2 reactivity assay

Serum samples of 28-33 week-old mice (diluted 1:100 in PBS) were incubated on HEp-2 slides (Bio-Rad Laboratories) for 60 minutes, as previously described (8). After washing with PBS, slides were incubated with Alexa Fluor-488 conjugated donkey anti-mouse IgM or IgG $F(ab')_2$ fragments (Jackson ImmunoResearch) for one hour. After washing and staining for 5 minutes with DAPI, slides were embedded in VectaShield (Vector Laboratories). The LSM 510 META confocal fluorescence microscope (Zeiss) was used to measure fluorescence intensity.

ELISA

Serum Ig subclasses were determined by sandwich ELISA. First, plates were coated with unlabeled anti-IgM, anti-IgG1 and anti-IgG2a (Southern Biotech) overnight at 4°C. The next day, serum and isotype standards (IgM, Bio-Rad; IgG1 and IgG2a, Southern Biotech) were serially diluted and incubated at RT for three hours. This was followed by an incubation of 30 minutes with biotinylated IgM, IgG1 and IgG2a-specific antibodies (Southern Biotech) and subsequently 30 minutes of incubation with streptavidin peroxidase-labeled antibodies (Jackson ImmunoResearch). Finally, we added 3, 3',5, 5'- Tetramethylbenzidine (TMB) substrate (SeraCare) and stopped the reaction by using sulfuric acid. Samples were read at an OD of 450 nm using the VersaMax Microplate Reader (Molecular Devices) to measure color intensity.

Line Immunoblot Assay (LIA) for extractable nuclear antigens

To measure extractable nuclear antigens, we used the INNO-LIA® ANA Update kit (Fujirebio) according to manufacturer's instructions. In short, LIA strips were incubated with serum samples of 28-33 week-old mice (diluted 1:200 in sample diluent (Fujirebio)) or with cut-off control containing human IgG positive control antibodies. Next, LIA strips were incubated with Alkaline Phosphatase (AP)-labeled anti-mouse IgG antibodies (Jackson ImmunoResearch). The cut-off control was incubated with the supplied Alkaline Phosphatase (AP)-labeled anti-human IgG antibodies (Fujirebio). Finally, we added 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate (Fujirebio) diluted in

substrate buffer and stopped the reaction by adding sulfuric acid (Fujirebio). The LIA strips were removed from the troughs and interpreted after they had completely dried. As reference, LIA strips from samples were compared to the supplied cut-off control and regarded positive when bands were stained more intensely than the cut-off control.

Statistical analysis

The non-parametric Mann-Whitney *U* test was used for statistical analyses. Log Rank test was used to calculate the significance for survival differences between indicated group of mice for the CLL experiments. Differences between groups with P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc).

RESULTS

CD19-hBtk transgenic B cells display increased signaling of the Akt pathway

Given the increased survival and activated phenotype of Btk-overexpressing (CD19-hBtk) B cells (8, 15), we first studied BCR signaling in splenic B cells from 8-week-old CD19-hBtk mice. To this end, we stimulated total wild-type (WT) and CD19-hBtk splenic cells with αIgM, the TLR9 ligand CpG or combinations thereof and determined the phosphorylation status of various signaling molecules by phosphoflow cytometry analysis of gated $B220^{+}CD3^{-}$ B cells (**Figure 1A;** gating strategy in **Figure S1A**). In these experiments, we used unstimulated gated $B220^{+}CD3^{-}$ B cells as a control.

Phosphorylation of Y182 in the ITAM of Ig-α/CD79a, the transmembrane protein that forms a complex with the BCR, was reduced in unstimulated CD19-hBtk B cells compared with WT controls (**Figure 1B)**. Expression of phosphorylated Y759 PLCγ2 (pPLCγ2) and Y348 Syk (pSyk) was similar in unstimulated WT and CD19-hBtk B cells (**Figure 1B**). As expected, in WT B cells phosphorylated CD79a (pCD79a) and pPLCγ2 were induced upon αIgM stimulation (**Figure 1A, 1B)**, but not upon CpG stimulation for CD79a and pPLCγ2 (**Figure S1B**). Both pCD79a and pPLCγ2 appeared somewhat reduced in αIgM-stimulated CD19-hBtk B cells, although this was not significant. BCR-induced pSyk was significantly reduced in CD19-hBtk B cells when compared with WT B cells (**Figure 1B**).

TLR9-induced phosphorylation (S240/S244) of S6, a ribosomal protein that is a downstream target of various signaling cascades, including the Akt pathway, appeared unchanged in CD19-hBtk B cells compared with WT controls (**Figure 1C**). However, significantly increased pS6 was seen in CD19-hBtk B cells upon BCR engagement alone or in combination with CpG stimulation (**Figure 1C**). Phosphorylation of Akt at S473/T308 upon stimulation with αIgM or CpG was comparable between CD19-hBtk and WT B cells, although CD19-hBtk B cells showed reduced pAkt upon combined BCR-TLR stimulation (**Figure 1C**). Taken together, these findings revealed limited effects of TLR stimulation on the BCR signaling pathway, whereas BCR stimulation induced S6 phosphorylation more strongly in CD19-hBtk than in WT B cells.

In addition, we investigated the expression levels of TLR7 and TLR9 protein as these TLRs are most relevant in the context of autoimmune disease (17-19, 23). Expression of TLR7 was similar in CD19-hBtk and WT splenic B cells *ex vivo* and following *in vitro* **αIgM stimulation (Figure 1D**). In contrast, TLR9 protein levels were decreased in CD19-hBtk B cells compared with WT controls, both *ex vivo* and upon 48 hours of *in vitro* stimulation with αIgM (**Figure 1E)**. This finding implies that CD19-hBtk and WT may have a different responsiveness to TLR9 ligands.

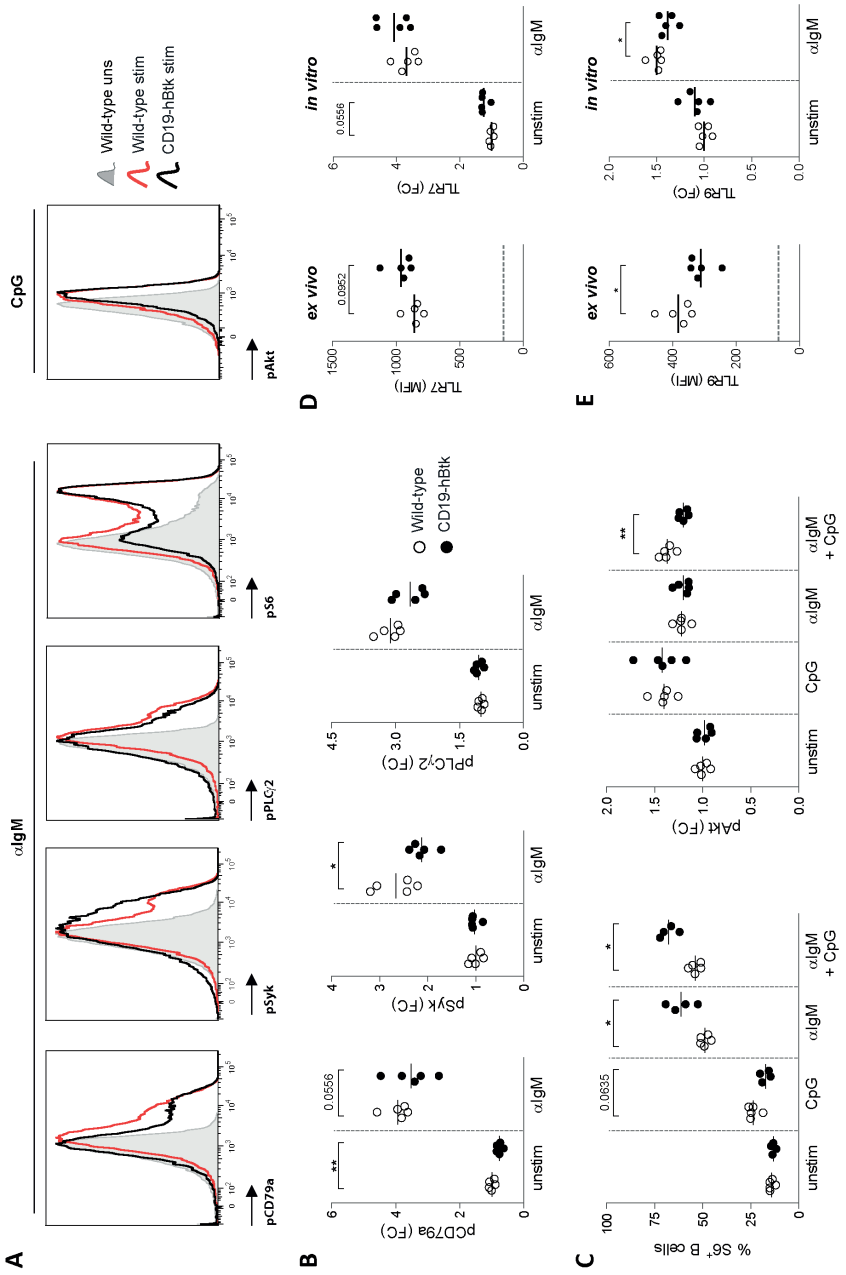

Figure 1. Increased S6 phosphorylation upon BCR engagement in CD19-hBtk B cells.
(**A**) Histogram overlays of representative examples of the phosphoprotein analyses are shown for αIgM-induced induction of pCD79a, pSyk, pPLCγ2 and pS6, as well as CpG-induced pAkt. (**B-C**) Splenic cells of wild-type and CD19-hBtk transgenic mice were stimulated for 5 minutes (CD79a, Syk and PLCγ2) or 3 hours (S6 and Akt)

and gated for B cells after the indicated *in vitro* stimulation. Fold change (FC) increase of median fluoresence intensity (MFI) values compared to WT unstimulated are shown for phosphorylation of CD79a, Syk, PLCγ2 (**B**) and Akt (**C**). Phosphorylation of ribosomal protein S6 was quantified using percentages of positive cells compared to unstimulated cells (**C**). (**D-E**) MFI values of TLR7 (**D**) and TLR9 (**E**) protein in splenic B cells *ex vivo* and MFI FC induction of these TLRs on MACS-purified B cells after 48 hours of stimulation with αIgM. Symbols represent individual mice and bars indicate mean values. Graphs represent one to two individual experiments, each with 4-5 mice per group; CD19-hBtk and WT mice were 8-10 weeks old; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

BCR and TLR9 signaling differentially affect CD19-hBtk B cell proliferation and survival

To study whether TLR9 responsiveness alters BCR-mediated activation of CD19-hBtk B cells, we MACS-purified naïve B cells from spleens of 8-week-old CD19-hBtk and WT mice and stimulated these fractions *in vitro* with αIgM, CpG or a combination thereof for 48 hours to evaluate cell cycle progression by propidium iodide (PI) staining. Consistent with our published findings (8), these analyses showed that upon BCR stimulation survival and proliferation was increased in B cells from CD19-hBtk mice, compared with WT littermates (**Figure 2A**). This effect was entirely dependent on Btk kinase activity, as the presence of the Btk small molecule inhibitor ibrutinib completely abrogated cellular proliferation in both WT and CD19-hBtk αIgM stimulated B cells (**Figure 2A**). In contrast, in single CpG-stimulation and combined αIgM/CpG-stimulation, Btk-overexpressing B cells showed reduced proliferation, compared with WT B cells (**Figure 2B,C**). This was dependent on Btk kinase activity, as Btk inhibition essentially leveled-out the differences between CD19-hBtk and WT B cells (**Figure 2B,C**). Intriguingly, addition of ibrutinib decreased the proliferation of CpG-stimulated WT B cells, suggesting that Btk kinase is part of the signaling pathway downstream of TLR9 that induces B cell proliferation. Therefore, both Btk inhibition and Btk overexpression resulted in reduced B cell proliferation following stimulation by CpG.

In summary, we found that Btk overexpression increases survival and proliferative responses upon BCR engagement, but limits the responsiveness to TLR9 stimulation.

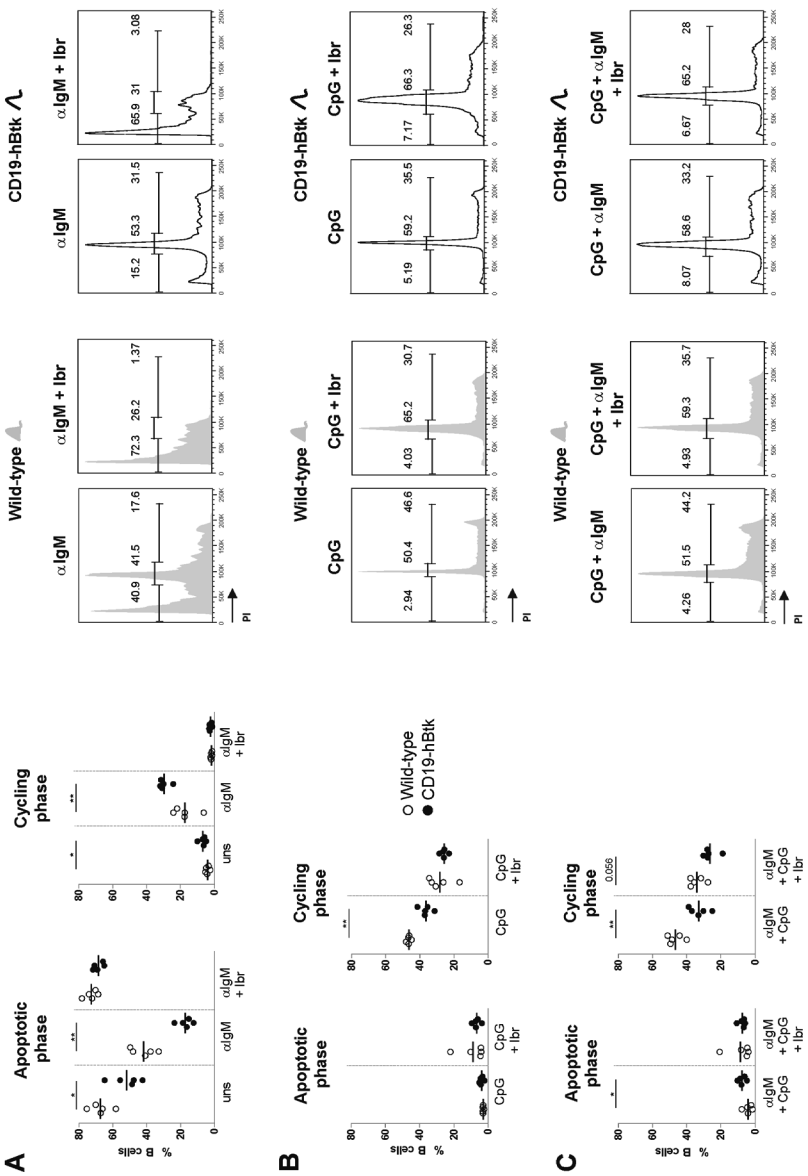

Figure 2. BCR and TLR9 stimulation have different effects on CD19-hBtk B cells.

Proportions of B cells in apoptotic or cycling fractions, as determined by propidium iodide (PI) DNA content staining of *in vitro* cultured purified naïve splenic B cells that were stimulated with 10 μg αIgM (**A**), 2μM CpG (**B**) or αIgM and CpG combined (**C**), with or without ibrutinib (Ibr, 1μM) as indicated (*left*). Symbols represent individual mice and bars indicate mean values. Representative flow cytometry graphs of WT and CD19-hBtk B cells are shown on the right, with the gating applied for apoptotic cells (sub-G1), resting cells (G0/G1) and dividing (S/G2/M) B cells. CD19-hBtk and WT mice were 8-10 weeks old; n = 4-5 per group; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

The activation status of CD19-hBtk B cells is increased following combined BCR and TLR9 stimulation

Next, we tested activation marker upregulation of naïve B cells of 8-week-old mice upon *in vitro* stimulation with αIgM, CpG and a combination thereof for 72 hours. No major differences were observed between WT versus CD19-hBtk B cells in the upregulation of CD86 expression (**Figure 3A**). *In vitro* stimulation with CpG or αIgM resulted in an induction of CD25/IL-2R and the costimulatory protein CD80 in B cells from both mouse groups (**Figure 3B,C**). Combined stimulation of αIgM and CpG decreased surface expression levels of CD86 and CD25 compared to αIgM alone, both in WT and in CD19-hBtk B cells (**Figure 3A,B**), whereas this was not the case for CD80 (**Figure 3C**). However, following CpG stimulation surface expression of CD25 and CD80 was significantly increased on CD19-hBtk B cells compared with WT B cells (**Figure 3B,C**). Interestingly, in CD19-hBTK B cells but not in WT B cells, simultaneous stimulation with CpG and αIgM resulted in an additional increase in CD25 and particularly CD80 expression compared to CpG alone (**Figure 3C**). Expression of the early activation marker CD69 was lower in CD19-hBtk B cells, compared with WT B cells upon stimulation with αIgM, CpG or combined stimulation (**Figure 3D**). As early activation marker CD69 is downregulated shortly after its induction, it is conceivable that its reduced expression on CD19-hBtk B cells resulted from rapid downregulation. Our finding of reduced CD69 expression would thus be consistent with enhanced activation of CD19-hBtk B cells following BCR or TLR9 stimulation. In these experiments, Btk inhibition by ibrutinib affected BCR-induced but not TLR9-induced changes in the expression of surface activation markers (**Figure 3A-D**).

Therefore, we conclude that BCR and TLR9 signaling act in synergy to induce a more activated surface phenotype in Btk-overexpressing B cells.

