Btk in autoimmunity and leukemia: too much of a good thing?

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ISBN

Cover design Laurens Kil
Printing Ridderprint BV, Ridderkerk
Lay-out Ridderprint BV, Ridderkerk

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Btk in auto-immuniteit en leukemie: teveel van het goede?

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 3 december 2013 om 15.30

door
Laurens-Paul Kil
geboren te Bergen op Zoom.

Erasmus
ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

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The studies described in this thesis were performed at the Department of Pulmonary Medicine, Erasmus MC, Rotterdam, the Netherlands.

This research was supported by the Dutch Arthritis Association.

Reumafonds
Dutch Arthritis Foundation

The printing of this thesis was supported by Molecular Medicine Postgraduate School and Erasmus MC

Molecular Medicine
Postgraduate School

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General introduction

Parts of this chapter have been published in reviews in International Reviews of Immunology and Current Pharmaceutical Design:

Aberrant B Cell Selection and Activation in Systemic Lupus Erythematosus
International Reviews of Immunology, Aug 2013, Vol. 32, No. 4: 445–470

The role of B cell receptor stimulation in CLL pathogenesis
Current Pharmaceutical Design, Aug 2012, Vol. 18, No. 23: 3335-3355

Introduction

All living organisms are continuously challenged by invasive disease-bearing microbes, including viruses, bacteria, fungi and parasites. If the host organisms for these microbes do not effectively repel these microbes, the integrity of the host body is threatened and serious illness or even death may occur. In higher vertebrates a complex immune system has evolved with specialized cell-types that recognize and combat these microbes in either a microbe-specific ('adaptive') or aspecific ('innate') manner.

To specifically recognize single microbial molecules ('antigens'), B and T lymphocytes belonging to the adaptive immune system express antigen receptors. To mount an immune response to the huge diversity of potentially invading microbes, a stochastic assembly and optimization through mutation of these antigen receptors assures the generation of a virtually infinite antigen receptor repertoire. For T and B cells, antigenic stimulation of their antigen receptor delivers crucial activation signals that determine B and T cell fate. While activated T cells mainly control cell-mediated immunity against microbes, upon activation B lymphocytes provide humoral immunity by secretion of their antigen receptor (the B cell receptor; BCR) as antibodies.

While the upside of antigen receptor diversity is the warranted presence of antigen-specific B and T cells upon encounter with foreign microbes, the downside of the ability to form antigen receptors with infinite variability is the inevitable formation of receptors that can bind self-antigens. If not properly

controlled, B and T cells expressing such autoreactive receptors can induce devastating autoimmune disease by attacking the host's own tissues. Furthermore, chronic antigen receptor stimulation by self-antigens may induce malignantly transform B and T cells into leukemic cells.

To prevent autoimmune disease and cancer, multiple checkpoints are incorporated in B and T cell differentiation to timely eliminate newly emerging autoreactive cells. Nevertheless, considerable fractions of peripheral B cells exhibit BCRs with some degree of autoreactivity. Therefore, the activation of B cells with dangerous antigen specificities is constrained by additional control mechanisms, some imposed by other immune cells and others acting in a B cell intrinsic fashion. The most direct and perhaps most powerful way for self-reactive B cells to assess and control their emergence and activation is simply by sensing self-antigens through the BCR and accurately responding to this self-antigen recognition. Proper control of BCR signaling strength and quality is therefore key to prevent B cell-driven autoimmune diseases or B cell malignancies.

This thesis will focus on how altered signaling of the BCR – and in particular its downstream signaling molecule Bruton's tyrosine kinase – may propel the development of rheumatic autoimmune diseases and chronic lymphocytic leukemia.

Signaling of the B cell receptor

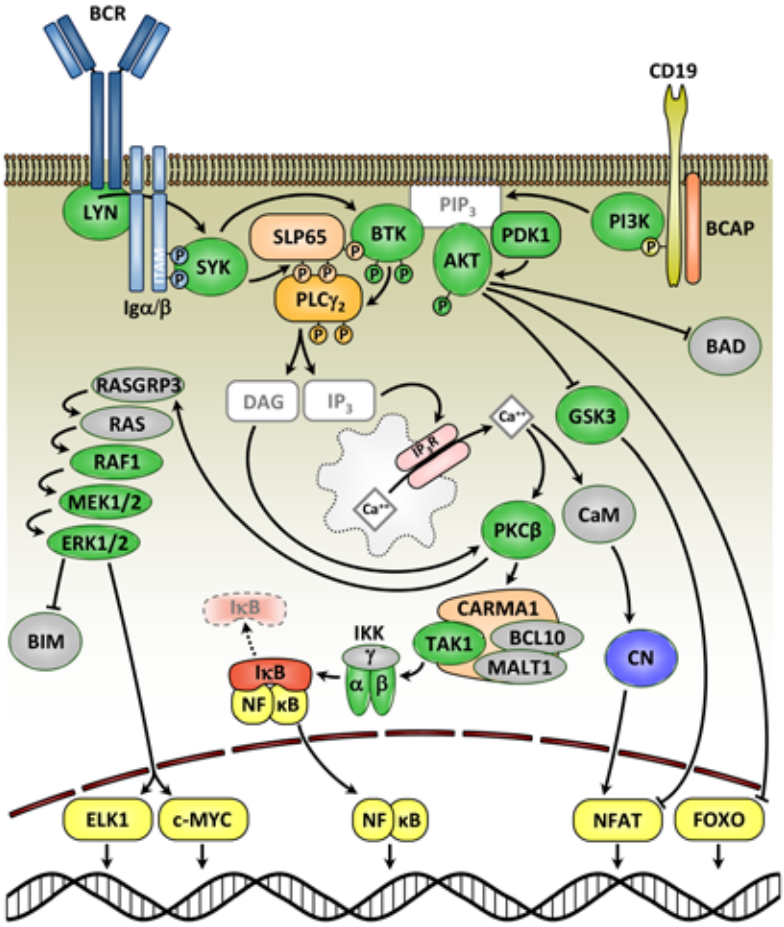
The BCR complex is composed of two IgH and IgL chains, associated with a Igα/β (CD79a/b) heterodimer whose cytoplasmic tail contains immunoreceptor tyrosine-based activation motifs (ITAMs) that are required to initiate BCR signaling. Since monovalent soluble antigens fail to activate B cells upon binding to the BCR, classically the onset of BCR signaling was thought to occur once BCRs are cross-linked by antigen. Interestingly, recent evidence however indicates that BCRs form dense clusters on the B cell membrane that in this configuration auto-inhibit BCR signaling, while antigen binding by the BCR can disperse these clusters and thereby facilitate the initiation of BCR signaling (1). When BCR signaling commences, a proximal signaling machinery is assembled that further orchestrates the activation of multiple divergent downstream signaling pathways.