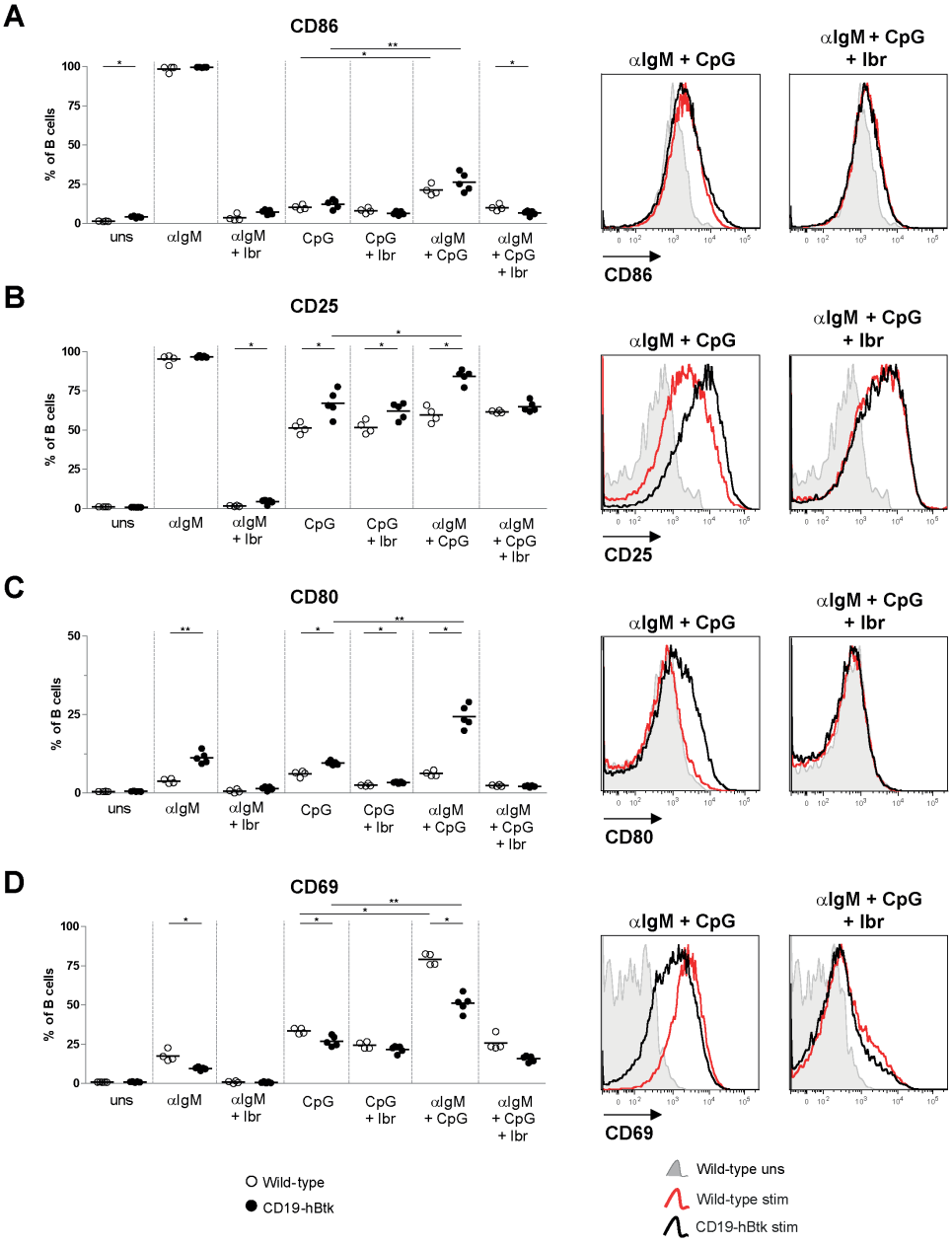

Figure 3. CD19-hBtk B cells show increased upregulation of activation markers upon synergistic BCR and TLR9 stimulation.

Proportions of B cells positive for CD86 (**A**), CD25 (**B**), CD80 (**C**) and CD69 (**D**) upon 72 hours of stimulation of MACS-purified naïve splenic B cells with 10μg αIgM (αIgM) and/or 2μM CpG, with or without ibrutinib (Ibr, 1μM), as indicated (*left*). Symbols represent individual mice and bars indicate mean values. Representative histogram overlays are depicted on the right. CD19-hBtk and WT mice were 8-10 weeks old; n = 4-5 per group; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

IL-10-production by CD19-hBtk B cells is synergistically increased following BCR and TLR stimulation

We have previously shown that autoimmune CD19-hBtk B cells of 30-week-old (aged) mice have an enhanced capacity to produce various cytokines, including IL-6, IL-10 and IFNγ (16). Accordingly, stimulation with PMA/ionomycin increased the proportions of IL-10-producing CD19-hBtk transgenic B cells to higher levels than WT control B cells (**Figure 4A**). This increase was observed in follicular (Fol) and marginal zone (MZ) B cells, but not in splenic $CD5^+$ B-1 cells, and was not affected by the presence of ibrutinib (**Figure 4B;** gating strategy in **Figure S2A,B**). To study B cell responsiveness to BCR and TLR signaling with respect to IL-10 production, we stimulated splenocytes from 30-week-old WT and CD19-hBtk mice with αIgM, poly-IC (pIC; recognized by TLR3), LPS (recognized by TLR4), Imiquimod (IMQ; TLR7) and CpG (TLR9), either alone or in various combinations in the presence of golgistop (monensin). In splenocyte cultures with monensin alone or with αIgM very few B cells were positive for IL-10, as measured by intracellular flow cytometry (**Figure 4C**). TLR stimulation with LPS and particularly CpG resulted in a clear induction of $IL\text{-}10^+$ B cells specifically in CD19-hBtk mice (**Figure 4C**). The proportions of $IL\text{-}10^+$ CD19-hBtk B cells - but not WT B cells - synergistically increased upon combined stimulation of BCR/TLR4 and BCR/TLR9 (**Figure 4C**). Stimulation with αIgM, together with TLR3 or TLR7 did not show a synergistic effect. This capacity of transgenic Btk was largely dependent on its kinase activity, because ibrutinib reduced IL-10 production in response to TLR and BCR ligands nearly to WT levels (**Figure 4C**).

Aged CD19-hBtk mice have increased numbers of splenic $CD5^+$ B-1 cells (8) and slightly decreased numbers of Follicular (Fol) and marginal zone (MZ) B cells, suggesting differential effects of Btk overexpression on these B cell subpopulations. Therefore, we investigated IL-10 production by splenic B cell subsets separately. The proportions of $IL\text{-}10^+$ Fol B cells were low (**Figure 4D**). Nevertheless, we noticed that $IL\text{-}10^+$ Fol B cells were increased in CD19-hBtk mice upon stimulation with CpG. Hereby, a synergistic effect was observed when B cells were additionally stimulated with αIgM (**Figure 4D**). MZ B cells of CD19-hBtk mice contained the highest proportions of $IL\text{-}10^+$ cells upon synergistic BCR and TLR9 stimulation (**Figure 4D**). We observed that splenic $CD5^+$ B-1 cells from CD19-hBtk mice did not show substantial differences, although significant, in proportions of $IL\text{-}10^+$ cells, when compared with $CD5^+$ B-1 cells from WT littermates (**Figure 4D**).

These data show that, compared to WT B cells, IL-10-production is increased following synergistic BCR and TLR9 stimulation in CD19-hBtk Fol and MZ B cells, but not in $CD5^+$ B-1 B cells. Hereby, CD19-hBtk transgenic MZ B cells have the highest capacity to produce IL-10.

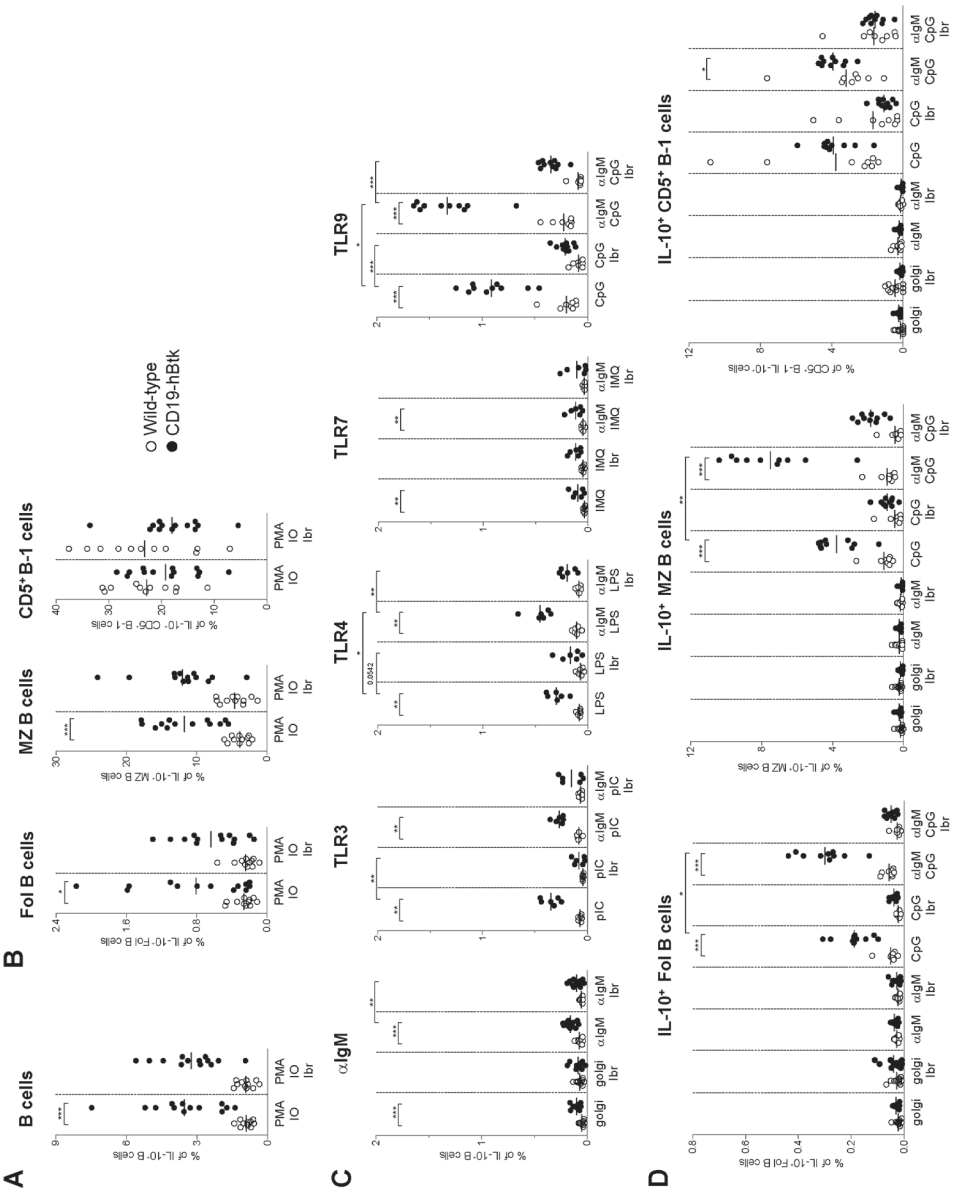

Figure 4. Increased IL-10 expression following synergistic BCR and TLR9 stimulation of CD19-hBtk B cells. (**A-B**) Proportions of IL-10-expressing B cells after 4 hours of *in vitro* stimulation of total splenocytes from the indicated mice with PMA/ionomycin in the presence of monensin (golgi), as determined by intracellular flow cytometry. Shown are data for gated total $CD19^+B220^+CD3^-$ B cells (**A**), or for gated B cell subpopulations (**B**), as indicated: follicular (Fol) B cells, marginal zone (MZ) B cells and $CD5^+$ B-1 cells. (**C**) Proportions of IL-10-expressing B cells after 4 hours of *in vitro* stimulation of total splenocytes from the indicated mice with αIgM, the indicated TLR ligands, or combinations thereof in the presence of monensin (golgi), as determined by intracellular flow cytometry.

(**D**) Proportions of IL-10-expressing B cells after 4 hours of *in vitro* stimulation of total splenocytes from the indicated mice with αIgM (αIgM), CpG, or combinations thereof, with or without ibrutinib (Ibr, 1μM), in the presence of monensin (golgi), as determined by intracellular flow cytometry. CD19-hBtk and WT mice were 28-33 weeks old. Symbols represent individual mice and bars indicate mean values. Graphs represent two to three individual experiments; ; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney *U* test.

Increased IFNγ and IL-6 production by CD19-hBtk B cells reflects *in vivo* B cell activation

Splenic B cells from aged CD19-hBtk mice have increased proportions of IL-6$^+$ and IFNγ$^+$ cells upon stimulation with PMA/ionomycin, compared to those from WT mice (16). A separate analysis of splenic B cell subsets showed that the increased IL-6$^+$ and IFNγ$^+$ production was present in CD19-hBtk transgenic Fol and MZ B cells, but not in CD19-hBtk transgenic CD5$^+$ B-1 cells (**Figure 5;** gating strategy in **Figure S2A-B).** Hereby, the proportions of IL-6$^+$ or IFNγ$^+$ cells were larger in the MZ B cell than in the Fol B cell fractions. We found that both for WT and for transgenic mice the proportions of IL-6$^+$ and IFNγ$^+$ cells were quite similar in cultures with monensin alone and in cultures stimulated by PMA/ionomycin or αIgM, irrespective of the presence of ibrutinib (**Figure 5**). Thus, addition of ibrutinib to these *in vitro* cultures did not reduce the proportions of IL-6$^+$ or IFNγ$^+$ CD19-hBtk Fol or MZ B cells to WT levels (**Figure 5A,B,D,E**).

In vitro CpG stimulation, either alone or in combination with αIgM or ibrutinib had limited effects on IL-6$^+$ or IFNγ$^+$ production by Fol B cell or CD5$^+$ B-1 cells (**Figure 5A,C,D,F**). In contrast, CpG stimulation increased the proportions of IL-6$^+$ cells by MZ B cells from both CD19-hBtk as WT mice, to levels higher than in PMA/ionomycin cultures (**Figure 5B**). Addition of ibrutinib *in vitro* did not appear to affect the production of IL-6 by MZ B cells from either mouse group; additional stimulation with αIgM reduced the frequencies of IL-6$^+$ cells, compared to CpG stimulation alone (**Figure 5B**). In contrast to IL-6 production, we noticed that CpG stimulation was associated with moderately reduced IFNγ production by MZ B cells, irrespective of the presence or absence of ibrutinib (**Figure 5E**).

In summary, the increase of the proportions of IFNγ$^+$ and IL-6$^+$ cells within the B cell population present in the spleen of CD19-hBtk mice could be attributed to Fol and MZ B cells and not to CD5$^+$ B-1 cells . This increase was largely independent of *in vitro* stimulation and thus essentially reflected *in vivo* B cell activation. Only IL-6 production by MZ B cells could be enhanced *in vitro* by CpG stimulation.

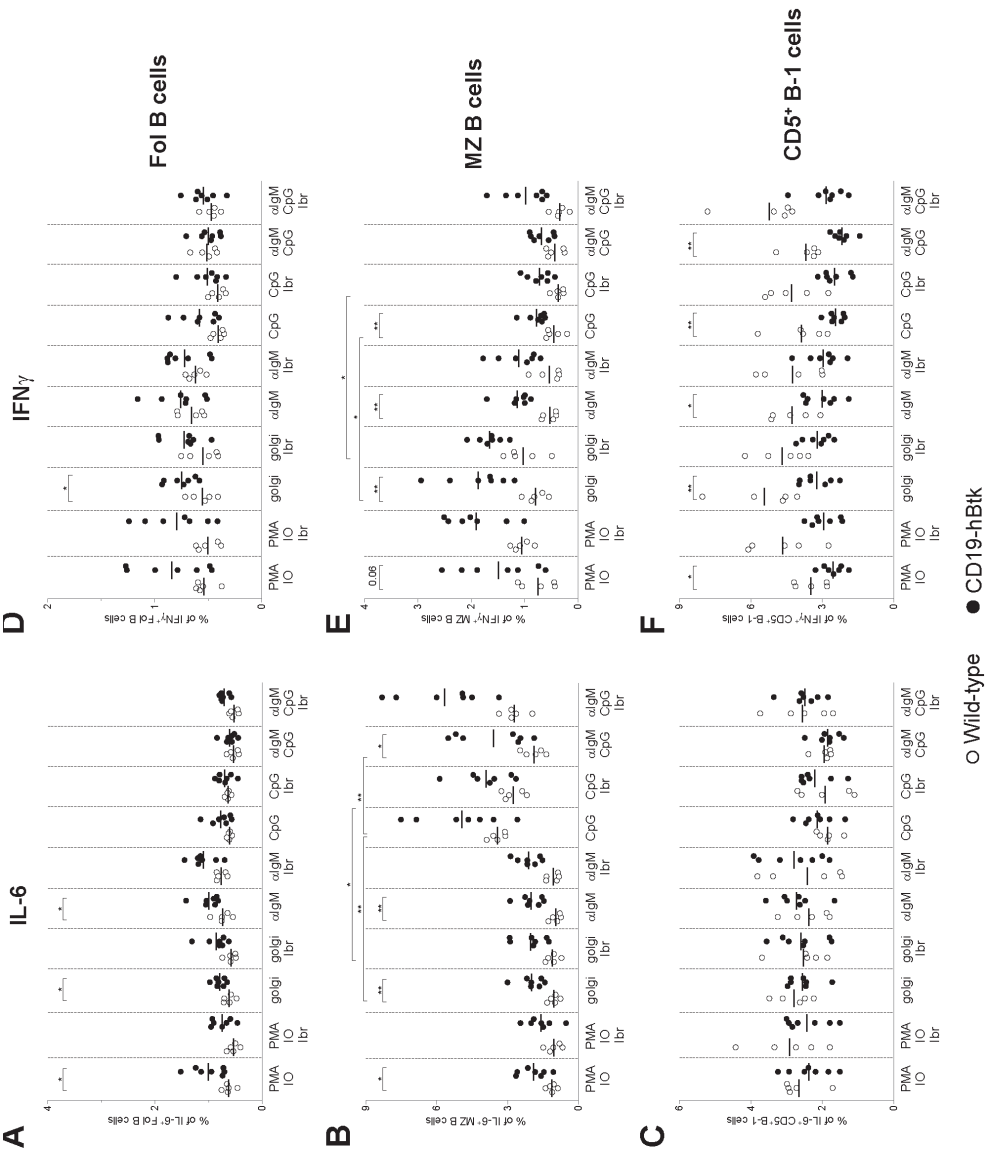

Figure 5. Analysis of IL-6 and IFNγ expression in B cell subsets from CD19-hBtk B cells.
Proportions of IL-6$^+$ (**A-C**) and IFNγ$^+$ (**D-F**) B cells upon stimulation with the indicated stimuli, with or without ibrutinib (Ibr, 1μM) in the presence of monensin (golgi), within gated B cell subsets: follicular (Fol) B cells (**A**,**D**), marginal zone (MZ) B cells (**B**,**E**) and CD5$^+$ B-1 cells (**C**,**F**). CD19-hBtk and WT mice were 28-33 weeks old. Symbols represent individual mice and bars indicate mean values. Graphs are representative for one to two individual experiments; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

Increased BCR responsiveness in CD19-hBtk B cells is independent of MyD88 expression

To study whether TLR signaling is important for the development of Btk-mediated autoimmune disease, we crossed CD19-hBtk mice onto a MyD88-deficient background and aged these mice to characterize their phenotype. At ~30 weeks of age, the total numbers of Fol and MZ B cells in the spleen were decreased in both CD19-hBtk and *Myd88*$^{-/-}$CD19-hBtk mice, compared to WT and *Myd88*$^{-/-}$ mice (**Figure 6A**). The absolute numbers of CD5^{+} B-1 cells showed the converse and were increased in CD19-hBtk and *Myd88*$^{-/-}$CD19-hBtk mice (**Figure 6A**).

When we investigated BCR responsiveness of total splenic B cells of 8-week-old mice, we found that the prolonged calcium influx and increased S6 phosphorylation upon αIgM stimulation of CD19-hBtk B cells was MyD88-independent (**Figure 6B,C**). *Myd88*$^{-/-}$CD19-hBtk B cells did not increase pS6 levels upon CpG stimulation (**Figure 6C**), confirming that these cells were TLR unresponsive. In addition, we noticed that the enhanced upregulation of the cell surface markers CD69, CD86 and CD25 upon αIgM stimulation was similar for CD19-hBtk and *Myd88*$^{-/-}$CD19-hBtk B cells (shown for 48 hours; **Figure 6D**). Cell cycle analysis by PI staining after two days of *in vitro* culture of MACS-purified B cell fractions, in the presence or absence of αIgM, revealed increased survival and proliferation of CD19-hBtk B cells in a MyD88-independent manner (**Figure 6E**). As expected, stimulation with LPS or CpG did not induce proliferation in *Myd88*$^{-/-}$ and *Myd88*$^{-/-}$CD19-hBtk B cell fractions (**Figure 6E**).

Although we did not detect any effects of MyD88-deficiency on αIgM-induced B cell proliferation and survival or expression of activation markers *in vitro*, it cannot be excluded that in an *in vivo* environment MyD88-deficiency may hamper or augment BCR-dependent survival or proliferation signals. Therefore, we investigated whether MyD88-deficiency would influence leukemia development in our *IgH.TE*μ chronic lymphocytic leukemia (CLL) mouse model, in which we previously showed that (i) CLL development is critically dependent on Btk and is accelerated in the presence of the CD19-hBtk transgene, and that (ii) malignant CLL B cells harbor high phosphorylation of Btk, Akt and S6 (34, 36). To this end, we crossed *IgH.TE*μ mice on the *Myd88*$^{-/-}$ background. Monitoring for the presence of increased frequencies of malignant CD5^{+} B cells in peripheral blood (see Materials and Methods) revealed that *IgH.TE*μ and *Myd88*$^{-/-}$*IgH.TE*μ mice had a comparable incidence of leukemic disease (**Figure S3A**). In addition, analysis of the CLL cells for phosphatidylcholine (PtC)-specificity of the BCR, indicative for a B-1 cell origin (37), showed that MyD88-deficiency had no major effect on the usage of the PtC-specific stereotypic BCR (**Figure S3B**).

From these *in vitro* and *in vivo* experiments, we conclude that the BCR responsiveness of CD19-hBtk B cells is increased, irrespective of the presence of MyD88.

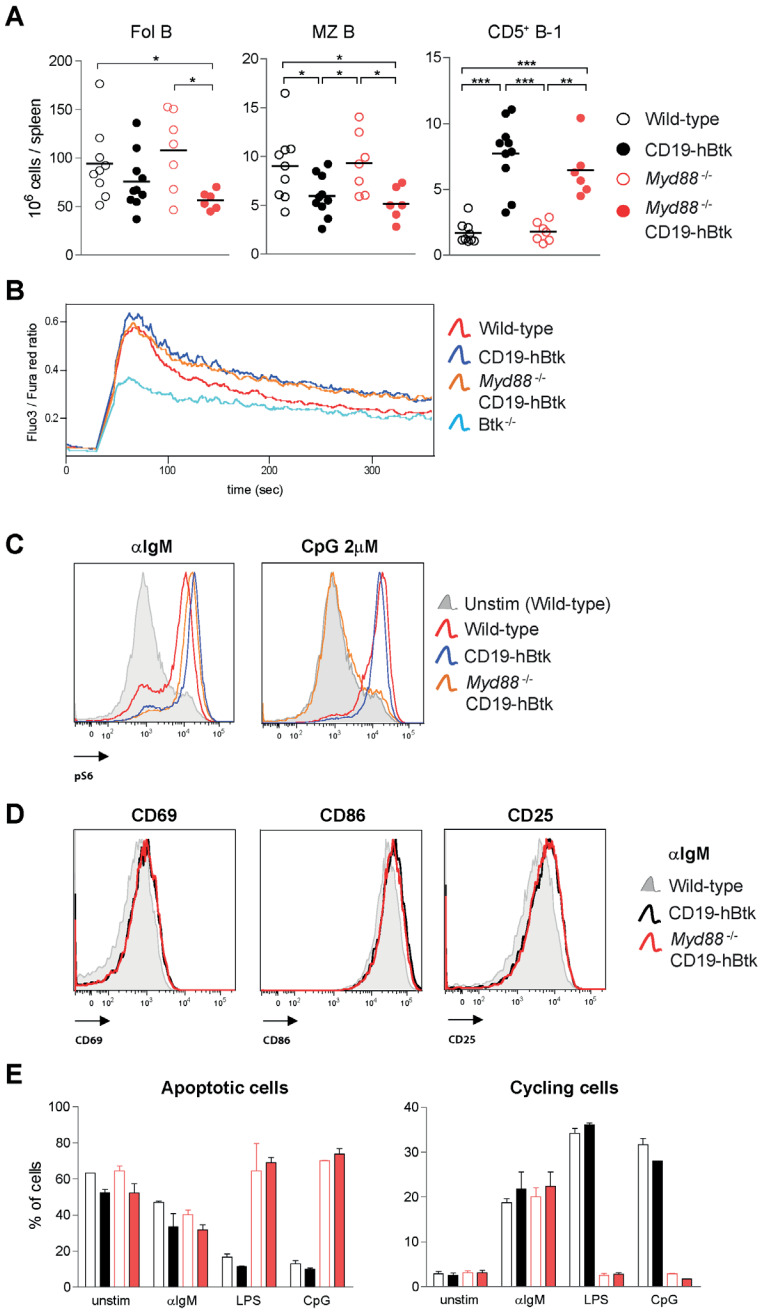

Figure 6. Increased BCR responsiveness in CD19-hBtk B cells is independent of MyD88 expression.
(**A**) Quantification of the absolute numbers of follicular (Fol) B cells ($CD19^+CD21^-CD23^+$), marginal zone (MZ) B cells ($CD19^+CD21^+CD23^-$) and $CD5^+$ B-1 cells ($CD19^{high}B220^{int}CD5^+$) in spleens from the indicated aged mice. (**B**) Ca^{2+} influx assay in B cells after stimulation with 25 μg $F(ab')_2$ anti-IgM in the indicated mouse groups. Data are representative for three mice analyzed.

(**C**) Representative histogram overlays of phosphorylated S6 (pS6) upon 20 μg/mL αIgM or 2 μM CpG stimulation in the indicated mouse groups. Data are representative for two to three mice analyzed. (**D**) Representative histogram overlays of the expression of activation markers CD69, CD86 and CD25 in *Myd88*$^{-/-}$CD19-hBtk (red), CD19-hBtk (black) and WT mice (gray). Data are representative for two mice analyzed. (**E**) Proportions of B cells in apoptotic (*left*) or cycling (*right*) fractions, as determined by propidium iodide (PI) staining for DNA content, after two days of *in vitro* stimulation with the indicated stimuli. CD19-hBtk and WT mice were 28-33 weeks old (**A**) or eight weeks old (**B-E**). Data (mean values + SD) represent one to three individual experiments; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney *U* test.

MyD88 is indispensable for Btk-mediated autoimmune disease

Investigating autoimmune parameters in aged mice revealed that CD19-hBtk mice had increased numbers of splenic GC B cells, follicular helper T (Tfh) cells and follicular regulatory T (Tfr) cells compared to WT littermates (**Figure 7A;** gating strategy in **Figure S2A,C,D**), as previously observed (8, 16). This increase was fully dependent on MyD88 expression, as *Myd88*$^{-/-}$CD19-hBtk mice had splenic GC B cell, Tfh and Tfr numbers similar to WT or *Myd88*$^{-/-}$ mice (**Figure 7A**). In addition, the increase of IgM^{+}, IgG1^{+}, and less prominently, IgG2bc^{+} plasma cells in the spleens of CD19-hBtk mice was MyD88-dependent (**Figure 7B;** gating strategy in **Figure S2A,E**). IgM^{+} plasma cells in the BM were also MyD88-dependently increased (**Figure S4A**). IgG1^{+} plasma cells in BM were increased in MyD88-deficient mice, as was previously described, and increased further in Btk-overexpressing MyD88-deficient mice (**Figure S4A**). Total serum IgM levels were not different between the four groups of mice (**Figure 7C**). Likewise, the increased numbers of IgG1^{+} plasma cells in the spleens of CD19-hBtk mice was not reflected by increased total IgG1 concentrations in the serum, compared with the three other groups of mice (**Figure 7B,C**). We observed increased total IgG1 and decreased IgG2c levels in the serum of *Myd88*$^{-/-}$CD19-hBtk mice, when compared with CD19-hBtk mice, which reflected the numbers of plasma cells in BM rather than spleen (**Figure 7B,C; S4A**).

Next, we investigated the cytokine producing capacity of splenic B and T cells. We observed that the increase of the proportions of IL-6^{+} and IFNγ^{+} cells in the splenic B cell populations of CD19-hBtk mice was MyD88-dependent (**Figure 7D**). In contrast, the increase in IL-10^{+} B cells in CD19-hBtk mice appears to be MyD88-independent (**Figure 7D**). A separate analysis of Fol and MZ B cells showed that the profiles for IFNγ^{+}, IL-6 and IL-10 across the four groups of mice were similar in the two B cell subsets (not shown). For CD5^{+} B-1 we observed that the production of the three cytokines was slightly reduced in CD19-hBtk mice, in line with our findings described above (**Figures 4 and 5**), as well as in *Myd88*$^{-/-}$CD19-hBtk mice (not shown). The observed increase of cytokine production, including IFNγ^{+} and IL-10, by splenic T cells from in CD19-hBtk mice was not observed in T cells from *Myd88*$^{-/-}$CD19-hBtk mice (**Figure 7E**).

Analysis of autoimmune pathology by immunohistochemistry showed numerous perivascular B and T lymphocyte infiltrates in the salivary glands of CD19-hBtk mice, which were completely absent in *Myd88*$^{-/-}$CD19-hBtk mice (**Figure S4A**), as well as in WT and *Myd88*$^{-/-}$ mice (data not shown). MyD88 was also required for IgM^{+} or IgG2c^{+} glomerular

immune complex depositions in the kidneys, which were present in CD19-hBtk but hardly in *Myd88*$^{-/-}$CD19-hBtk mice (**Figure S4B**). The production of IgM autoantibodies (mainly cytoplasmic) and anti-nuclear IgG autoantibodies, as seen in CD19-hBtk mice, was absent in *Myd88*$^{-/-}$CD19-hBtk mice (**Figure 7F**). It is of note that the anti-nuclear auto-antibodies in CD19-hBtk mice were reactive to dsDNA and chromatin (8, 16), RNA polymerase RNP-A, RNP-C, and Smith antigen SmB (**Figure S4D**; **Table S2**), representing auto-antibody specificities that were shown to be dictated by TLR7 and TLR9 (17).

Taken together, these data show that MyD88 is required for the development of all hallmarks of autoimmune pathology in CD19-hBtk mice with increased BCR responsiveness.

DISCUSSION

Btk is a critical kinase in the BCR signaling pathway and is known to interact with various proteins that are downstream of TLRs. The interplay between the BCR and TLR signaling is thought to be crucial for the pathogenesis of autoimmune disease. Our findings provide evidence that TLR signaling is critical in systemic autoimmunity driven by overexpression of Btk in transgenic mice, which is characterized by the induction of TLR7/9-associated auto-antibody specificities, including dsDNA, histone, RNP-A, RNP-C, and SmB.

Analysis of the functional consequences of BCR and TLR stimulation in our CD19-hBtk mouse model revealed that substantial complexity exists regarding the interplay of the two signaling pathways. Btk overexpression amplified S6 signaling in the context of IgM stimulation, but no effects were observed on signaling levels upon TLR stimulation, even though we detected decreased protein levels of TLR9 in CD19-hBtk B cells compared to WT controls. In parallel, we found that Btk overexpression increased survival and proliferation of B cells when these were stimulated through BCR engagement, but reduced proliferation after CpG or CpG/αIgM stimulation. Nevertheless, using different functional readouts, clear additive or synergistic effects were observed. These include upregulation of surface markers such as CD25, CD80 and CD86 and the capacity of CD19-hBtk B cells to produce significantly increased IL-10 levels after CpG stimulation, as compared to wild-type B cells. Nevertheless, enhanced IL-10 expression was only seen in Fol and MZ B cells and not in CD5^{+} B-1 cells. Along these lines, we found that in aging CD19-hBtk mice Fol B cells and CD5^{+} B-1 cells expressed increased levels of IL-6 and IFNγ, but this was essentially not increased upon *in vitro* stimulation with PMA/ionomycin, αIgM or CpG. We also noticed that TLR9 engagement by CpG increased the capacity of MZ B cells to produce IL-6 to levels that were beyond those reached by PMA/ionomycin stimulation. In contrast, CpG stimulation of MZ B cells appeared to decrease their capacity to produce IFNγ. Despite the observed complexity of TCR and BCR interplay exposing both synergistic and opposite outcomes, we established that MyD88-deficient CD19-hBtk transgenic mice did not develop autoimmune symptoms. Therefore, we conclude that TLR signaling is crucial for the induction of Btk-driven autoimmune disease.

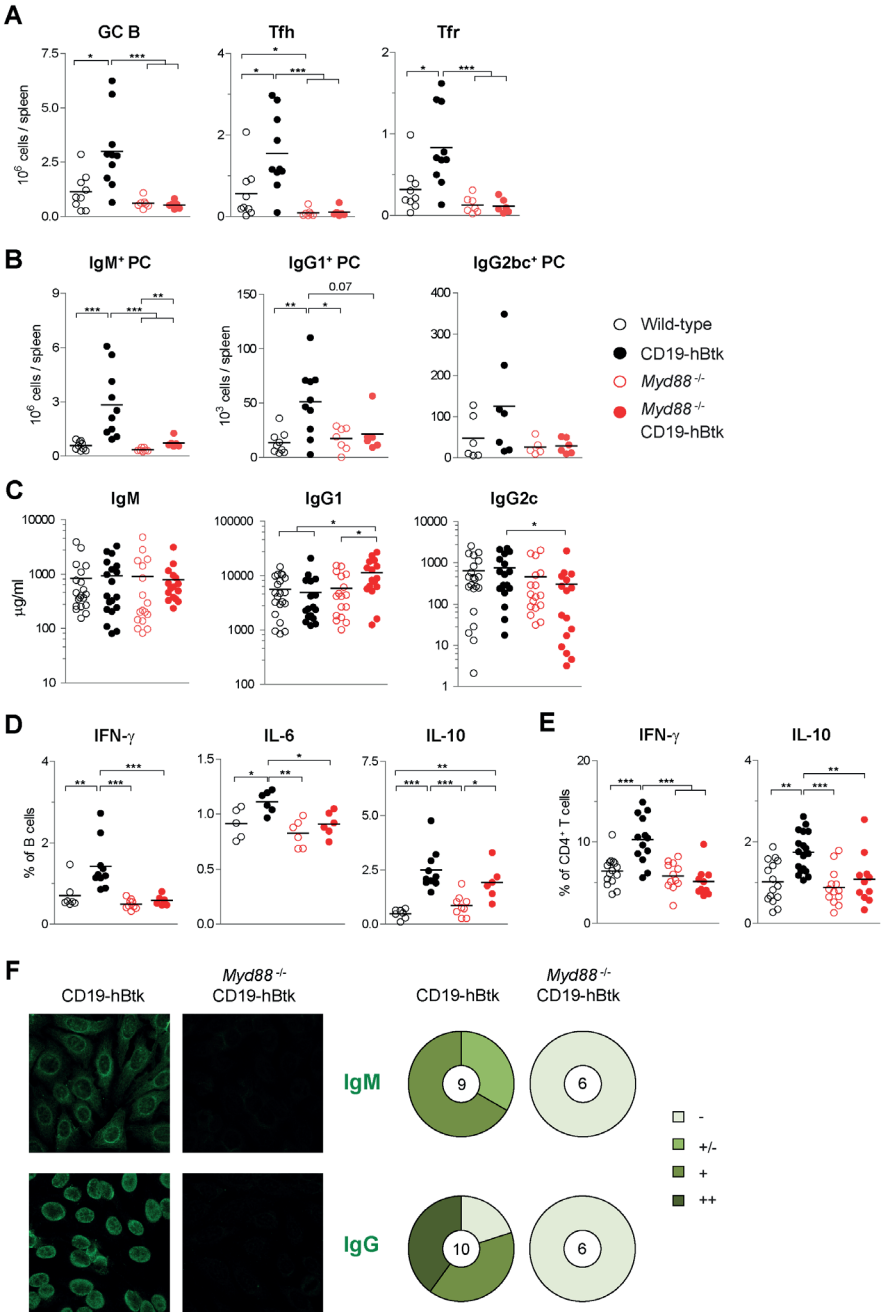

Figure 7. MyD88 is required for Btk-mediated autoimmune disease.

(**A**) Absolute numbers of splenic germinal center B cells (GC; $CD19^+IgD^-CD95^+$), follicular T helper cells (Tfh; $CD3^+CD4^+CXCR5^+PD1^+FoxP3^-$) and follicular T regulatory cells (Tfr; $CD3^+CD4^+CXCR5^+PD1^+FoxP3^+$). (**B**) Absolute numbers of splenic IgM^+, $IgG1^+$ and $IgG2bc^+$ plasma cells (PC; $CD11b^-IgG1^-IgGbc^-IgM^+CD138^+$, $CD11b^-IgG1^+CD138^+$ and $CD11b^-IgG2bc^+CD138^+$ respectively). (**C**) Serum concentrations of IgM, IgG1 and IgG2c, as determined by

ELISA. (**D,E**) IFNγ, IL-6 and IL-10 expression in gated B cell fractions (**D**) and $CD3^+CD4^+$ T cell fractions (**E**) upon *in vitro* stimulation with PMA/ionomycin for 4 hours in the presence of monensin. Symbols represent individual mice and bars indicate mean values. Graphs represent two to three individual experiments. (**F**) Representative pictures of serum IgM (upper panel) and IgG (lower panel) reactivity with HEp-2 cells and quantification of autoreactivity. Total number of mice analyzed are indicated within the pie charts; -, no staining; +/-, mild staining; +, moderate staining; ++, strong staining. CD19-hBtk and WT mice were 28-33 weeks old; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney *U* test.