Proximal BCR signaling

Upon antigen binding to the BCR, Src-family protein tyrosine kinase Lyn phosphorylates the Igα/β ITAMs, thereby creating docking sites for Syk (spleen tyrosine kinase) (2, 3) (Figure 1). In parallel Lyn phosphorylates tyrosine residues in the cytoplasmic tail of the BCR co-receptor CD19, enabling the binding and activation of phosphatidylinositol-3 kinase (PI3K) and Vav (4-6). Vav binds and activates Rac1, which subsequently can increase enzymatic activity of PI3K by inducing a conformational change of PI3K (7). Besides CD19, cytoplasmic adapter BCAP (B cell adapter for PI3K) can recruit PI3K as well (8), indicating a redundancy in sites for docking and activating PI3K following BCR triggering. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3) that attracts the Tec-family kinase Bruton's tyrosine kinase (Btk) (9, 10) to the cell membrane via interaction through its PH domain (4, 11, 12).

After localization of Btk to the cell membrane, Syk and Lyn transphosporylate Btk in its kinase domain at the Y551 position (13, 14), followed by Btk autophosphorylation at Y223 (15). Although disruption of the Y223F autophosphorylation site does not hamper B cell development significantly

Figure 1. Overview of BCR signaling pathways.



Upon antigen binding by the B cell receptor, signaling is initiated by LYN-mediated phosphorylation of ITAMs in the cytoplasmic tail of Igα/β and CD19, resulting in recruitment of SYK and PI3K, respectively. PI3K generates PIP3 to enable membrane recruitment of Btk. Syk then phosphorylates Slp65 to create a docking platform for Btk and PLCγ2, leading to phosphorylation of PLCγ2 by Btk. The formation of this BCR micro-signalosome forms the branching point from which 4 major signaling pathways originate, finally resulting in activation of ELK/c-Myc, NF-κB, NFAT and FOXO transcription factors.

(16), Btk autophosphorylation might exert a regulatory role on Btk activity (15) or alter Btk's SH3 domain binding properties and thereby change interactions of Btk with other proteins (14).

For the transphosphorylation of Btk and further signal propagation to downstream effectors, recruitment of Slp-65 (SH2 domain-containing leukocyte protein of 65 kDa, alternatively named BLNK or BASH) is essential, as Slp-65 serves as a scaffold for several tyrosine kinases including Syk and Btk (17-22).

Activated Syk generates Slp-65 docking sites by phosphorylation (21) that enable binding of Btk and phospholipase $C\gamma_2$ ($PLC\gamma_2$) to Slp-65 (23, 24). This final recruitment of $PLC\gamma_2$ completes the formation of so-called microsignalosomes, composed of Vav, PI3K, Btk, Slp-65 and $PLC\gamma_2$, that initiate BCR signaling upon antigen binding (25).

BCR signaling pathways in mature B cells largely overlap with those downstream of the pre-BCR consisting of an IGH chain that is expressed at the pre-B cell stage, together with the non-rearranging surrogate light chain constituents 14.1 l-like (called l5 in the mouse) and VpreB, upon successful IGH V(D)J recombination. As the pre-BCR functions as a crucial checkpoint during development (see for review (26, 27), humans or mice with a deficiency for important BCR signaling molecules, such as Syk, Slp65, Btk or PLCγ show a (partial) block at the pre-B cell stage. Remarkably, detailed analyses of compound knock-out mice have indicated that homologous signaling proteins that were previously thought to be not expressed in the B cell lineage, but in other hematopoietic cell populations, make important contributions to pre-BCR signal transduction (28-30). These proteins include the Syk family member ZAP70 which is often expressed in CLL cells and was previously regarded as T-cell specific, the LAT/SLP-76 adapter molecules, which enable pre-B cell differentiation in the absence of Slp-65/BLNK, the Tec kinase (31), which has the ability to partially compensate for Btk, and PLCg1 are expressed in the B cell lineage.

The detailed characterization of downstream BCR signaling pathways during the last decades has revealed that a few major signaling pathways diverge at different levels from the signaling cascade initiated by the BCR microsignalosome. The first major branching point is marked by activation of the Akt signaling pathway by PI3K, which occurs downstream of Syk but does not require $PLC\gamma_2$ activation. All other signaling routes are dependent on $PLC\gamma_2$, but further branching of signaling routes is directed by the two PIP_2 cleavage products DAG and IP_3 generated by $PLC\gamma_2$. Whereas IP_3 induces Ca^{2+} influx that is particularly important for activation of NFAT transcription factors and JNK, DAG-mediated activation of PKCβ is required for p38, ERK and NF-κB activation (Figure 1).

Akt and pro-survival signals

The serine/threonine kinase Akt (alternatively called PKB) can be attracted to the cell membrane by interaction of its PH domain with PIP_3 generated by PI3K (32). PI3K signaling to Akt represents a separate branch in BCR signaling, since Akt activation is dependent on PI3K activity, but not $PLC\gamma_2$ (33). After phosphorylation of Akt at the cell membrane by 3-phosphoinositide dependent kinase (PDK1; (34)), fully activated Akt returns to the cytoplasm to orchestrate an anti-apoptotic program (reviewed in (35)). Akt can control cell survival by targeting several transcription factors. One of the primary targets of Akt are forkhead transcription factors (FOXOs), which can be phosphorylated by Akt leading to their release from DNA and their subsequent degradation (36, 37). This abrogates transcription of the pro-apoptotic proteins Bim (Bcl-2 interacting mediator of cell death), a BH3-only protein that can inhibit the pro-survival family members Bcl-2, Bcl-$_{XL}$ and Mcl-1 (38). Akt can further regulate NFAT transcription via inhibition of glycogen synthase kinase-3 (GSK-3) (39) or activate NF-κB transcription (40). Akt can also promote pro-survival pathways by blocking pro-apoptotic proteins, including the BH3-only protein Bad, releasing Bad from Bcl-$_{XL}$ (41, 42). Inhibition of GSK-3 by Akt leads to stabilization of Mcl-1 and thus enhanced cell survival, because GSK-3 has the capacity to phosphorylate Mcl-1 to target it for ubiquitination and

proteosomal degradation (43). Finally, activated Akt can also stabilize X-linked inhibitor of apoptosis (XIAP; (44), and directly prevent apoptosis by phosporylating caspase-9 (45). Taken together, Akt is a central component in pro-survival signaling downstream of the B cell antigen receptor.

PLCγ2 signaling to NFAT and NF-kB transcription factors

Following Btk transphosphorylation by Syk and Lyn, Btk activates $PLC\gamma_2$ by phosphorylating residues Tyr753 and Tyr759 (46). Activated $PLC\gamma_2$ generates second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3) through hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), the same substrate used by PI3K (Figure 1). To prevent early abrogation of BCR signaling due to exhaustion of PIP_2, Btk associates via its PH domain with phosphatidylinositol-4-phosphate 5 kinase (PIP5K) that readily regenerates PIP_2 from PIP (47).

The two second messengers generated by $PLC\gamma_2$ activate partially overlapping downstream signaling pathways. The IP_3 generated by $PLC\gamma_2$ binds to IP_3 receptors located on the endoplasmatic reticulum (ER) membrane, leading to release of Ca^{2+} from the ER (48). The depletion of ER Ca^{2+} stores induces aggregation of STIM1 molecules in the ER membrane and positioning of these STIM1 multimers in close proximity of Orai1 plasma membrane Ca^{2+} channels (49, 50), resulting in activation and opening of these Ca^{2+} channels and thus Ca^{2+} influx (51, 52). Intracellular Ca^{2+} can activate calmodulin and subsequently calmodulin activates calcineurin phosphatase, which in turn activates NFAT transcription factors (reviewed in (53)). B-cell specific ablation of the regulatory b1 subunit of calcineurin, thereby interfering with NFAT transcription factor activation, revealed a requirement of NFAT factors for BCR induced B cell proliferation, B1 cell development and regulation of plasma cell differentiation by IRF4 (54). Nonetheless, individual NFAT transcription factors may exert different or even opposing functions, as NFATc1 is essential for BCR induced proliferation and prevention of BCR induced cell death, while absence of NFATc2 B lymphocytes resistant to BCR induced apoptosis (55).

Both intracellular Ca^{2+} and DAG generated by $PLC\gamma_2$ can activate another downstream kinase, protein kinase C beta (PKCβ) (Figure 1). Activated PKCβ phosphorylates CARMA1 (also called CARD11 or BIMP1), which leads to assembly of a scaffold complex with Bcl10 and MALT1 (56). In addition, PKCβ is responsible for recruiting Tak1 (TGF-β-activated kinase 1) and IkB kinase (IKK) to this complex ((56, 57). This brings Tak1 and IKK in close proximity, allowing the phosphorylation of IKK by Tak1 (57). Activated IKK then induces ubiquitination of IkB, resulting in targeting of IkB for proteasomal degradation (reviewed in (58, 59)). The removal of IkB permits the nuclear translocation of p50/RelA and p50/c-rel NF-κB heterodimers that initiate a transcriptional program favoring both cell survival and proliferation.

NF-κB regulates transcription of many genes that are directly involved in cell cycle progression and anti-apoptotic pathways (reviewed in (60)). For example, NF-κB can support progression during early phases of the cell cycle by stimulating transcription of E2F3, a transcription factor involved in G1/S phase transition (61), and c-myc (62). Survival of B cells upon BCR stimulation can be instructed by NF-κB through transcription of the Bcl-2 related pro-survival factors such as $Bcl\text{-}_{XL}$ (63) and A1/Blf1 (64).

ERK and other MAP kinases

DAG produced by $PLC\gamma_2$ can recruit PKCβ to the cell membrane, as well as the C1-domain harboring

guanine nucleotide exchange factor RasGRP3 (65). This co-localization of PKCβ and RasGRP3 allows PKCβ to phosporylate RasGRP3 at position Thr133, which is necessary for full activation of RasGRP3 (66, 67). Activated RasGRP3 promotes GTP acquisition by Ras, which is only active in a GTP-bound state ((68, 69). Ras subsequently activates Raf1, a mitogen-activated protein 3 kinase (MAP3K), and in concert with B-Raf they phosphorylate MEK1/2 (mitogen-activated protein kinase 1/2), that in turn phosphorylates ERK1/2 (extracellular signal-regulated kinase1/2; reviewed in (70)) (Figure 1). As a dimer, ERK1/2 can translocate to the nucleus, where it can phosphorylate transcription factors including Elk-1 and c-Myc (reviewed in (71) to promote cell survival and especially cell cycle entry. Apart from phosphorylation of transcription factors, another mechanism by which ERK1/2 can counteract apoptosis is via phosphorylation of BH3-only protein Bim (particularly the extra long Bim isoform, Bim_{EL}), thereby dissociating Bim from Bcl2 family members such as Mcl-1 and $Bcl\text{-}_{XL}$ (72).