Various molecular mechanisms may connect Btk to both BCR and TLR signaling. First, Btk is phosphorylated downstream of the BCR and can directly interact with several components of the TLR signaling pathway. These include the Toll/IL-1R homology (TIR) domain, which is the intracellular signaling module of TLR, the adapters MyD88 and MyD88 adapter-like (MAL) and IL-1R-associated kinase-1 (IRAK-1) (9, 27). In particular, Kenny et al. showed that Btk is essential for co-localization of the BCR and TLR9 within an auto-phagosome-like compartment (30). The authors found synergistic upregulation of activation markers, which is in concordance with our findings. However, we did not observe a kinase-independent role for Btk in synergistic IL-6 production in response to CpG and αIgM in splenic B cells. Second, upon BCR engagement Btk phosphorylates the B-cell adaptor for phosphoinositide 3-kinase (BCAP), which provides a binding site for PI3K (38). Interestingly, BCAP also links TLR signaling to PI3K activation (39, 40) and contains a TIR domain, which is used by TLR signaling adapters including MyD88. As a result, it is conceivable that BCAP reduces the availability of MyD88 for activation of NF-κB. Third, PLCγ2, which is a direct substrate of Btk, can interact with another TIR-domain containing adapter molecule, the B cell adaptor protein with ankyrin repeats (BANK1) (41) which shows genetic association with SLE in GWAS (42). BANK1 augments TLR7/TLR9 signaling and was reported to control CpG-induced IL-6 secretion (43). B cell IL-6 production, which can be enhanced by IFNγ, promotes Tfh cell differentiation and initiates spontaneous germinal center formation (44). Therefore, it is likely that increased IL-6 production by Btk-overexpressing B cells is a major driver of autoimmunity in CD19-hBTK mice. In our crosses with MyD88-deficient mice we could show that enhanced expression of both IL-6 and IFNγ in CD19-hBtk mice is TLR-dependent.

IL-10-producing B cells are important in autoimmune diseases, as IL-10 is a proliferation factor for B cells and has well-known regulatory functions (45-47). We observed that in PMA/ionomycin stimulation experiments IL-10 production by CD19-hBtk B cells was independent of MyD88 (**Figure 7D**) and independent of CD40L expression (16). However, stimulation with TLR ligands strongly induced IL-10-producing CD19-hBtk B cells, but WT B cells showed low responsiveness. This is in line with the previously reported finding that Btk is required for TLR-induced IL-10 production by B cells (48, 49) and that B cells from lupus-prone mice upregulate IL-10 production in response to TLR stimulation, but not to BCR or CD40 engagement (50). Furthermore, we found that CD19-hBtk B cells specifically increased IL-10 production upon combined BCR-TLR stimulation over TLR stimulation alone, whereas this remained unchanged in WT B cells. This finding demonstrates that regarding IL-10 production autoimmune CD19-hBtk B cells are very sensitive to dual ligation, which is in stark contrast with the limited responsiveness of WT B cells.

We also found that all splenic B cell subsets produced IL-10. Peritoneal $CD5^+$ B-1 cells were previously shown to rely on Btk for the production of IL-10, as B cells with low Btk expression had decreased levels of IL-10 mRNA, compared with B cells with physiological Btk levels (51). Although the number of $CD5^+$ B-1 cells is significantly increased in CD19-hBtk mice, we did not observe increased proportions of IL-10^+ cells within the splenic $CD5^+$ B-1 population in CD19-hBtk mice, compared with WT. It was recently reported that Btk is not required for the maintenance of the B-1 cell pool (52). Therefore it is very well possible that Btk overexpression mainly affects the generation of $CD5^+$ B-1 cells during development and not the IL-10 production by $CD5^+$ B-1 cells in adult mice. The increase in IL-10 production and the synergistic responsiveness of CD19-hBtk Fol and MZ B cells might point to an attempt of B cells to control the autoimmune response, potentially via TLR9 signaling (17, 23, 53). However, elevated IL-10 serum levels were found in SLE patients with active disease (54), suggesting that IL-10-producing autoimmune B cells could be disease-promoting, e.g. by the effects of IL-10 on B cell survival (45).

The increase in IFNγ^+ and IL-6^+ CD19-hBtk B cells upon PMA/ionomycin stimulation was fully dependent on MyD88 expression and *in vitro* Btk inhibition for several hours did not reduce IFNγ and IL-6 to WT levels. This suggests that *in vivo* TLR-mediated B cell activation is crucial for the increase in IFNγ^+ and IL-6^+ CD19-hBtk B cells. Further experiments are required to identify the molecular mechanisms that are responsible for the altered cytokine production capacity upon long-lasting *in vivo* B cell activation, apparently resulting in rewiring of signal transduction pathways and turning these B cells refractory to Btk inhibition.

We demonstrated that TLR signaling is important in hBtk-mediated autoimmune disease, although the *in vitro* BCR responsiveness of CD19-hBtk B cells, including downstream signaling, survival and proliferation, was enhanced in a MyD88-independent fashion. However, spontaneous GC formation, increased plasma cell differentiation, induction of Tfh cells, increased production of IFNγ and IL-6, and the presence of anti-nuclear autoantibodies in serum, were clearly abrogated in the absence of MyD88. It was recently reported that circulating chromatin in apoptotic cell micro-particles induced SLE, which was dependent on MyD88 expression (24). This suggests that autoreactive BCRs bind chromatin in apoptotic material, providing a strong survival signal by synergistic signaling with nucleic acid-sensing TLRs. In addition, BCR-TLR synergism can drive AID expression and thereby promote class-switching in T cell independent responses (55). TLR signaling was also shown to be required for amplification of GC responses, whereby B-cell-intrinsic TLR responsiveness was upregulated during GC reactions (56), implicating TLR signaling also in later stages of B cell activation and differentiation.

Our findings demonstrate that enhanced BCR signaling in CD19-hBtk B cells on its own is not sufficient for the development of autoreactive responses. TLR expression in cell types other than B cells could well contribute to Btk-driven autoimmunity, particularly because TLR signaling in dendritic cells (DCs) and lymph node stromal cells is relevant for activation of (autoreactive) B cells (22, 57, 58). Thus, the activation of autoreactive B cells relies on TLR signaling in B cells and potentially also in other cells, such as DCs and lymph node stromal cells.

In summary, we have shown that TLR-mediated activation is important for Btk-driven autoimmune disease, partly by synergistically enhancing signaling responses of autoreactive B cells. Next to mouse studies concerning Btk inhibition in autoimmune diseases, currently clinical trials are ongoing with several Btk inhibitors in autoimmune disease patients, including RA (CC-292, HM71224, M2951 and GS-4059) and SLE (BIIB068, MSC2364447C and M2951) (www.clinicaltrails.gov). Based on our current findings, together with the observed increase in BTK protein expression levels in circulating B cells from patients with RA and SjS (15), it is attractive to speculate that Btk inhibition will dampen both BCR pathway activation and synergistic BCR/TLR signaling in patients with systemic autoimmune disease.

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DISCLOSURE

The authors declare to have no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

JR designed the research studies, performed experiments, analyzed the data, and wrote the manuscript. MdB, SPS, and MA performed experiments and analyzed the data. OC and RH contributed to the research design and the writing of the manuscript and supervised the study. All co-authors approved the final manuscript.

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SUPPLEMENTAL MATERIAL

Table S1A. Antibodies used for FACS

Antigen	Fluorochrome	Clone	Manufacturer
Flow cytometry			
B220	AF700	RA3-6B2	BD Biosciences
CD1d	BV421	1B1	BD Biosciences
CD3	APC-efl780	17A2	eBioscience
	BV421	145-2C11	BD Biosciences
CD4	AF700	GK1.5	eBioscience
	PCP-cy5.5	RM3-5	eBioscience
CD5	APC	Ly-1	eBioscience
CD11b	AF700	M1/70	eBioscience
CD19	APC-ef780	1D3	BD Biosciences
	PCP-Cy5.5	eBio1D3	eBioscience
CD21/35	FITC	eBio4E3	eBioscience
	BV605	7G6	BD Biosciences
CD23	PE-Cy7	B3B4	BD Biosciences
CD25	BV605	PC61	Biolegend
CD43	PE	S7	BD Biosciences
CD80	PCP-cy5.5	GL1	BD Biosciences
CD86	PE-cy7	GL1	BD Biosciences
CD95	PE-CF594	Jo2	BD Biosciences
CD138	APC	281-2	BD Biosciences
IgD	FITC	11-26c.2a	eBioscience
	BV711	11-26c.2a	BD Biosciences
IgM	PE-Cy7	II/41	BD Biosciences
IgG1	FITC	A85-1	BD Biosciences
CTLA4	PE	UC10-4F10-11	BD Biosciences
CXCR5	Biotin	2G8	BD Biosciences
FoxP3	FITC	FJK-16s	eBioscience
ICOS	APC	C398.4A	eBioscience
IFN-γ	PE-Cy7	XMG1.2	eBioscience
IL-6	PE	MP5-20F3	BD Biosciences
IL-10	FITC	JES5-16E3	eBioscience
PD-1	BV421	J43	BD Biosciences
TLR7	PE	A94B10	BD Biosciences
TLR9	PE	J15A7	BD Biosciences
Streptavidin	APC-efl780	-	eBioscience
	PE-Cy7	-	eBioscience
	BV711	-	BD Biosciences

Table S1B. Antibodies used for Phosphoflow

Antigen	Fluorochrome	Clone	Manufacturer
Phosphoflow			
CD3	BV421	145-2C11	BD Bioscience
B220	AF700	RA3-6B2	BD Biosciences
IgD	BV711	11-26c.2a	BD Biosciences
IgM	PE-Cy7	II/41	BD Biosciences
pCD79a (Y182)	AF647	D1B9	Cell signaling Technologies
pSyk (Y348)	PE	L120-722	BD Biosciences
pAkt (T308)	-	D25E6	Cell signaling Technologies
pAkt (S473)	-	D9E	Cell signaling Technologies
pPLCγ2 (Y759)	AF647	K86-689.37	BD Biosciences
pS6 (S240/S244)	-	D68F8	Cell signaling Technologies
anti-Rabbit	PE	-	Jackson ImmunoResearch
Streptavidin	BV786	-	BD Biosciences

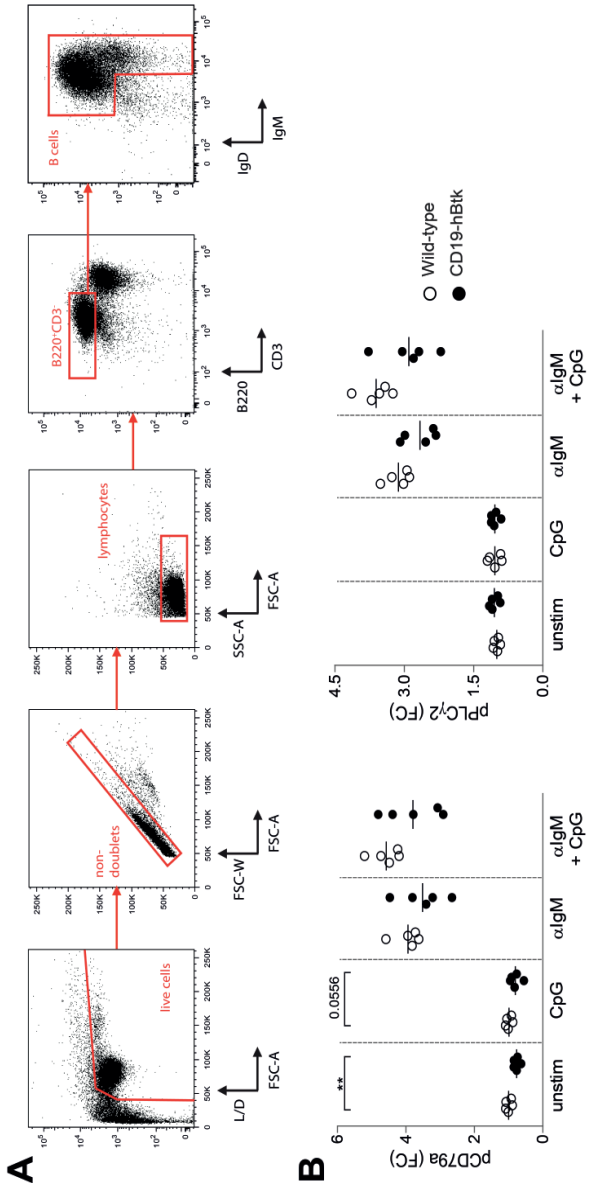
Table S1C. Antibodies used for MACS procedures

Antigen	Fluorochrome	Clone	Manufacturer
CD5	Biotin	53-7.3	BD Bioscience
CD11b	Biotin	M1/70	eBioscience
CD138	Biotin	281-2	BD Bioscience
CD43	Biotin	eBioR2/60	eBioscience
CD95	Biotin	Jo2	BD Bioscience
Gr-1	Biotin	RB6-8C5	eBioscience
TER-119	Biotin	TER119	eBioscience

Table S2. Autoantibody reactivity in aged WT, CD19-hBtk mice and *Myd88*$^{-/-}$ CD19-hBtk mice.

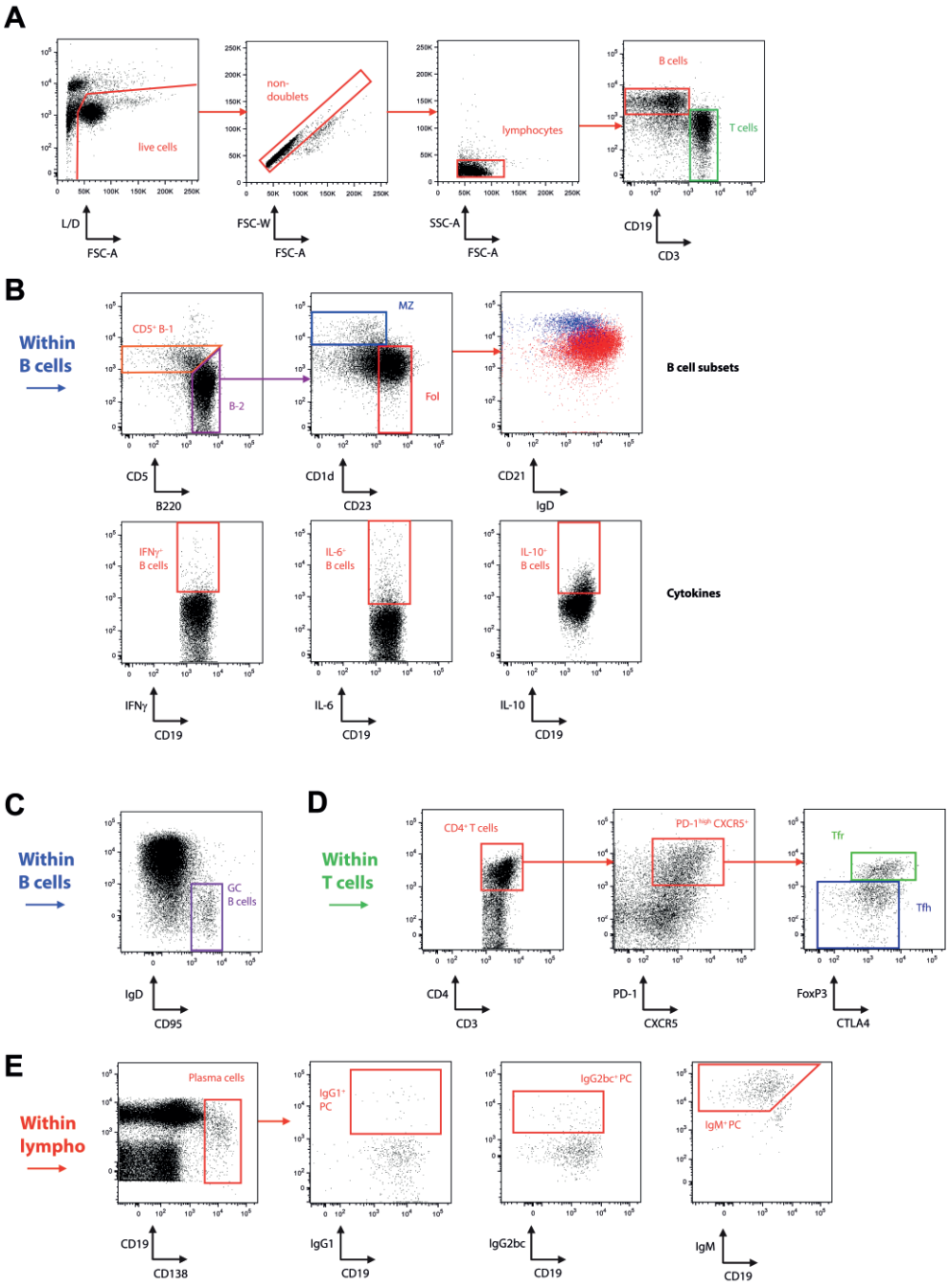
Genotype		Reactivity of autoantibodies*)
Wild-type	#1	-
	#2	-
	#3	-
CD19-hBtk	#1	Histones (++), RNP-A (++), RNP-C (++)
	#2	RNP-A (++), RNP-C (+)
	#3	Histones (++), RNP-A (++), RNP-C (++), SmB (+)
	#4	RNP-A (+)
	#5	Histones (++), RNP-A (++), RNP-C (++)
	#6	Histones (++), RNP-A (++), RNP-C (++)
	#7	Histones (++), RNP-A (+), RNP-C (+)
Myd88$^{-/-}$ CD19-hBtk	#1	-
	#2	-
	#3	-

*) Autoantibody reactivity in 28-33 week-old mice, as measured by Line Immunoblot Assay.