Whereas Ras signaling is required to induce activation of ERK1/2, other MAP kinases p38 and JNK can be induced by $PLC\gamma_2$ without intermediate signaling via Ras (73). JNK phosphorylation required the presence of IP_3 receptors and thus was dependent on intracellular mobilization of Ca^{2+}, while p38 phosphorylation proved to be (largely) DAG and PKCβ dependent. Although some overlap exists in the gene targets of the transcriptional programs indirectly induced by ERK, p38 and JNK (reviewed in (74)), in particular JNK is capable of promoting activation-induced cell death (AICD). JNK can phosphorylate the long isoform of Bim (Bim_L), releasing BimL from microtubule motor protein dynein light chain 1 (DLC1), thereby inducing apoptosis (75). Moreover, JNK can activate c-Jun (76), a transcription factor that can drive the expression of pro-death genes (77, 78).

Although the pathways described above partly overlap in their signaling cascades and have partly overlapping targets (e.g. transcription factors commonly activated by MAP kinases, or anti-apoptotic factors affected by both Akt and MAPK signaling), it is the balance of these BCR signaling routes that determines the effect on B cell fate, ranging from proliferation and survival to apoptosis. How this balance is precisely fine-tuned remains to be fully elucidated, but this balance is likely determined both by B cell intrinsic mechanisms that largely depend on the stage of B cell differentiation and activation (reviewed by (79)) and by B cell extrinsic factors. These external factors not only comprise simultaneous signals from co-stimulatory or inhibitory receptors that may employ the same signaling pathways, but importantly the nature of the antigen and thus its interaction with the BCR may greatly determine the signaling strength of individual signaling routes downstream of the BCR. For example, differences in Ca^{2+} influx frequency and length are reflected by differences in activation patterns of NF-kB versus NFAT ((80, 81)).

B cell intrinsic tolerance checkpoints: purging the B cell repertoire

Signaling of the BCR is not only critical for the activation of fully mature B cells, but also provides essential clues for the negative selection of autoreactive B cells during their differentiation. Genetic defects affecting the selection of developing B cells or the activation of these cells after completing their differentiation can predispose to the development of B cell-driven autoimmune disease. Systemic lupus erythematosus (SLE) is considered to be the most prototypic B cell-driven autoimmune disease, since the production of autoantibodies recognizing nuclear self-antigens (antinuclear autoantibodies;

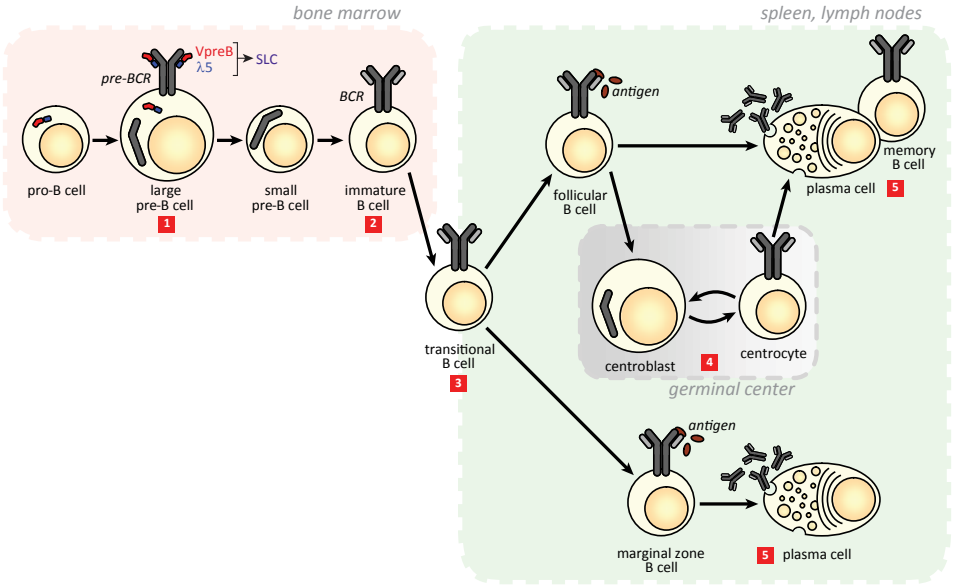
ANAs) represents an almost indispensable prerequisite for diagnosis (82, 83). The true pathology in SLE is thought to be triggered by circulating immune complexes formed by these ANAs that inflict inflammation upon deposition in multiple organs including the kidneys, joints, skin, lungs, eyes, and central nervous system (84-86). As ANA production usually precedes the onset of SLE symptoms for many years, an initiating role in disease development is therefore attributed activated self-reactive B cells (87). Therefore, many researchers have aimed to uncover defects in B cell selection processes that may predispose to SLE by facilitating the emergence of autoreactive B cells.

The development of each B lymphocyte is characterized by sequential events that lead to the assembly of its unique B cell receptor (BCR). To generate a B cell pool with a highly diverse repertoire of BCRs that can bind any newly encountered pathogen, the stochastic nature of the recombination of V(ariable), D(iversity) and J(unction) gene segments that encode the BCR heavy and light chains will ensure a huge variety in the antigen specificities and affinities of the BCR repertoire. This variety is even further diversified by the random insertion of non-template nucleotides (N-nucleotides) at these recombination sites (88). Inevitably, the random nature of V(D)J recombination will generate large numbers of BCRs that can recognize self-antigens; an estimate of the occurrence of polyreactive B cells ranges from ~50-75% among immature B cells (89). Therefore, during B cell development multiple autoreactivity checkpoints are implemented for the stringent counterselection of autoreactive B cells (Figure 2). These B cell intrinsic checkpoints comprise clonal deletion of autoreactive cells, BCR editing, and the induction of anergy, and these checkpoints have been extensively studied in (transgenic) mouse models that we will discuss below (90).

A first assessment of fitness and possible autoreactivity of the immunoglobulin (Ig) heavy chain (IgH) occurs instantly after its formation and expression in conjunction with the surrogate light chain (SLC) proteins VpreB and lambda5 (λ5) as the pre-BCR (91, 92). Not only is the mere ability of the IgH to be expressed at the cell surface determined, but as a binding template the SLC may test for sufficient future pairing capacity of the IgH to the yet unformed Ig light chain (IgL) (93). To provide the pre-B cell with positive feedback on the successful expression of a stable pre-BCR, the pre-BCR has the capacity to signal autonomously (94). This capacity is conferred by the polyreactive nature of the SLC as a whole but in particular of the non-immunoglobulin tail of λ5, allowing the pre-BCR to bind multiple self-antigens including heparan sulfate and galectin-1 on bone marrow stromal cells (95-98). Recent research however demonstrated that the IgH itself provides an N-glycosylation site that is dispensable for pre-BCR expression but indispensable for pre-BCR signaling, indicating that an internal IgH epitope serves as autoantigen that enables pre-BCR signaling (99).

The concept of the pre-BCR as an autoreactive receptor whose signaling instructs positive selection of pre-B cells with functional IgH chains is further supported by the observation that in the absence of the non-immunoglobulin tail of λ5 the incorporation of a IgH complementarity determining region (CDR) 3 of an autoreactive IgH can rescue pre-BCR function (98). In addition, transgenic expression of a self-reactive BCR at the pre-B cell stage on a SLC-deficient background proved to rescue pre-B cell differentiation (100). Apart from a role in the positive selection of pre-BCR expressing cells, a recent report demonstrated spontaneous ANA production in mice deficient for the SLC, suggesting a role for pre-BCR signaling in the negative selection of pre-B cells expressing autoreactive IgH chains (101).

Figure 2. B cell autoreactivity checkpoints during B cell development.



The stepwise rearrangements and adaptations of IgH and IgL chains prompt the need for implementation of BCR autoreactivity checkpoints at multiple developmental stages of B cell development. At the large pre-B cell stage the expression of the pre-BCR is primarily intended to positively select those pre-B cells with a proper rearranged IgH chain (1), but since defective expression of the autonomously signaling SLC components VpreB and λ5 will favor the selection of intrinsically autoreactive IgH chains, the SLC thus exerts a role in the counterselection of selfreactive IgH chains. Upon rearrangement of an IgL chain immature B cells will assess the self-reactivity of their fully formed BCR to bone marrow antigens (2). Self-sensing through the BCR at this developmental stage will instruct either revision of the BCR (receptor editing) or clonal deletion, allowing only those B cells to egress the bone marrow with no or low selfreactivity. Arriving in new antigenic environments including peripheral blood and secondary lymphoid organs, transitional B cells will again determine their BCR autoreactivity (3). At this stage self-reactive B cells may be silenced through clonal deletion or the induction of antigen unresponsiveness (anergy). B cells that surpass these checkpoints and complete their maturation can respond to foreign antigens, but BCR editing processes as SMH that occur in germinal center B cells (centroblasts and centrocytes) may jeopardize selective alloreactivity of the BCR, demanding tightly controlled positive and negative selection exerted by T lymphocytes (4) . Finally, after completing their differentiation in memory B cells or plasma cells, persistence of possibly escaping autoreactive effector cells can be controlled by several mechanisms, including the competition for survival factors as BAFF and APRIL (5).
BCR, B cell receptor; SLC, surrogate light chain; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain.

Though this seems as assigning yet another function to pre-BCR signaling, this finding of impaired censoring of autoreactive pre-B cells actually extends the concept of the pre-BCR as selfreactive receptor, since it is conceivable that in SLC deficient mice the only pre-B cells passing the pre-BCR checkpoint are those cells that express a strongly autoreactive IgH chain, whose signaling will substitute for the lacking autonomous SLC signals. Indeed, in the absence of λ5 expression the numbers of immature and

mature B cells are up to 20-fold reduced, implying a dramatically changed selective pressure on IgH chains expressed in λ5-deficient murine pre-B cells (102). This altered selective pressure at the pre-BCR checkpoint could indeed be confirmed by autoreactive characteristics of IgH chains isolated from a λ5-deficient patient (103).

Besides a role for shaping the IgH chain repertoire of developing B cells, the SLC complex may contribute to or control autoimmunity even in mature B cells. A decade ago SLC-expressing mature B cells have been described both in healthy individuals and patients with rheumatic disease (104). Whereas these cells constitute only a minor fraction (up to ~1%) of circulating mature B cells in healthy controls, these cells were enriched in inflamed joints of rheumatoid arthritis (RA) patients. Further analysis of these cells demonstrated that they more prominently (re)expressed VpreB than λ5, that these B cells exhibited BCRs with polyreactive specificities and signs of more extensive BCR revisions (104-106). Although the SLC may provide autoreactive mature B cells with ligand-independent BCR signals that may support their activation, the dilution of membrane IgL chains by SLC complex expression as well as their overt attempts to revise their BCR may indicate that these SLC-expressing cells actually try to correct the expression or production of their self-reactive BCR. Future studies on SLC-expressing mature B cells are required to provide more insight into the role of SLC signaling in mature B cells in the context of autoimmunity, and to elucidate whether SLC+ mature B cells are not only involved in the pathogenesis of RA but also other rheumatic diseases as systemic lupus erythematosus (SLE).