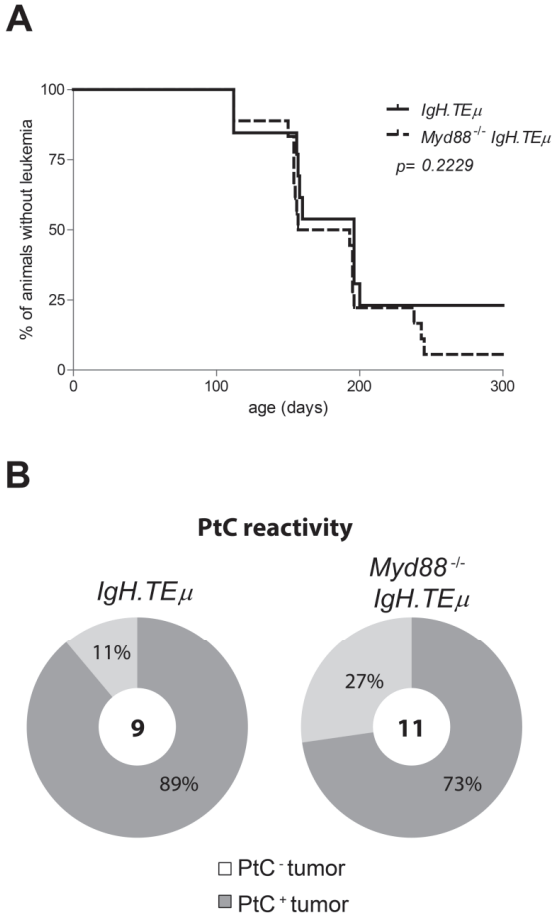
Supplementary Figure 1. Gating strategy phosphoflow and analysis of pCD79a and pPLCγ2 in B cells after stimulation.

(**A**) Gating strategy for live cells by viability marker staining, followed by gating for single lymphocytes. From live lymphocytes, we gated for splenic B cells ($B220^+CD3^-IgD^+IgM^+$). (**B**) Fold Change (FC) increase of median fluoresence intensity (MFI) values compared to WT unstimulated (which was set to 1.0) for phosphorylation of CD79a and PLCγ2 in B cells that were activated by the indicated stimuli. Symbols represent individual mice and bars indicate mean values.CD19-hBtk and WT mice were 8-10 weeks old; n = 4-5 per group; **p<0.01 by Mann-Whitney U test.



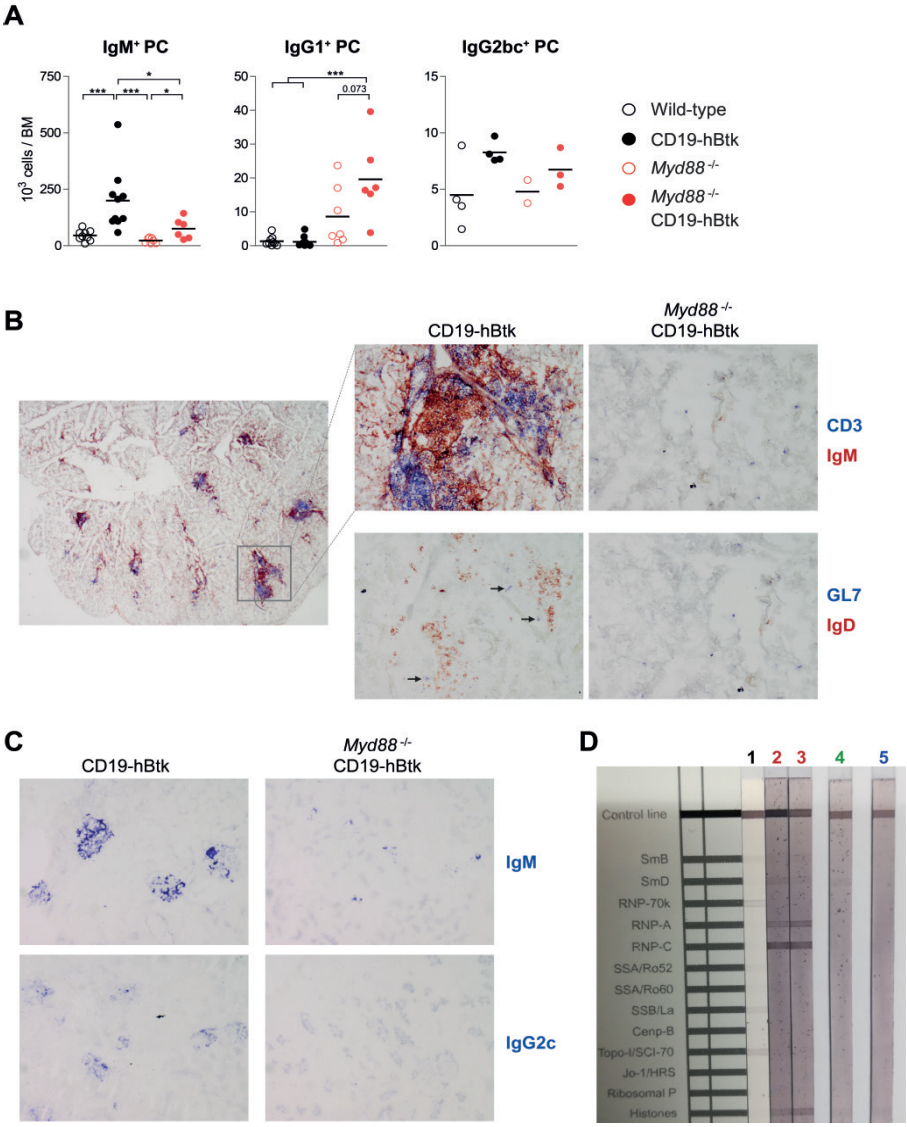
Supplementary Figure 2. Gating strategy for cytokine stainings, B cell subsets and plasma cells.
(**A**) For all stainings, we first gated for single live B and T cells, based on CD19 and CD3 expression. (**B**) For B cell subset cytokine staining, we gated within $CD19^{+}CD3^{-}$ B cells for $CD5^{+}$ B-1 cells ($CD19^{+}B220^{int}CD5^{+}$), marginal zone B cells ($CD19^{+}B220^{+}CD1d^{high}CD21^{high}CD23^{-}IgD^{-}$) and follicular B cells ($CD19^{+}B220^{+}CD1d^{-}CD21^{+}CD23^{+}IgD^{+}$). Expression

of IFNγ, IL-6 and IL-10 within total B cells are shown as examples. (**C**) From B cells, we gated for germinal center B cells ($CD19^+IgD^-CD95^+$). (**D**) From T cells, we gated on CD4+ cells that highly express PD-1 and are CXCR5-positive. Foxp3 and CTLA4 were used to separate T follicular helper cells (Tfh; $CD3^+CD4^+CXCR5^+PD1^+FoxP3^-$) and T follicular regulatory cells (Tfr; $CD3^+CD4^+CXCR5^+PD1^+FoxP3^+$); representative flow cytometry graphs for these gating strategies. (**E**) From CD11b-negative lymphocytes, we gated for IgM^+, $IgG1^+$ and $IgG2bc^+$ plasma cells (PC; $CD11b^-IgG1^-IgG2bc^-IgM^+CD138^+$, $CD11b^-IgG1^+CD138^+$ and $CD11b^-IgG2bc^+CD138^+$ respectively).



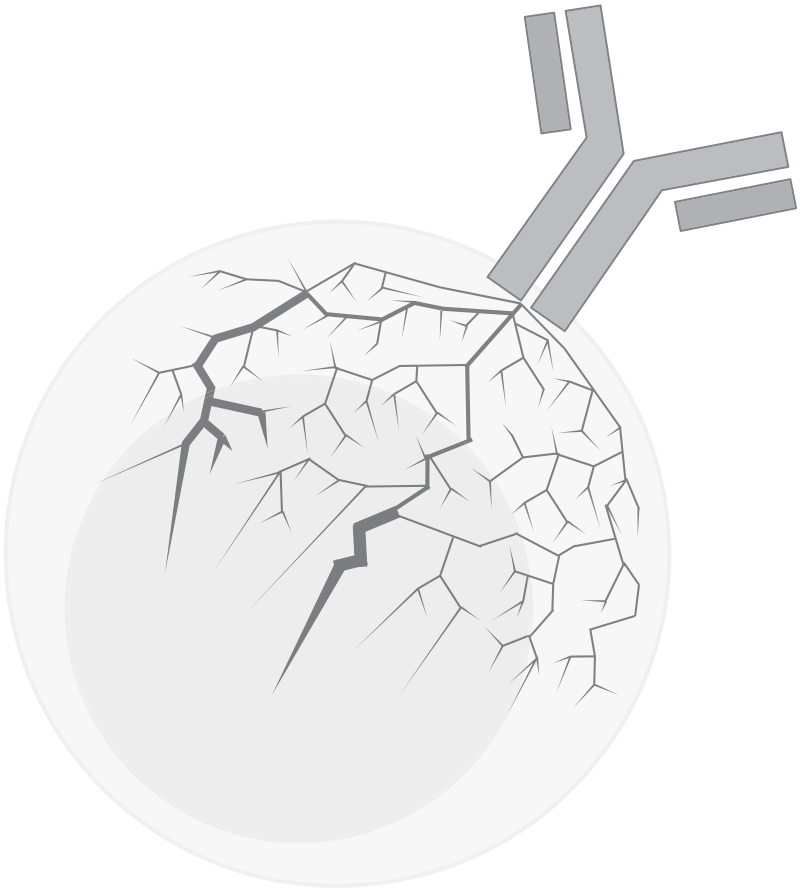
Supplementary Figure 3

(**A**) Retrospective Kaplan-Meier incidence curve showing *IgH.T*Eμ mice (solid line) and Myd88 $^{-/-}$ IgH.TEμ mice (dotted line). (B) Pie chart summarizing frequencies of splenic PtC-recognizing (PtC^+) and non PtC-recognizing (PtC^-) CLL cases from nine IgH.TEμ mice (left) and eleven Myd88 $^{-/-}$ IgH.TEμ mice (*right*), as indicated. Log Rank test was performed to calculate the significance for survival differences between indicated groups.



Supplementary Figure 4. The autoimmune phenotype of CD19-hBTK mice, compared with *Myd88*$^{-/-}$ CD19-hBtk mice.

(**A**) Absolute numbers of bone marrow (BM) IgM$^+$, IgG1$^+$ and IgG2bc$^+$ plasma cells (PC; CD11b$^-$IgG1$^-$IgGbc$^-$IgM$^+$CD138$^+$, CD11b$^-$IgG1$^+$CD138$^+$ and CD11b$^-$IgG2bc$^+$CD138$^+$ respectively). Numbers are per single femur. (**B**) Histological analysis of salivary gland sections for CD3$^+$ T cells (blue) and IgM$^+$ B cells (red) (top panel) and GL7$^+$ GC B cells (blue) and IgD$^+$ B cells (red) (lower panel) at 28-33 weeks of age. Representative pictures are shown, n= 3 per group. (**C**) Histological analysis for IgM (upper panel) and IgG2c (lower panel) immune complex deposition in kidney glomeruli at 28-33 weeks of age. Representative pictures are shown, n= 3 per group. (**D**) Line Immunoblot Assay (LIA) for extractable nuclear antigens. The indicator reference is located on the left. On top of the LIA strips, five immunoblots are indicated by numbers: the cut-off control (1) and representative pictures of CD19-hBtk (2,3), wild-type (4) and *Myd88*$^{-/-}$ CD19-hBtk (5) mice. Only CD19-hBtk mice stained positive for autoantibodies, in these specific cases for RNP-A, RNP-C and histones.



CHAPTER 8

Transcriptomic deconvolution reveals the contribution of multiple signaling pathways to the CLL transcriptional signature in mice

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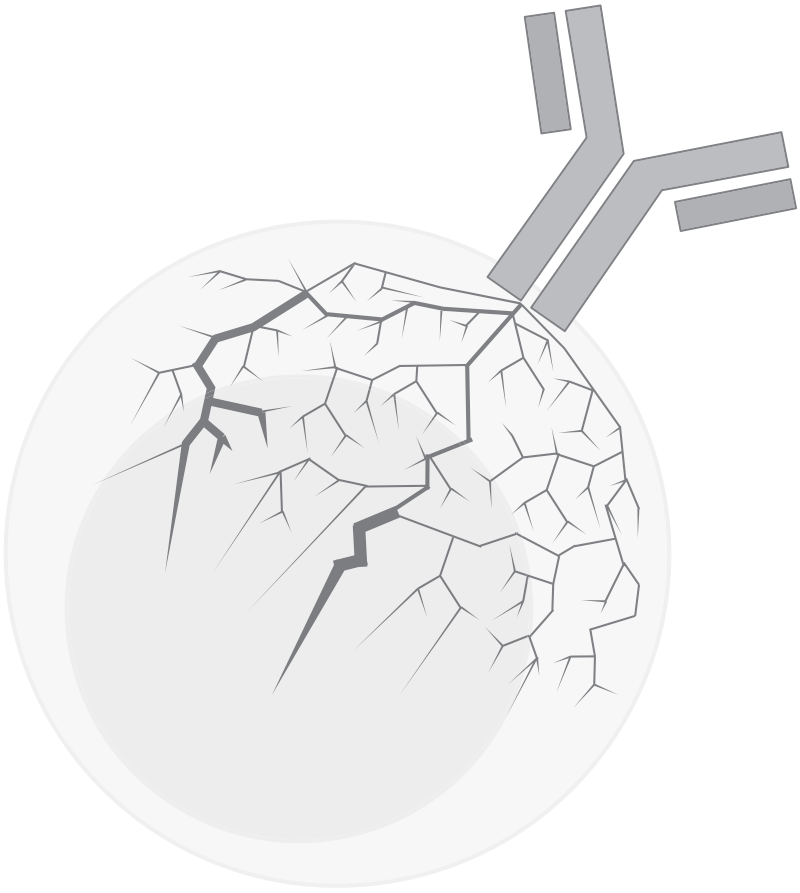
Submitted



CHAPTER 9

Differences in network connectivity of B cell receptor signaling molecules between mutated and unmutated chronic lymphocytic leukemia

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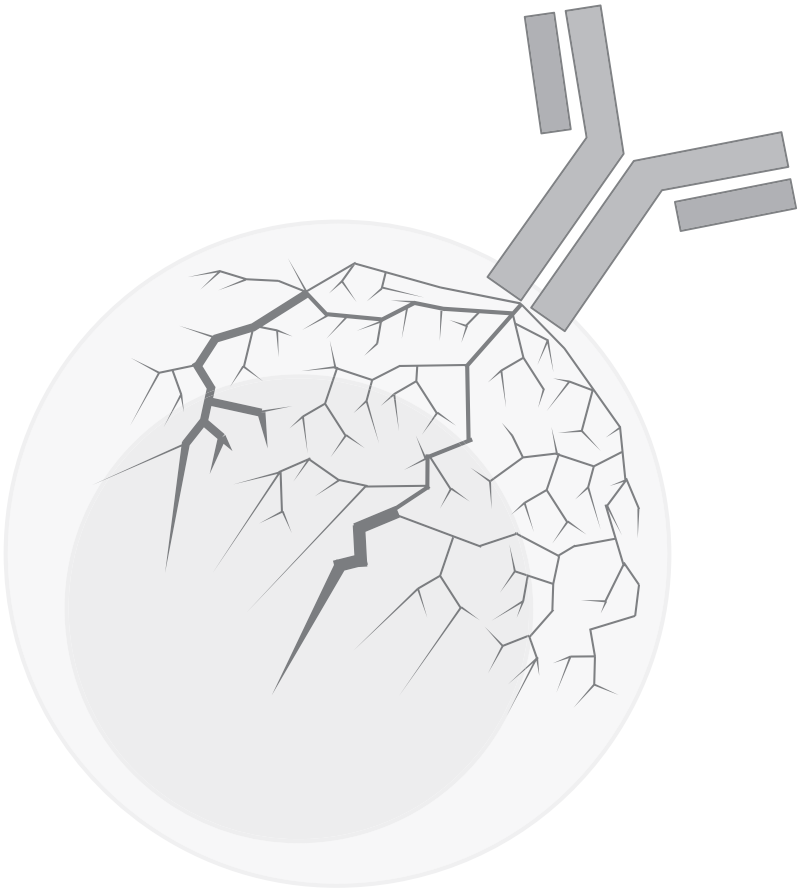

CHAPTER 10

General discussion

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General discussion

In this thesis, we addressed the role of BTK in BCR signaling and other pathways that contribute to B cell activation and survival. For these studies, we employed the CD19-hBtk model for Btk-mediated autoimmune disease, the *IgH.TE*μ model for chronic lymphocytic leukemia (CLL) and primary samples from CLL patients. To investigate the role of Btk in various B cell signaling cascades, we performed experiments using (phospho)flow cytometry and transcriptomic analysis on various crosses with other mouse models and *in vitro* and *in vivo* treatment with signaling inhibitors.
We identified the importance of the interplay between Tec and Btk in regulating downstream signaling of the BCR signaling pathway and the role in activation of Akt signaling (**Chapter 4**). Using phosphoflow cytometry (**Chapter 3**), we showed that Btk kinase not only regulates downstream BCR signaling pathways but also provides feedback to upstream BCR molecules (**Chapter 5**). Next to BCR signaling, we aimed to investigate the contribution of other key pathways contributing to Btk-mediated autoimmune disease. By crosses on CD40 ligand-deficient and Myeloid Differentiation Factor 88 (MyD88)-deficient mice, we found that T cell help and TLR signaling were crucial for the development of autoimmunity in Btk-overexpressing mice (**Chapter 6 and 7**). Transcriptomic analysis of CLL B cells revealed the contribution of multiple independent signaling pathways to the CLL-specific gene signature (**Chapter 8**). Finally, we observed that the mutational status of the IGHV gene of CLL patients determines the BCR signaling response (**Chapter 9**).

Regulation of Bruton tyrosine kinase

Regulation of Btk at protein level

Regulation of BTK expression levels is vital for B cells survival and differentiation as lack of BTK causes immunodeficiency and overexpression of Btk leads to development of autoimmune disease[1-4].Upon BCR activation in mouse and human B cells, BTK protein levels are upregulated[3, 5], although the mechanisms involved are not fully understood (**Chapter 2**). It has been shown that BTK can induce its own transcription in an NF-κB-dependent fashion, involving an NF-κB binding site in the BTK promotor[6]. In addition, B cell specific overexpression of BTK was previously shown to lead to development of an autoimmune phenotype[3]. We found that the autoimmune phenotype does not solely rely on enhanced BCR signaling but also depends on T cell interaction and TLR signals (**Chapter 6 and 7**). In human setting, we also observed increased BTK expression in B cells from patients with Rheumatoid Arthritis, Sjörgren's syndrome and granulomatosis with polyangiitis (GPA)[5, 7]. BTK was specifically increased in rheumatoid arthritis patients with anti-citrullinated protein antibodies (ACPA)[5], suggesting that targeting BTK could be sensible in diseases with antibody-mediated pathology. However, increased BTK levels in autoimmune disease

patients might be the consequence rather than the cause as inhibition of T cell activation with CTLA4 Ig abatacept lead to normalized BTK expression in B cells from Sjögren syndrome patients. In addition to the role of BTK's in autoimmune disease, increased expression and constitutive activity of BTK were observed in patients with chronic lymphocytic leukemia (CLL). BTK-overexpression does not spontaneously lead to the development of CLL, however, does accelerate the onset of disease when crossed on a CLL mouse model[8].