Pre-BCR signaling following the successful production of a IgH chain will instruct the termination of SLC expression, a short proliferative burst to expand positively selected pre-B cells, and finally differentiation of these large cycling pre-B cells into small pre-B cells that will attempt to rearrange their kappa (Igκ) or lambda (Igλ) light chain (107) loci. Once a IgL chain has been produced it is readily expressed together with the preformed IgH chain, leading to the expression of a complete BCR whose antigen specificity and thus possible autoreactivity can finally be evaluated. A vast majority of these immature B cells expresses a BCR with some selfreactivity (89) and self-antigen sensing through the BCR will trigger apoptosis of these cells (108), unless they can avert their clonal deletion by altering their BCR specificity through receptor editing (109, 110). This receptor editing involves ongoing V(D)J recombination of still available distally located V gene segments to the used J segment. Once recombination possibilities at this locus are exhausted the V(D)J recombination machinery is directed towards another IgL locus while the originally recombined locus is inactivated (111). The process of receptor editing is a major force purifying the emerging immature B cell pool from autoreactive clones, since ~25% of all peripheral B cells displays edited IgL chains (112-114). In both the MRL/lpr SLE mouse model and human patients the process of receptor editing is hampered, mostly mirrored by lower frequencies of B cells expressing edited IgL chains and consequently less stringent removal of autoreactive BCRs from developing B cells (115, 116).

Though intended to remove autoreactive BCR specificities from the B cell repertoire, ironically receptor editing may also facilitate the persistence of selfreactive B cells when recombinational silencing of the originally recombined Ig locus fails. In this scenario termed allelic inclusion a B cell may co-express two Ig (light) chains and thus two or more BCRs, one of which is still selfreactive (117-120). Importantly, dual IgL chain expression in B cells has not only been demonstrated in mice but also in the normal human

B cell repertoire (121, 122). Peripheral B cells that express dual IgL chains do pose a serious threat on immune tolerance since they actively participate in anti-self immune responses, secrete autoantibodies, and account for approximately half of all memory and plasmablast cells in aging MRL/lpr mice (123). It is believed that such dual-reactive B cells escape silencing because the co-expression of a non-selfreactive BCR will dilute the membrane expression of the autoreactive BCR, thereby reducing BCR-mediated activation upon self-antigen binding and thus preventing the activation of other silencing.

Whereas receptor editing represents the primary means to remove self-reactive cells among developing immature B cells, during their final maturation as transitional B cells the principal tolerance mechanisms to control them are clonal deletion and especially anergy, a state of reversible antigen-specific unresponsiveness that is maintained by continuous antigenic exposure (124-126). The underlying molecular changes that impose anergy on B cells have been studied using a plethora of mouse models that transgenically express BCRs with variable affinities to different self-antigens (127). When integrating the divergent and sometimes opposing data obtained from these different mouse models, it is evident that the outcome of anergic B cell activation and differentiation is governed by multiple factors including BCR affinity, antigen abundance, and antigen valence. Despite the abundant phenotypical differences these transgenic mouse models have exposed defects in anergy that drive SLE-like pathology in murine SLE models. Autoreactive BCRs or individual Ig chains cloned from diseased MRL/lpr mice could be transgenically expressed in autoimmune-resistant wild-type strains without hampering B cell selection and development while fully developed transgene-expressing B cells could not be activated (e.g. V_H3H9 or V_H2-12 transgenic mice) (128-130). In contrast, introduction of such transgenes on the original MRL/lpr background rapidly induced production of the transgenically introduced auto-antibodies, demonstrating a defect in maintaining tolerance through anergy (131).

While the artificially limited BCR repertoire of the aforementioned BCR-transgenic mice allowed the identification of anergic subphenotypes of peripheral B cells, the study of naturally occurring anergic cells with more diverse chronic antigenic stimulation patterns was impossible until the identification of transitional stage 3 (T3) peripheral B cells as a subpopulation harboring anergic B cells ("An1" B cells) rather than B cells in a maturational stage (132). Additionally, functionally silenced self-reactive cells may also be enriched in other peripheral B cell subpopulations such as pre-plasma cells in wild-type mice, while this block in function and further differentiation of autoreactive pre-plasma cells in MRL/lpr mice was incomplete (133). Despite the concentration of anergic cells in these naturally occurring B cell subpopulations, the presence of anergic B cells is most likely not restricted to the T3 or pre-plasma cell stages. It probably extends to many more mature B cell subpopulations, since peripheral B cells in mice that express a GFP transgene under control of the gene regulatory region of BCR signaling target *Nur77* exhibit a large gradient of GFP expression (134). The authors elegantly demonstrated that GFP expression tightly correlates with the antigen dose used to trigger BCR signaling and that the level of GFP expression correlates with hallmark characteristics of anergic B cells, including high basal Ca^{2+} signaling, reduced Ca^{2+} mobilization upon BCR triggering and a more autoreactive BCR repertoire reflected by higher production of ANAs upon *in vitro* stimulation.

Recently, great advances have been made in our understanding of the reversible rewiring of BCR signaling that imposes a functional paralysis on anergic B cells. Although the incomplete activation of

downstream BCR signaling pathways has been described in anergic cells (80, 124, 135), the upstream signaling adaptations that orchestrate the preferential activation of ERK and NFAT over NF-kB and JNK are now being uncovered. The failed activation of NF-kB and JNK in anergic cells may be explained by the reduced generation of phosphatidylinositol-3,4,5-triphosphate (PIP_3) that can potently activate the NF-kB and JNK pathways (136). The reduced production of PIP_3 may result from enhanced PTEN upregulation, and although this mechanism may effectively downregulate BCR signaling for sustained periods of time, its kinetics are incompatible with the speed at which the state of anergy in B cells can be reverted (124). The upregulation of PTEN now preferably seems to occur in anergic models characterized by high affinity BCR-binding to self-antigens, whereas in several other transgenic anergic models and in natural An1 cells the phosphorylation mode of the CD79 BCR signal transduction module is decisive in the differential activation of divergent BCR signaling pathways (137). The authors show that in anergic B cells the CD79 phosphorylation pattern is biased towards monophosphorylation that selectively activates SHIP-1 and thus promotes PIP_3 hydrolysis, providing a quick and non-permanent signaling route that can nearly instantly control PIP_3 levels in anergic cells.