In order to better understand the upregulation of Btk upon activation *in vivo*, we infected wild-type mice, Btk-overexpressing mice with X-31 H3N2 influenza virus and evaluated upregulation of Btk protein levels at the peak of disease activity. After 5 days post infection, we observed upregulated Btk protein levels in wild-type (WT) and CD19-hBtk splenic B cells compared to B cells derived from PBS-treated controls (**Figure 1A**). Although we observed similar upregulation in local lung and peritoneal WT B cells, we did not detect local upregulation Btk protein in CD19-hBtk B cells upon X-31 infection (**Figure 1B,C**). We did not find upregulated Btk protein levels upon infection in the draining (mediastinal) lymph nodes (**Figure 1D**), suggesting that regulation of Btk protein levels are differentially regulated in this anatomical compartment. This would be in line with previous findings in autoimmune CD19-hBtk mice, which shows that development of autoimmune disease mainly presents in the spleen but not in lymph nodes of aging CD19-hBtk mice[3]. The differences in Btk protein regulation in lung and pleural cavity B cells from CD19-hBtk mice could be due to the presence or absence of regulatory elements in the human Btk construct, however, this would require further studies in the regulation of Btk protein levels in CD19-hBtk mice. As such, we next tried to reduce the human Btk overexpression to check whether low dose overexpression is sufficient to drive Btk-mediated autoimmunity. To reduce Btk overexpression to one additional copy, CD19-hBtk mice were crossed on CMV-cre mice (CMV-scBtk). CMV-scBtk splenic B cells showed reduced Btk protein levels compared to CD19-hBtk counterparts but significantly increased compared to WT controls (**Figure 2A**). Key parameters of Btk-mediated autoimmune disease are spontaneous GC formation and increased differentiated B cells in the spleens of CD19-hBtk mice[3]. We observed that the relative increase of GC B cells and T follicular helper (Tfh) cells was not significantly increased in CMV-scBtk mice compared to WT mice, whereas this was the case for CD19-hBtk controls (**Figure 2B**). This was also the case for the GC output, as percentages of plasma cells and memory B cells in CMV-scBtk mice were unchanged (**Figure 2C**). Analysis of autoantibodies in the serum by Hep2 assay revealed that CMV-scBtk mice do not have increased IgM or IgG autoreactive antibodies (**Figure 2D**), suggesting that these mice do not develop autoimmune disease. Further characterization of the CMV-scBtk mice would be required to draw definitive conclusions, however, these data suggest that the amount of Btk overexpression is crucial and that the autoimmune phenotype does not solely rely on the ectopic expression of human Btk.

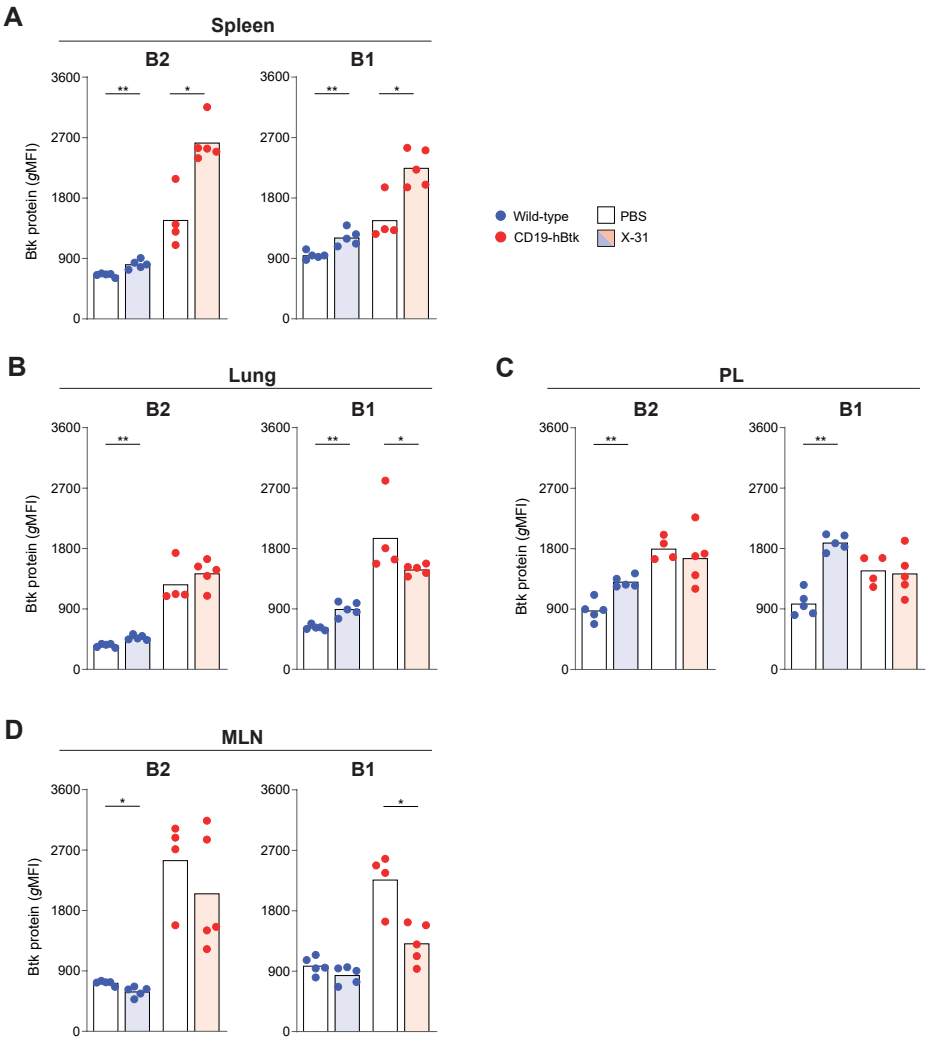

Figure 1. Impaired Btk protein upregulation in CD19-hBtk B cells upon infection with X-31 influenza virus. (**A-D**) Quantification of Btk expression level by geometric mean fluorescence intensity (*g*MFI) in B2 and B1 cells in spleen (**A**), lung (**B**), pleural cavity lavage (PL; **C**) and the lung-draining mediastinal lymph node (MLN; **D**). Symbols represent individual mice and bars indicate mean values, colored bars represent X-31 challenged mice; each group contained 4-5 mice; CD19-hBtk and WT mice were 8-12 weeks old; *p<0.05, **p<0.01 by Mann-Whitney *U* test.

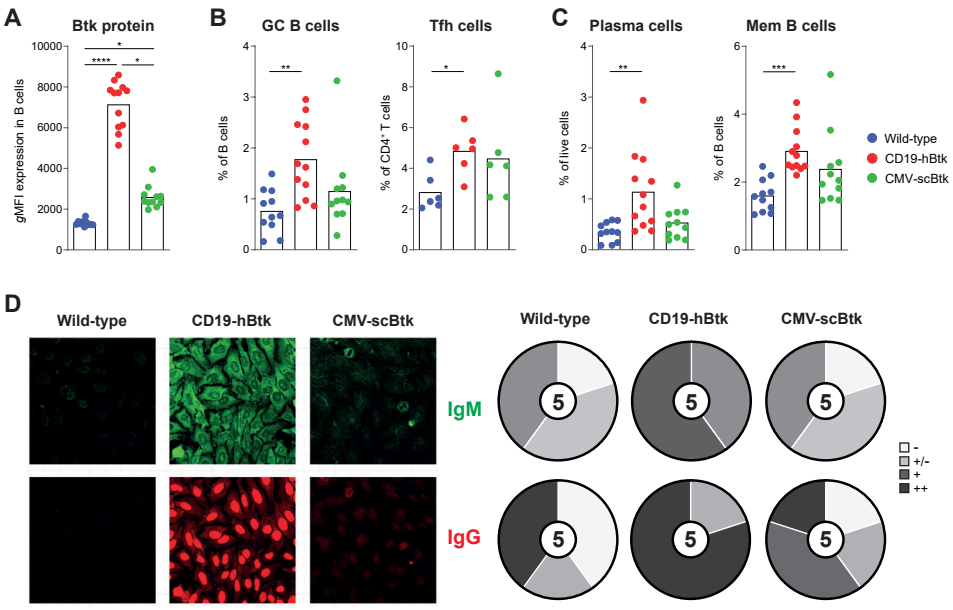

Figure 2. Limited Btk overexpression show a reduced autoimmune phenotype.
(**A**) Quantification of Btk expression level by geometric mean fluorescence intensity (*g*MFI) in total B cells in spleen. (**B**) Relative number of germinal center (GC) B cells of total B cells and follicular Thelper (Tfh) cells of CD4-positive T cells in the spleen. (**C**) Percentage of plasma cells (PC) B cells of live cells and memory (mem) B cells of total B cells in the spleen. (**D**) Representative microscopy pictures (*left*) of serum IgM (*top panel*) and serum IgG (*bottom panel*) autoreactivity to HEp-2 cells. Total mice analyzed is included in the individual pie charts included for quantification (*right*), –, no staining; +/–, mild staining; +, moderate staining; ++, strong staining. Symbols represent individual mice and bars indicate mean values; Graphs are representative of 2 individual experiments, each group contained 4-6 mice; CD19-hBtk and WT mice were 24-32 weeks old; *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001, by Mann-Whitney *U* test.

Targeting Btk kinase function

Ibrutinib is an orally bioavailable, irreversible BTK inhibitor that binds specifically to a cysteine residue (C481) in the ATP-binding region of the BTK kinase domain and thereby inhibits its enzymatic activity[9]. Ibrutinib treatment has shown clear clinical anti-tumor effects in CLL and various other B cell malignancies[10-12], even though this compound is not highly specific: it also binds to EGFR, TEC and ITK[9], potentially compromising its therapeutic index. It remains unclear what the physiological effects would be of putative inhibition of TEC activity by ibrutinib in CLL cells. On the one hand it has been reported in mice that Tec can partly compensate for the absence of Btk in B cells, but on the other hand we recently found that Akt signaling was increased in Tec-deficient B cells (**Chapter 4**). Nevertheless, it is not very likely that the effectiveness of ibrutinib in CLL is partially due to its non-specific nature, because the more selective BTK inhibitor acalabrutinib has also shown high effectiveness in relapsed CLL in clinical trials[13]. A phase III trial comparing the efficacy of ibrutinib and acalabrutinib is currently ongoing, of which the results will be crucial to determine whether acalabrutinib is as effective as ibrutinib. Of the next-generation BTK inhibitors, acalabrutinib performed particularly well due to the high overall response rate in CLL patients, also including del(17p) CLL subjects[13]. We showed that specific inhibition of BTK kinase activity by acalabrutinib influences both up- and downstream BCR signaling pathways (**Chapter 5**). Remarkably, the exact mechanism how ibrutinib or acalabrutinib ameliorates CLL disease is currently unknown. It is believed that BTK inhibition has a direct effect on B cell survival, but there is also evidence that it has major effects on B cell migration and adhesion and thereby on B cell homing and retention in a favorable micro-environment.[4, 10, 14]

The use of BTK inhibitors is not limited to cancer, as there is a growing interest for BTK inhibitors for treatment of autoimmune diseases with autoantibody involvement. Targeting BTK activity has proven effective in several mouse models[9, 15] and there are currently clinical trials several trails with BTK inhibition are ongoing (**Chapter 2**). Ibrutinib treatment might enhance immune responses or autoimmune symptoms in patients, as it has been found that by targeting ITK in T cells ibrutinib drives differentiation of T helper cells to a Th1 phenotype[16]. To specifically target Btk, there are currently some trials ongoing using next-generation Btk inhibitors for treatment of autoimmune diseases. A very recent clinical trial with evobrutinib significantly lowered enhancing lesions forming in patients suffering from Multiple Sclerosis[17]. In addition, treatment with acalabrutinib also seems promising for treatment of patients suffering from coronavirus disease 2019 (COVID-19) inflammation[18], although the effectiveness might be due to inhibition of monocyte activation instead on B cell activity. This would together indicate that Btk inhibition could be a promising therapeutic option is several autoimmune diseases, however, more patients trials are required to make solid conclusions on the efficacy and safety profile in autoimmune disease setting.

Unfortunately, continuous Btk inhibition treatment was reported to lead to selection or outgrowth of resistant CLL clones, as described in a subset of CLL patients who showed disease relapse upon ibrutinib therapy. This phenomenon was attributed to either a BTK C481S

mutation (the site of attack of ibrutinib), or to activating mutations in PLCγ2 (R665W, S707Y and L845F), an important downstream substrate of BTK[19]. The progression of disease while on ibrutinib therapy is associated with a poor outcome, as some patients experience rebound lymphadenopathy and symptoms upon discontinuation. This demands the need to develop novel therapeutic strategies, in particular the development of rational treatment combination strategies that could improve the current success rates without increasing the toxicities.

Epigenetic regulation of Btk

Next to presenting with constitutive active BCR signaling on protein level, several epigenetic aberrations are also frequently observed in CLL. CLL patients were previously shown to be hypomethylated DNA compared to normal B cells[20]. The BCR mutational status also played a role, as U-CLL cells have a more open and accessible chromatin than M-CLL cells[21]. In addition, the authors also showed a distinct chromatin signature in CLL patients with cytogenic aberrancies or mutations in MYD88[21]. A recent study showed that ibrutinib treatment in CLL patients reduces H3K27 acetylation and trimethylation marks compared to naive CLL cells, which also related to altered gene expression of chromatin modifier EZH2 in these treated patients[22].

Furthermore, about 50% of CLL patients have deletions at 13q14, which are associated with downregulation of miR-15a and miR-16[23]. Loss of this miRNA cluster results in overexpression of survival genes, including BCL-2 and MCL-1, thereby inducing resistance to apoptosis. It has also been shown that several miRNAs, including miR-155, are overexpressed in CLL, diffuse large B cell lymphoma (DLBCL) and multiple myeloma (MM) patients[24]. Because the inositol phosphatase SHIP1 is a primary target of miR-155[25], HDACi may lead to lower expression of SHIP1, which negatively regulates BCR signaling by dephosphorylating PIP_3. The direct effects of HDACi on *BTK* locus accessibility, e.g. through promoter or enhancer elements in the *BTK* locus, has not been extensively studied thusfar. However, the observed robust downregulation of BTK protein levels by HDACi clearly indicates that the influence of such direct effects on BTK expression is negligible[26].

As previously discussed, microRNAs, including miR-185, play an important role in the post-translational regulation of BTK expression. Therefore, it is conceivable that not only BTK function but also BTK protein expression levels are very relevant for normal B cell function, indicating that BTK dysregulation could play an important role in B cell malignancies. This is in agreement with recent findings showing that increased expression of BTK is associated with autoimmune disease. Belver *et al.* showed that targeted deletion of Dicer in B cells in mice results in lower levels of miR-185, accumulation of BTK and an autoimmune phenotype[27].

Combination therapies adjusting BCR signaling

Combination therapies leukemia and lymphoma

Similar to other cancer treatments, selection or outgrowth of resistant clones is a major problem in clinical management of CLL. Although monotherapy strategies have a better safety profile, patients will indefinitely undergo progression or will experience other toxicity issues. Combining or replacing conventional chemotherapeutics with novel targeted drugs could provide new treatment options that could pose considerable selection pressure for the CLL cells to escape elimination. Treatment combinations of chemotherapy and B cell depletion therapy showed strongly improved outcome in CLL[28, 29]. As most small-molecule inhibitors have fewer side-effect compared to chemotherapy, treatment with multiple small-molecule inhibitors combined with B cell depleting agents is gaining broad interest (see **Figure 3** for overview). Acalabrutinib together with B cell depletion therapy using Obinutuzumab was also proven superior to B cell depletion with chemotherapy[30], indicating that combination therapy could provide a chemotherapy-free treatment option to CLL patients. There have been multiple trails studying the combined effectivity of CD20-targeting B cell depleting agents together with BTK or PI3K inhibition. The outcome of these first clinical trials showed that BTK inhibition and B cell depletion was effective as first line treatment[31], however, relapsed/refractory CLL patients did not benefit from the addition of B cell depleting therapy to ibrutinib treatment alone[32]. In contrast, PI3K inhibition with rituximab was effective in relapsed CLL patients[33]. This together shows that the dual treatment of small molecule inhibitors with B cell depleting agents holds great promise. Furthermore, there are also strategies combining several signaling targets in order to breach the microenvironment niche that is a major issue in CLL disease. Treatment with a dual SYK/JAK inhibitor cerdulatinib induced apoptosis in CLL cells and reduced capacity to produce CCL3 and CCL4[34, 35], two chemokines that are induced upon interaction with nurse-like cells. Combination therapy targeting both BCR signaling modules and the microenvironment interaction of CLL cells hold great promise, however, would require further evaluation by clinical trials.

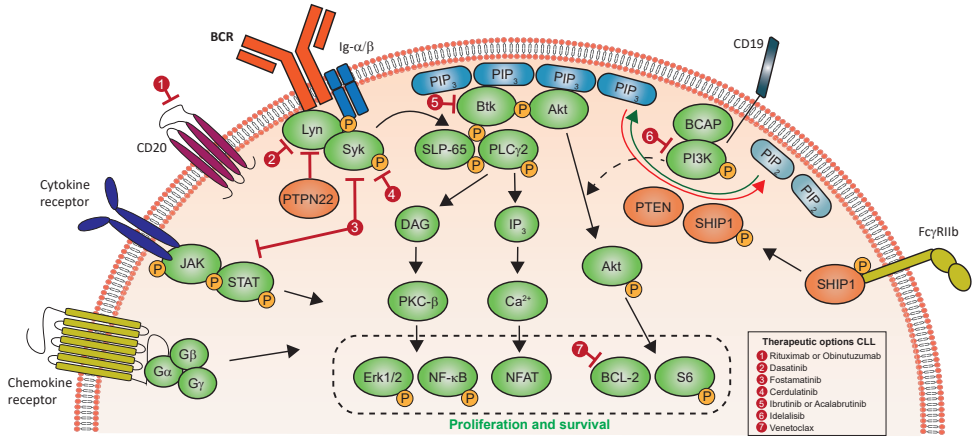

Figure 3. Signaling in B cells and therapeutic intervention strategies in CLL.
Overview of BCR signaling and other important signaling modules for B cells. Upon BCR engagement, LYN will activate and phosphorylate Ig-α and Ig-β, subsequently activating SYK. Together with CD19-mediated activation of PI3K, this leads to activation of SLP-65, BTK and PLCγ2. This in turn activates downstream signaling pathways crucial for proliferation and survival, including engagement of ERK, NF-κB and AKT downstream mediators like S6 and anti-apoptotic proteins like BCL-2. Other receptor signaling pathways like cytokine receptor signaling and chemokine receptor signaling also contribute to the proliferation and survival signature of healthy and diseased B cells. On the other hand, inhibitory signals from Fc receptors are able to control signaling by activation of phosphatases, like SHIP1. Therapeutic interventions for CLL patients are shown by red circles and mentioned on the lower right bottom.

Next to targeting BTK, PI3Kδ inhibition using idelalisib was previously shown to be effective in treating CLL[36, 37]. Antagonists of anti-apoptotic protein BCL-2, which is overexpressed in CLL – improved CLL disease activity, in patients with U-CLL and patients presenting with chromosome 17p deletions[38]. Interestingly, it was also reported that acalabrutinib treatment increased the effectivity of BCL-2 antagonist venetoclax[39] and that venetoclax treatment is effective in refractory MCL patients after initial treatment with BTK inhibitors[40]. This would suggest it would be useful to see if venetoclax as second-line treatment to BTK inhibition works for subgroups of CLL patients. Lastly, several clinical trials are currently ongoing that evaluate the efficiency of combination therapy using BTK inhibitors together with venetoclax and Obinutuzumab (**Table 1**).

Table 1. Clinical trials for therapeutic combination therapy in CLL including BTK-inhibitors.

Therapy line	Therapeutic regimen	Trial phase	Status	Reference (NCT)
	Ibrutinib			
First line	Ibrutinib + Daratumumab (anti-CD38)	I	Recruiting	NCT03447808
RR/CLL	Ibrutinib + Daratumumab (anti-CD38)	II	Recruiting	NCT04230304
First line	Ibrutinib + Venetoclax	III	Active, not recruiting	NCT03462719
First line (elderly)	Ibrutinib + Obinutuzumab + Venetoclax	III	Recruiting	NCT03737981
First line	Ibrutinib + Obinutuzumab + Venetoclax	III	Recruiting	NCT03701282
First line	Ibrutinib – Obinutuzumab – Venetoclax (sequential therapy)	II	Recruiting	NCT03755947
	Acalabrutinib			
First line	Acalabrutinib + Obinutuzumab	II	Recruiting	NCT04505254
First line	Acalabrutinib + Obinutuzumab	II	Recruiting	NCT03516617
R/R CLL	Acalabrutinib + Venetoclax post initial Venetoclax + Rituximab/Obinutuzumab	II	Not yet recruiting	NCT04523428
First line	Acalabrutinib + Venetoclax + Obinutuzumab	II	Recruiting	NCT03580928
First line	Acalabrutinib + Venetoclax + Obinutuzumab	III	Recruiting	NCT03836261
R/R CLL	Acalabrutinib + Venetoclax + Obinutuzumab	II	Recruiting	NCT04169737

It is also attractive to explore combination therapies of HDACi with acalabrutinib, which has a promising safety profile. Combination therapy with ibrutinib and HDACi's affected the survival of CLL cells in the *Eμ*TCL*-1 CLL mouse model and also induced synergistic cytotoxicity in primary cells from CLL patients[26]. This would together suggest that targeting BCR signaling and HDAC activity simultaneously would be a promising therapeutic options targeting survival of CLL cells. Next to combining HDACi with BTK inhibitors, combinations with a BCL-2 inhibitor or inhibitors that target other BCR signaling molecules, such as PI3K or SYK, would be very interesting with respect to treatment of CLL or other B cell malignancies. Treatment with CUDC-907, which is a dual inhibitor of both PI3K and HDAC activity, resulted increased apoptosis of MCL cells[41].

Previous studies provided evidence that effective reduction of BTK expression by HDAC inhibitors and targeting enzymatic activity of BTK may be promising as a therapeutic modality that suppresses survival signals in CLL[26]. This raises an important question: should ibrutinib and HDACi be given simultaneously, before small-molecule inhibitor treatment or after resistance arises? Because of current problems of toxicity in CLL patients with HDACi and side-effects from ibrutinib treatment, it might not be wise to give the therapeutics simultaneously. The rationale of giving HDACi as second-line therapy after ibrutinib may be more sensible, since the authors showed that C481S-mutant BTK can still be targeted. Additional studies are required to establish the best treatment strategy and, importantly, should show whether HDACi is beneficial for patients that show a limited response to BTK inhibition. Furthermore, other leukemic diseases, such as MM, mantle cell leukemia, and activated B cell-like DLBCL, could very well benefit from combination therapy with small-

molecule inhibitors, HDACi or other new epigenetic modulators. The combination of these drugs with other effective therapeutic options like B cell depleting agents or venetoclax should be studied in clinical trial setting, also looking into the optimal sequential order of therapy. Difference in regulation and activation of BCR signaling between unmutated and mutated CLL should also give new directions to better treat these two CLL subsets.

In addition to existing therapeutic modalities, it would be worthwhile to develop compounds that efficiently and selectively target PLCγ2, because of the ibrutinib-induced gain-of-function mutations in PLCγ2[19]. Although CLL cells with C481S-mutant BTK can be very well targeted by HDACi, patients with an ibrutinib-induced constitutive active PLCγ2 mutation will not benefit from HDACi because it does not affect PLCγ2 protein expression. Furthermore, it was previously shown that these activating PCLγ2 mutants are increasingly sensitive to activation via Rac2[42], suggesting that Rac2 inhibition could be beneficial for treatment of ibrutinib-induced PLCγ2 mutations. We studied the effect of Btk inhibition on BCR signaling and found feedback mechanisms that upregulate specific signaling pathways as consequence of the loss of Btk kinase function (**Chapter 5**). Additional studies on BCR rewiring induced by BTK inhibition therapy are essential to identify additional pathways that are receptive upon ibrutinib-induced mutations. Furthermore, there is a need for additional mechanistic studies on the role of different pathways in CLL as this might be altered in different subtypes (**Chapter 9**).

Combination therapeutic strategy autoimmune diseases

Combination therapy targeting BCR signaling might also be beneficial for patients with autoimmune diseases, as there are indications that several other BCR signaling molecules than BTK hold promise for therapeutic targeting. Inhibition of SYK kinase function has been quite successful in mouse models of rheumatoid arthritis and SLE[43, 44], however, fostamatinib treatment in patients did not prove to be very effective over standard therapy[45]. There is a search for other, more selective, SYK inhibitors for autoimmune disease treatment to also limit the occurrence of adverse events that are commonly observed using fostamatinib. A single-nucleotide polymorphism in PTPN22, a phosphatase important in regulation of Src kinase activity, has also been found in several autoimmune diseases[46, 47]. This R620W polymorphism alters BCR signaling activity and also leads to increased expression of genes like *CD40* and NF-κB signaling molecules[48]. Interplay of enhanced BCR signaling and co-receptor activity has often been reported, certainly for the BCR with and TLR signaling[49, 50]. We also confirmed the role of CD40 and TLR interplay in our Btk-mediated AID model (**Chapter 6&7**), confirming that intrinsically enhanced BCR expression does require licensing of co-receptor signaling to develop autoimmunity. Also CD40 expression is crucial for B cell maturation, as it was shown that CD40-deficiency further hampered maturation of $Btk^{-/-}$ B cells compared to CD40-sufficient $Btk^{-/-}$ B cells[51]. BTK targeting therapy could potentially target both signaling pathways and thereby dampen synergistic activation. However, combination targeting of BTK together with other routes - like signaling involved in PTPN22 and SYK activation – could be very promising to correct B cell tolerance in autoimmune disease patients.

Concluding remarks and future directions

Treatment with BTK inhibitors have shown to be important in treatment of patients with autoimmune disease, leukemia and lymphoma. In this thesis, we aimed to investigate BTK signaling and interaction with other molecules and signaling cascades than BCR signaling. An important finding of our studies is that BTK and Tec have distinct functions in activating Akt signaling in B cells. Moreover, we showed that Btk-mediated autoimmunity not solely dependent on BCR signaling, as TLR signaling and CD40 signaling proved to be required for the autoimmune phenotype. This was also the case for a mouse model of CLL, as both BCR, T cell help and TLR signaling showed additional contribution to the activated phenotype of these leukemic cells.

Inhibition of other BCR molecules seem promising, also in combination therapy with BTK inhibitors. Although this strategy is gaining a lot of interest for treatment of CLL, the effectiveness of BCR signaling inhibition and combination therapy in the field of autoimmunity still requires much fundamental and clinical interest. For autoimmunity, it is crucial to first check the efficiency of dual targeting in mouse models and subsequently in clinical trial. Combinations of BTK and PI3K/AKT inhibitors or NF-κB could be very interesting with respect targeting both central BCR signaling and key survival pathways.

Further investigations into the B cell signaling molecules and the effect of specific pathways inhibition will be crucial to understand the underlying mechanism of B cell survival and selection. To better understand the molecular mechanism of BTK regulation, it would also be very worthwhile to better study the interaction between Btk and Tec protein in B cell activation. Deep profiling signaling studies could prove to be very insightful, certainly when looking into differences between Btk-deficient and Tec-deficient B cells compared to WT controls. This should lead to better understanding of the interplay between the respective signaling branches in both BCR and co-receptor signaling pathways and could eventually help in determining the most optimal order of the combination therapy will strongly impact the effectivity of treatment.

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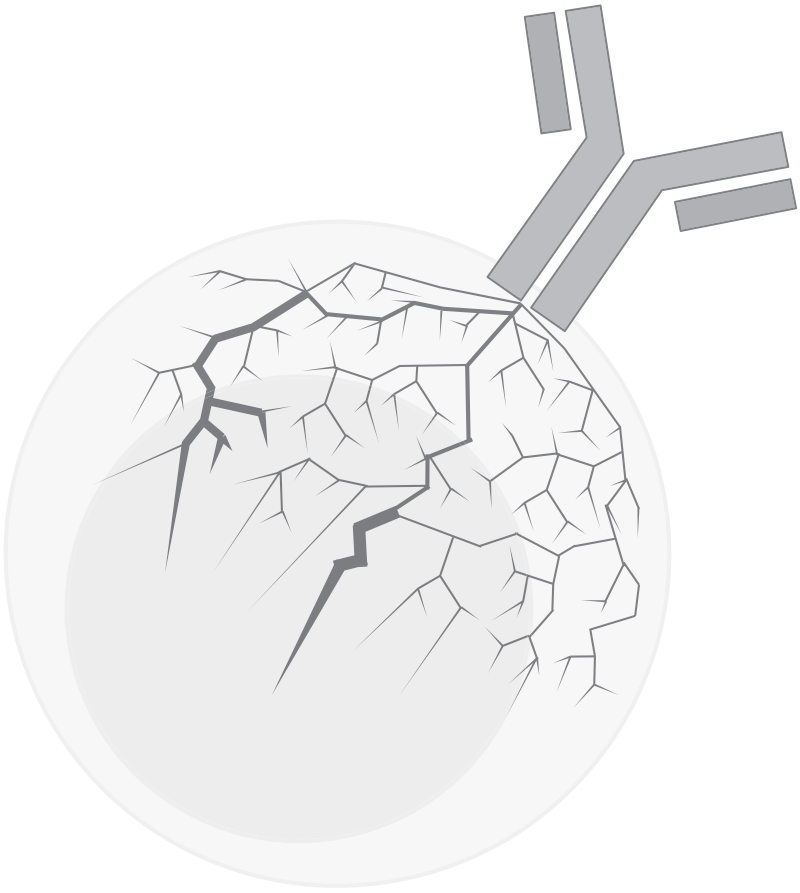

ADDENDUM

Summary

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SUMMARY

SUMMARY

B cells are lymphocytes that can recognize antigen via their antigen-specific B cell receptor (BCR). This process is crucial in the immune response against pathogens, as proper activation of these B cells eventually leads to the production of antibodies that can help clearing the infection. On the other hand, antibodies can also cause harm when they are directed to self-antigens, and thereby contribute to autoimmune disease. Rheumatoid arthritis and Sjogren's syndrome are autoimmune diseases marked by autoantibody-mediated pathology, providing a rationale for a distorted B cell response in these patients.

Signaling via the BCR is crucial for survival, proliferation and differentiation of B cells. Altered expression or regulation of proteins involved in the BCR signaling cascade has a drastic effect on B cell selection and on the responsiveness of B cells to particular antigens. Bruton's tyrosine kinase (Btk) is a crucial component of the BCR signaling pathway, but it is also involved in other signaling routes, including CD40 signaling initiated by T cell help, Toll-like receptor (TLR) to recognize additional danger signals and chemokine receptors. Tight regulation of Btk expression is required for normal B cell function, as lack of Btk expression leads to immunodeficiency and increased levels of Btk in B cells is associated with autoimmunity. In this thesis, we focused on the regulation of Btk expression and activity in B cells in health and in development of disease. In **Chapter 2**, we provide an overview of the role of Btk in several immune cells and distinct signaling routes and highlight the relevance of regulating Btk protein levels with respect to development of autoimmunity.

Studying BCR signaling in primary cells has become increasingly feasible due to the rise of phosphoflow, a flow cytometry-based technique to detect protein phosphorylation with high sensitivity at single-cell level. As we aimed to study Btk function and BCR signaling kinetics, our first objective was to reliably measure phosphorylation of signaling molecules in human and murine B cell populations. We therefore set-up a phosphoflow protocol to detect signaling protein phosphorylation in conjunction with B cell surface marker. A detailed description of procedures to measure phosphorylation is given in **Chapter 3.1 and 3.2**, which was essential for our studies on BCR signaling**.**

Although the relevance of Btk protein in B cells is obvious, the role of its most closely related family member, Tec kinase, in BCR signaling remained elusive. Therefore, we used mice with a targeted deletion of the *Tec* gene to identify the role of Tec protein and the interplay with Btk in **Chapter 4**. We showed that Tec kinase is dispensable for B cell development in the presence of Btk, but also found that mature Tec-deficient B cells showed increased activation and survival upon BCR stimulation, compared with wild-type (WT) control B cells. The increased survival of Tec-deficient B cells was likely due to enhanced Akt kinase signaling, which could be restored to WT levels by treatment with the Btk inhibitor ibrutinib. Taken together, these findings suggested that Tec and Btk may compete for activation of the Akt signaling route, and that the ability of Btk to activate Akt is limited by Tec kinase.

Targeting of Btk enzymatic activity has shown high efficacy in the clinic in the treatment of B cell malignancies and may be a promising therapeutic strategy in several autoimmune disorders. The precise effect of Btk kinase inhibition on the functionality of BCR signaling, however, has not been well studied. In **Chapter 5**, we therefore aimed to investigate the effects of Btk kinase activity on BCR signaling kinetics. We compared BCR signaling in Btk-deficient and WT B cells and found, as expected, that downstream survival pathways were decreased upon BCR stimulation in absence of Btk. Interestingly, we also observed that upstream signaling was increased in the absence of Btk protein. This was also observed after prolonged Btk inhibitor treatment of wild-type mice or mice engrafted with leukemic B cells, indicating that BCR signaling undergoes extensive rewiring upon Btk kinase inhibition.

Previous reports by our group showed that B cell-specific overexpression of Btk in mice leads to spontaneous development of an autoimmune phenotype. Although the role of BCR signaling in the increased survival of Btk-overexpressing B cells was evident, the involvement of additional receptors that have the capacity to promote B cell activation and survival, such as CD40 and TLRs, in the development of the autoimmune symptoms remained elusive. To investigate the importance of these signaling modules, we crossed Btk-overexpressing mice with CD40L-deficient mice in **Chapter 6** and thereby explored whether B-T cell interaction was crucial for the autoimmune phenotype. In addition, we also crossed Btk-overexpressing mice on myeloid differentiation factor 88 (MyD88)-deficient mice in **Chapter 7** to investigate the role of TLR signaling. We observed that the autoimmune phenotype was dependent on CD40 as well as TLR signals, because disease development of disease was abrogated in both mouse models. Whereas MyD88-deficient Btk-overexpressing mice did not show circulating autoantibodies, CD40L-deficient counterparts still produced some autoreactive IgM antibodies. From these observations, we concluded that the Btk-driven autoimmune phenotype relies on both T cell help and TLR signals.

Chronic lymphocytic leukemia (CLL) is a B cell malignancy that is marked by enhanced BCR signaling. However, the relative contribution of other receptor signaling pathways to survival and proliferation of CLL cells remained largely unknown. In **Chapter 8**, we performed transcription profiling on primary CLL B cells derived from the *IgH.TE*μ CLL mouse model previously generated in our lab, which is based on sporadic expression of the SV40 large T oncogene in mature B cells. We identified a CLL gene signature comprised of 2,105 genes differentially expressed between primary CLL cells and WT splenic B cells. This CLL signature was subsequently compared to the transcriptome of B cells activated by BCR, CD40/IL-4 and TLR stimulation. We observed that a major part of the CLL gene signature could be explained by general activation properties shared between these three pathways, mainly relating to cellular proliferation. Furthermore, we observed a unique set of CD40/IL-4-regulated genes within the CLL signature that strongly contributed to proliferation. The CLL signature also contained genes specifically regulated by TLR4 stimulation, but they were relatively low in number. Finally, a diverse group of genes was identified, which were specifically up or downregulated in Btk-overexpressing splenic B cells. Taken together, this transcriptomic deconvolution approach could link ~70% of the CLL signature genes to the investigated stimulatory signals. Therefore, we concluded that additional signals from the

CLL microenvironment were also critically involved and that - taken together - multiple independent signaling modules contribute to the CLL-specific gene expression signature.

Survival of CLL patients is dependent on the mutational status of the BCR, as patients with an unmutated BCR status (U-CLL) have a poorer prognosis than patients with a mutated BCR status (M-CLL). In **Chapter 9**, we used phosphoflow to investigate whether U-CLL and M-CLL cells show differences in BCR responsiveness. We observed that basal phosphorylation and responsiveness was generally quite comparable between the two CLL subtypes. However, basal phosphorylation in M-CLL cells showed lower connectivity between individual signaling molecules than U-CLL cells. These differences in signaling connectivity may imply that treatment strategies that target signaling molecules differentially affect U-CLL and M-CLL patients.

In **Chapter 10**, we discuss the findings in this thesis in light of recent advances in the treatment landscape for CLL and autoimmune diseases. Inhibition of Btk kinase activity in combination with other therapeutics targeting B cell survival could be the preferred mode of treatment in patients suffering from CLL and might also be effective in autoimmune disease patients.