In SLE it is unclear how and to what extent anergy and clonal deletion are affected at the final maturation stages from new bone marrow emigrant (immature) B cells to fully mature naïve B cells. It is clear however that the regulation of these B cells is defective in SLE, since in SLE patients no decline in the proportion of selfreactive cells can be observed between immature and mature B cells (both populations comprise ~40% autoreactive cells), whereas in healthy donors half of the originally ~40% selfreactive immature B cells are eliminated during their final maturation stages (138). This defective silencing of transitional B cells in SLE is probably reflect an intrinsic defect in B cell tolerance during B cell development rather than a loosened counterselection of peripheral selfreactive B cells in a pro-inflammatory environment, since the fraction of selfreactive cells among mature B cells remains high in SLE patients in clinical remission (139). As an increase in transitional and "pre-naïve" B cell numbers is commonly observed in SLE patients irrespective of disease activity, this increase may thus reflect the persistence of autoreactive maturating cells that fail to be removed (140-142).

Once autoreactive B cells have erroneously passed all self-tolerance checkpoints and have fully matured into naïve B cells in SLE patients, they may be recruited in autoimmune responses and differentiate into memory B cells and plasma cells via differentiation routes either dependent or independent of germinal center (GC) reactions (see chapter 3 and 4) (Figure 2). In SLE, exhaustion of the mature naïve B cell pool due to continuous differentiation of these cells into effector cells may be reflected by the characteristic decrease in naïve B cell numbers in peripheral blood (143). This hypothesis is strengthened by the observed (relative) increase in plasmablasts that accompanies the decline in naïve B cell numbers (143, 144), and the increase in plasmablasts indeed correlates with an increase in disease activity (145). In parallel to disturbances in frequencies of plasmablasts and plasma cells, memory B cells also circulate with increased frequencies in active disease (143) and display different BCR properties. Although in healthy controls autoreactive memory B cells can be found (146), in SLE the increase in memory B cells is not mirrored by a concomitant significant increase in the proportion of selfreactive IgG memory B cells, but the BCRs expressed by IgG memory B cells in individual SLE patients do correlate with their serology, suggesting that these memory B cells fuel the chronic production

of autoantibodies in these patients (147). Apart from these BCR characteristics, these memory B cells also differ phenotypically from memory cells found in healthy donors. Whereas CD27-negative memory B cells represent a rare fraction in healthy controls (148), these cells are enriched in SLE patients and may correlate with disease activity (149, 150). Within this CD27- fraction, a subpopulation of these cells expresses CD95, indicative of very recent B cell activation and probably a GC origin of these cells (150, 151). This is in line with the postulated substantial contribution of dysregulated GC reactions in breaking B cell tolerance in SLE (see chapter 3).

Solo Activation of Self-reactive Mature B Cells: The BCR and TLRs

Although BCR signals during B cell differentiation effectively purge the repertoire of newly emerging B cells, a considerable fraction of mature B cells still expresses a B cell receptor with some affinity for self-antigens (89). While anergy may largely control those persistent self-reactive mature B cells, it does not definitely safeguard against autoimmunity, as the reversible state of B cell anergy crucially depends on the quality and strength of BCR signaling. This inertness can easily be undone by activating signals or simply temporary antigenic deprivation (124). Therefore, it is not surprising that in mouse models single genetic defects affecting only BCR signaling have proven to be sufficient to permit activation of selfreactive mature B cells, sometimes even culminating in systemic auto-immune disease. In mouse models with continuously increased BCR signaling, B cell development is often severely hampered due to massive elimination following BCR signaling during critical developmental autoreactivity checkpoints. These models include transgenic B-cell targeted expression of molecules with constitutive signaling properties, e.g. latent membrane protein 2A (LMP2A), mutant forms of BCR signaling molecules such as Btk or overexpression of the co-stimulatory receptor CD19 (152-156). The remaining peripheral B cells however are usually easily activated and driven either into GC B cell (152) or plasma cell differentiation (155) by these continuous BCR signals, whereby the BCR signaling strength seems to program which differentiation route is promoted (157).

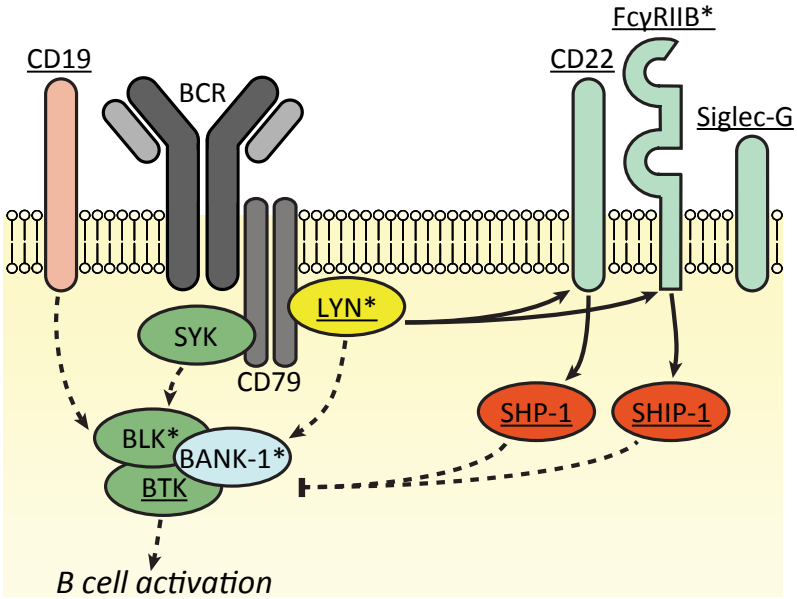
Whereas spontaneous autoimmunity is absent in LMP2A transgenic mice, is restricted to IgM autoantibody production in E41K-Btk transgenic mice, or is limited to low level IgG autoantibody production in CD19 transgenic mice, overt auto-immune pathology that closely resembles SLE does develop in mouse models with conditionally rather than continuously increased BCR signaling. These models include mice deficient for inhibitory co-receptors as FcγRIIB, CD22 or Siglec-G, mice deficient for inhibitory BCR signaling molecules as Lyn, SHP-1, or SHIP-1, and mice overexpressing wild-type Btk (137, 158-163) (Figure 3). B cell differentiation in these models is not hampered by large-scale clonal deletion of BCR-activated developing B cells. Therefore, there is no strong reduction in the numbers of emerging auto-reactive B cells that can contribute to auto-immunity once they are aberrantly activated through their BCR. Although autoimmunity in some of these models may be further propagated by additional defects in non-B cells or by simultaneous defects in multiple B cell signaling pathways, some of these models with B-cell restricted genetic deletion or overexpression of these targeted genes have demonstrated that a mere increase in BCR signaling can be sufficient to induce SLE-like disease (163).