NEDERLANDSTALIGE SAMENVATTING

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B cellen vormen een gespecialiseerde groep binnen de witte bloedcellen en spelen een cruciale rol in de afweer tegen ziekteverwekkers. Met behulp van hun B cel receptor (BCR) kunnen B cellen specifieke stukjes van een pathogeen, antigenen genoemd, herkennen. Als een dergelijk antigeen ook wordt herkend door een T cel, een ander type witte bloedcel, kan de T cel de B cel helpen om volledig geactiveerd te geraken. Als een B cel met behulp van zijn BCR deze antigenen heeft herkend en T cel hulp heeft ontvangen, wordt de B cel tot celdeling aangezet. Vervolgens kunnen de ontstane B cellen zich verder ontwikkelen tot memory B cellen, die belangrijk zijn voor immunologisch geheurgen, of tot een plasma cellen, die grote hoeveelheden antigeen-herkennende antistoffen kunnen produceren. Deze antistoffen kunnen aan de antigenen op ziekteverwekkers binden en dragen vervolgens bij aan de klaring van de infectie. De selectie van de BCR tijdens de B cel ontwikkeling en de sterkte van het signaal dat de BCR doorgeeft aan de celkern via BCR signalering zijn tezamen van groot belang voor de opbouw van een B cel compartiment met een zeer divers en functioneel BCR repertoire. Wanneer dit proces niet goed verloopt of uit balans raakt kan het voorkomen dat BCR lichaamseigen structuren herkennen, ook wel auto-reactiviteit genoemd. In gezonde mensen worden deze cellen uitgeselecteerd, maar als dit proces niet goed verloopt kan dit leiden tot de ontwikkeling van een auto-immuunziekte. Wanneer er veranderingen optreden in de regulatie van eiwitten die een rol spelen in BCR signalering kan dit een drastisch effect hebben op de selectie van B cellen en hoe een B cel reageert op antigene stimulatie.

Bruton's tyrosine kinase (Btk) is een enzym dat een fosfaatgroep kan aanbrengen aan een ander eiwit, dat daardoor meestal wordt geactiveerd. Btk heeft een cruciale positie binnen de BCR signaleringscascade, wat betekent dat Btk belangrijk is voor het doorgeven van het BCR signaal, uiteindelijk naar de celkern. Btk eiwit speelt ook een belangrijke rol in andere signalering routes die belangrijk zijn in B cel activatie, zoals in CD40 signalering die wordt geactiveerd door middel van T cel hulp. Het belang van Btk is evident, gezien een aangeboren tekort aan functioneel Btk eiwit leidt tot X-gebonden agammaglobulinemie, een immuundeficiëntie ziekte gekenmerkt door grote problemen in de B cel gemedieerde afweer en daardoor ernstige en terugkerende infecties. Ook de regulatie van expressie en activiteit van Btk eiwit is belangrijk voor het behoud van gezonde B cel functie, gezien patiënten met bepaalde soorten leukemie of auto-immuunziekten verhoogde activiteit of expressie hebben van Btk in B cellen. Dit tezamen illustreert het belang van strikte regulatie van het Btk eiwit voor normale B cel functionaliteit. In dit proefschrift hebben we de processen die bijdragen aan de regulatie van Btk activiteit in B cellen onderzocht, zowel in de gezonde situatie als in de ontwikkeling van verscheidene ziektebeelden. In **Hoofdstuk 2** bespreken we de functie en het belang van Btk in verschillende immuuncellen en signaleringsroutes. Daarnaast bespreken we ook de rol van Btk eiwit in de ontwikkeling van auto-immuunziekten.

Door recente technologische ontwikkelingen kan de BCR signaleringscascade beter en diepgravender worden onderzocht. De BCR signaleringscascade bestaat uit een netwerk van verschillende eiwitten, die net als Btk, na BCR stimulatie elkaar kunnen activeren, vaak door een fosforgroep aan een aminozuur te zetten. Door deze fosforgroep raken deze signaleringseiwitten geactiveerd en kunnen vervolgens bij andere eiwitten ook een fosforgroep

plaatsen, een proces wat fosforylering wordt genoemd. Phosphoflow is een flowcytometrie-gebaseerde techniek om fosforylering van eiwitten sensitief te kunnen kwantificeren. Gezien wij de exacte rol van Btk in BCR signalering wilden bestuderen, was ons primaire doel om betrouwbare kwantificering van signalering te kunnen meten in verschillende B cel subpopulaties in muis en mens. Om dit sensitief te kunnen detecteren hebben wij een eigen phosphoflow protocol opgezet in **Hoofdstuk 3.1 en 3.2**. De ontwikkeling van dit protocol was essentieel om onze vervolgstudies te verrichten omtrent BCR signalering.

De rol van het Btk eiwit in B cellen in BCR signalering is redelijk goed gedefinieerd, echter is over de functie van andere eiwitten binnen de Tec kinase familie, waartoe ook Btk behoort, nog weinig bekend. Eén van deze Tec kinase familie eiwitten die ook in B cellen tot expressie komt is Tec kinase. Vandaar dat we in **Hoofdstuk 4** hebben onderzocht of Tec kinase belangrijk is voor B cel functie en wat de rol is van dit eiwit naast Btk in BCR signalering. Uit onze studies is gebleken dat Tec kinase niet nodig is voor een normale B cel ontwikkeling als Btk aanwezig is. Echter observeerde we versterkte activatie en overleving van Tec-deficiënte B cellen na stimulatie via de BCR. De betere overleving van Tec-deficiënte B cellen is waarschijnlijk het gevolg van versterkte Akt signalering in deze B cellen, een fenomeen dat kon worden genormaliseerd door cellen te remmen met de Btk remmer ibrutinib. Dit tezamen wijst erop dat Tec en Btk competeren voor de activatie van de Akt signaleringscascade na BCR stimulatie en suggereert dat Btk-gemedieerde Akt signalering wordt gelimiteerd door de aanwezigheid van Tec eiwit.

De remming van Btk kinase activiteit is een effectieve therapeutische behandeling in verschillende oncologische ziektebeelden en ook veelbelovend voor de behandeling van patiënten met auto-immuunziekten. De effecten van Btk kinase inhibitie op de functionaliteit van BCR signalering is tot dusverre onderbelicht. Daarom hebben we in **Hoofdstuk 5** de rol van Btk eiwit en Btk remmers op BCR signalering in B cellen in detail onderzocht. Hier vergeleken we BCR signalering in Btk-deficiënte en normale, wild-type (WT) B cellen en observeerde verlaagde activatie van signaleringsroutes na BCR stimulatie onder Btk, zoals verwacht. Maar, de activatie van signaleringseiwitten direct onder de BCR was echter versterkt, wat een onverwachte bevinding is gezien deze eiwitten zich boven Btk in de signaleringscascade bevinden. Dit fenomeen konden we ook waarnemen in B cellen verkregen uit WT muizen en chronische lymfatische leukemie (CLL) cellen na behandeling met Btk remmers. Deze bevinding duidt erop dat dit mechanisme ook kan worden geïnduceerd door therapeutische behandeling. Deze veranderingen in BCR signalering na behandeling zou gevolgen kunnen hebben voor de gevoeligheid van antigene stimulatie, met eventuele consequenties voor de B cel response in patiënten die deze therapie ontvangen.

Eerder studies van onze groep beschreven een muismodel met B-cel-specifieke overexpressie van Btk die leidt tot ontwikkeling van een auto-immuun ziektebeeld. BCR signalering is van cruciaal belang voor de ontwikkeling van auto-immuniteit in dit muismodel, maar de rol van andere receptoren en hun samenhang met de BCR voor B cel activatie is nog nauwelijks bekend. Om de rol van andere signaleringsroutes te onderzoeken hebben we de Btk-overexpressie muis gekruist op CD40 ligand (CD40L)-deficiënte muizen in **Hoofdstuk 6.** Dit stelde ons in staat te bestuderen of de rol van de interacties tussen B en T cellen cruciaal was.

Daarnaast hebben we in **Hoofdstuk 7** gekeken of Toll-like receptor (TLR) signaleringsroutes een rol spelen door het Btk-overexpressie model te kruisen met muizen die deficiënt zijn voor myeloid differentiation factor 88 (MyD88), een molecuul dat een centrale functie heeft in de signaleringsroute van bijna elke TLR. Deze analyse was van belang omdat TLR signalering een belangrijke rol lijkt te spelen in de ontwikkeling van auto-immuunziekten in andere muismodellen. Uit onze experimenten bleek dat de Btk-gemedieerde auto-immuniteit in ons muismodel afhankelijk is van zowel B-T cel interacties als TLR signalering.

CLL is een ziektebeeld waarin maligne B cellen met versterkte en ongeremde BCR signalering een centrale rol spelen. Het belang van andere signaleringroutes, die - naast de BCR - belangrijk zijn in de overleving en proliferatie van CLL cellen is echter minder goed gekarakteriseerd. Om de contributie van verschillende signaleringroutes te bestuderen hebben we de genoombrede genexpressie profielen bepaald van CLL cellen afkomstig uit de milten van een CLL muismodel in **Hoofdstuk 8.** Een specifieke genexpressie signatuur kon worden bepaald en deze hebben we vervolgens vergeleken met de activatie signatuur van B cellen na BCR stimulatie, verkregen T cel hulp van CD40 en TLR stimulatie. CLL cellen bleken een activatie signatuur te bevatten die voornamelijk was toegespitst op celdeling en proliferatie, waar al deze drie routes aan bijdragen. Eén set genen gereguleerd door T cel hulp sprong eruit, gezien dit een unieke set vertegenwoordigde in de proliferatie signatuur van CLL cellen. Ook vonden we een fractie van de activatie signatuur die niet werd verklaard door deze signaleringroutes, wat kan duiden op andere receptoren die deze signatuur ook deels reguleren. Tezamen laat deze studie zien dat naast BCR signalering ook andere signaaltransductie routes belangrijk zijn voor de CLL-specifieke activatie signatuur.

Na antigene stimulatie en T cel hulp, heeft een geactiveerde B cel de mogelijkheid om mutaties in de BCR aan te brengen, waardoor deze nog specifieker en sterker zou kunnen binden aan een antigeen. In CLL is eerder gevonden dat patiënten met een laag aantal van dergelijke BCR mutaties (ongemuteerd CLL) een slechtere prognose hebben in vergelijking met patiënten met een meer gemuteerde BCR status (gemuteerd CLL). In **Hoofdstuk 9** hebben we met behulp van phosphoflow onderzocht of de BCR mutatiestatus geassocieerd is met andere signaleringseigenschappen in CLL cellen. We observeerden dat de signalering was verlaagd in CLL ten opzichte van gezonde B cellen, maar zagen geen verschillen op basis van de BCR mutatiestatus in CLL. Echter was de connectie tussen de BCR signaleringsmoleculen onderling verschillend in gemuteerd en ongemuteerd CLL. Dit zou kunnen impliceren dat therapeutische interventies gericht op inhibitie van BCR signalering anders kunnen uitpakken in gemuteerd en ongemuteerd CLL.

Ten slotte bespreken we in **Hoofdstuk 10** onze bevindingen in het licht van de meeste recente observaties in het veld van leukemie en auto-immuniteit. Remming van Btk kinase activiteit is effectief in CLL patiënten, en daarbij lijkt combinatietherapie van Btk remmers met een aantal andere remmers van BCR signaleringseiwitten betrokken bij overleving van B cellen de klinische respons te versterken. Daarnaast lijkt remming van Btk en combinatietherapie gericht op andere signaleringseiwitten ook interessant voor behandeling in verschillende auto-immuunziekten. Maar zeker in dit veld is nog veel nieuw onderzoek nodig omtrent de klinische effectiviteit van BCR modulatie.

PHD PORTFOLIO

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Jasper Rip

Erasmus MC Department	Pulmonary Medicine
Research School	Molecular Medicine
PhD period	September 2016 – October 2020
Promotor	Prof.dr. Rudi W. Hendriks
Co-promotor	Dr. Odilia B.J. Corneth

PhD training

Courses

Analysis of gene expression data using R (MolMed)	2016
Galaxy for NGS data (Molmed)	2017
Advanced Immunology course (MolMed & Dept. of Immunology)	2017
Research Integrity course (Erasmus MC)	2019
High Dimensional Analysis for Flow Cytometry Data (MolMed)	2020

(Inter)national conferences

BSI/NVVI joint meeting (Liverpool, United Kingdom)	Poster	2016
Midwinter Conference 2017 (Seefeld, Austria)	Poster	2017
15th B cell Forum Meeting (Budenheim, Germany)	Poster	2017
47th Annual meeting DGfI (Erlangen, Germany)	Oral	2017
16th B cell Forum Meeting (Masserberg, Germany)	Oral	2018
Keystone Symposia: B cells in immunity and autoimmunity (Dresden, Germany)	Poster	2018
Midwinter Conference 2019 (Seefeld, Austria)	Poster	2019
17th B cell Forum Meeting (Glashütten, Germany)	Poster	2019
NVVI Lunteren Symposium (Lunteren, The Netherlands)		2019
TRR130 Symposium (Freiburg, Germany)	Poster	2019
NVVI annual meeting (Noordwijkerhout, The Netherlands)	Poster	2019
Keystone Symposia: B cell renaissance (Banff, Canada)	Poster	2020

Teaching

Involved in educational activities for the MSc Infection and Immunity program	2018 - 2020
Commission member of the MolMed postgraduate school - Organization of MolMed courses and MolMed Day (yearly conference)	2016 - 2020
Teaching in MolMed course: How to Design and Pitch a Good Poster	2020
Supervized MSc thesis project of Marjolein Appelman (MSc. Infection and Immunity)	2017 - 2018
Supervized BSc internship of Jelle Bolier (BSc. Biology and Medical Laboratory Sciences, Hogeschool Rotterdam)	2018 - 2019
Supervized MSc internship of Tobias Defesche (MSc. Molecular Medicine)	2019 - 2020

Awards

Poster prize at the NVVI Annual meeting (Noordwijkerhout, The Netherlands)	2019

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DANKWOORD

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LIST OF PUBLICATIONS

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2021 **Rip J**, Hendriks RW, Corneth OBJ. A Versatile Protocol to Quantify BCR-mediated Phosphorylation in Human and Murine B Cell Subpopulations. Bio-protocol 2021.

2020 **Rip J**, de Bruijn MJW, Kaptein A, Hendriks RW, Corneth OBJ. Phosphoflow Protocol for Signaling Studies in Human and Murine B Cell Subpopulations. J Immunol. 2020.

2020 Pal Singh S, de Bruijn MJW, Velaso Gago da Graca C, Corneth OBJ, **Rip J**, Stadhouders R, et al. Overexpression of SH2-Containing Inositol Phosphatase Contributes to Chronic Lymphocytic Leukemia Survival. J Immunol. 2020;204(2):360-74.

2019 von Borstel A, Abdulahad WH, Sanders JS, **Rip J**, Neys SFH, Hendriks RW, et al. Evidence for enhanced Bruton's tyrosine kinase activity in transitional and naive B cells of patients with granulomatosis with polyangiitis. Rheumatology (Oxford). 2019;58(12):2230-9.

2019 **Rip J**, de Bruijn MJW, Appelman MK, Pal Singh S, Hendriks RW, Corneth OBJ. Toll-Like Receptor Signaling Drives Btk-Mediated Autoimmune Disease. Front Immunol. 2019;10:95.

2018 **Rip J**, Van Der Ploeg EK, Hendriks RW, Corneth OBJ. The Role of Bruton's Tyrosine Kinase in Immune Cell Signaling and Systemic Autoimmunity. Crit Rev Immunol. 2018;38(1):17-62.

2017 **Rip J***, Singh SP*, Hendriks RW. Targeting Bruton's tyrosine kinase expression levels through microRNAs in chronic lymphocytic leukemia treatment. Translational Cancer Research. 2017:S502-S7.
(**contributed equally*)

2017 de Bruijn MJ, **Rip J**, van der Ploeg EK, van Greuningen LW, Ta VT, Kil LP, et al. Distinct and Overlapping Functions of TEC Kinase and BTK in B Cell Receptor Signaling. J Immunol. 2017;198(8):3058-68

2017 Corneth OBJ, Verstappen GMP, Paulissen SMJ, de Bruijn MJW, **Rip J**, Lukkes M, et al. Enhanced Bruton's Tyrosine Kinase Activity in Peripheral Blood B Lymphocytes From Patients With Autoimmune Disease. Arthritis Rheumatol. 2017;69(6):1313-24.

2016 Corneth OB, de Bruijn MJ, **Rip J**, Asmawidjaja PS, Kil LP, Hendriks RW. Enhanced Expression of Bruton's Tyrosine Kinase in B Cells Drives Systemic Autoimmunity by Disrupting T Cell Homeostasis. J Immunol. 2016;197(1):58-67.

2016 **Rip J**, Hendriks RW, Corneth OBJ. BTK. In: Choi S, editor. Encyclopedia of Signaling Molecules. New York, NY: Springer New York; 2016. p. 1-10.

CURRICULUM VITAE

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Jasper Rip was born on December 17th 1992 in Gouda, the Netherlands and grew up in Zevenhuizen. Upon completing his secondary education at the Coenecoop College in Waddinxveen in 2011, he performed his bachelor studies in Health and Life Sciences at the Vrije Universiteit in Amsterdam. As part of the bachelor studies, he performed an internship at the department of Immunology with Dr. Menno van Zelm at the Erasmus MC in Rotterdam. Here he studied the BCR repertoire and mutational load in B cells derived from healthy donors and periodontitis patients. After obtaining the Bachelor degree in 2014, he was admitted to the research master Infection and Immunity at the Erasmus University in Rotterdam. During the master program, he performed an internship at the department of Pulmonary Medicine under supervision of Prof. Dr. Rudi Hendriks and Dr. Odilia Corneth, studying the contribution of B cell receptor and co-receptor interplay to the development of autoimmunity. In 2016, he went abroad for several months to perform an internship at the group of Prof.dr. Lars Nitschke at the department of Cell Biology of the University of Erlangen in Germany. During this internship, he continued to work on B cells in autoimmune disease and got acquainted with various different techniques. After completing the Master program in 2016, he returned to the department of Pulmonary Medicine for his PhD studies. Here he continued to work on BCR signaling in autoimmunity and leukemia, again under supervision of Rudi Hendriks and Odilia Corneth. The results of the PhD studies are described in this thesis, and will be defended in June 2021. In October 2021, Jasper started his postdoctoral studies in the group of Dr. Ramon Arens at the department of Immunology of the Leids Universitair Medisch Centrum (LUMC) in Leiden. His postdoctoral studies are focusing on the role of memory B and T cells in COVID-19 disease.