Enhanced BCR signaling may thus already suffice to break tolerance in autoreactive B cells, but the classical two-signal activation model originally postulated by Bretscher and Cohn dictates that the

activation of self-reactive cells should normally be prohibited by the absence of a second co-stimulatory signal to self-antigen stimulated B cells (164). A powerful way to deliver two separate activation signals to self-reactive B cells is the simultaneous engagement of their BCR and toll-like receptors (TLRs) by chromatin-containing immune complexes (165). Self-reactive B cells expressing an IgG-specific BCR (rhemautoid factor, RF) can bind these immune-complexes, internalize them and deliver the chromatin content to intracellularly located TLR9 (166). In mouse models these RF expressing B cells efficiently form memory B cells upon stimulation with chromatin-containing immune complexes, establishing a pool of cells that can contribute to flares in autoimmunity (167).

While RF antibodies do not represent the most predominant or pathogenic autoantibodies in SLE patients, this concept of dual receptor stimulation could be extrapolated to self-reactive B cells

Figure 3. BCR signaling molecules associated with SLE in patients and murine models.



The start of BCR signaling is characterized by parallel signaling of co-stimulatory receptor CD19 and activation of Src family kinase Lyn and Syk family kinase Syk. Whereas Syk exclusively exerts an activating role downstream of the BCR, Lyn also activates inhibitory co-receptors as CD22 and FcγRIIB that dampen BCR signaling through the activation of phosphatases SHP-1 and SHIP-1, respectively. Downstream of Syk B cell activation is accomplished through complex signaling cascades composed of numerous signaling molecules, including kinases as BLK and BTK and adaptor molecules as BANK-1. Arrows indicate stimulatory interactions, blunt arrows represent inhibitory interactions, and dotted lines indicate indirect interactions. Underlined molecules have been shown to be able to contribute to murine lupus when overexpressed, deleted or mutated. Asterisks indicate molecules that have been associated with an elevated risk for SLE development in humans. SYK, spleen tyrosine kinase; BLK, B lymphocyte kinase; BTK, Bruton's tyrosine kinase; BANK-1, B cell scaffold protein with ankyrin repeats; SHP-1, Src homology domain 2-containing phosphatase-1; SHIP-1, phosphatidylinositol-3,4,5-triphosphate 5-phosphatase-1.

expressing anti-DNA or anti-RNA BCRs (168, 169). Moreover, DNA or RNA specific B cells can present other self-peptides in chromatin-containing immune complexes to T_H cells that recognize these different chromatin epitopes, thereby in turn facilitating epitope spreading in the B cell compartment (170). The deleterious role of TLR signaling in SLE development is exemplified by the beneficial effect of TLR signaling deficiencies on ANA production in MRL/lpr mice and FcγRIIB deficient mice (171-174) or the aggravation of lupus phenotypes upon overexpression of TLR7 (175-177). Despite this deleterious role of TLR signaling in breaching tolerance of self-reactive B cells even in the absence of T_H cell co-stimulation (178), other findings demonstrate that TLR signals may additionally exert some regulatory functions in autoimmunity (172, 179-181). In line with the importance of TLR signaling in SLE pathogenesis in mice, firstly polymorphisms TLR7 and TLR9 have been identified in SLE patients of Asian origin (182). Secondly, in genome-wide association studies (GWAS) not only associations with polymorphisms in TLRs themselves were found, but also in the ubiquitin-editing enzyme A20, which negatively regulates TLR signaling (183). Thirdly, a connection was observed between SLE and mutant alleles of IRAK1, a signaling molecule downstream of multiple TLRs including TLR7 and TLR9 (184, 185). Finally, a contribution of dysregulated TLR signaling to lupus development is also reflected by associated polymorphisms in interferon regulatory factor 5 (IRF5) (186, 187), a key transcription factor in TLR signaling targeted by TLR7 and TLR9 (188).

In summary, an extensive body of evidence demonstrates that intrinsic aberrancies affecting BCR and TLR signaling may severely hamper the counterselection of autoreactive B cells during developmental autoreactivity checkpoints. Additionally, after erroneously passing these checkpoints BCR and TLR signaling pathways may either directly or in conjunction activate self-reactive B cells. Future translational and clinical studies should therefore test whether in the treatment of SLE compounds are effective that can selectively block TLR or BCR signaling, such as the new small molecule inhibitors that can target downstream signaling molecules as Btk (189). But despite the clear defects in intrinsic B cell activation, translational research should not exclusively be limited to B cell oriented therapies since help of other immune cells as T_H cells or myeloid cells may strongly propel SLE-like disease by establishing mutual activation loops between different immune cell types. In the next section we will outline the molecular basis of those immune cell interactions that have implications for SLE pathogenesis.

B-T cell crosstalk that shapes autoantibody responses

For generating high affinity antibodies, the introduction of extra mutations into the variable regions of Ig chains is required through somatic hypermutation (SHM), a process mediated by the enzyme activation-induced cytidine deaminase (AID) and orchestrated by various co-stimulatory signals (190, 191). Although SHM is not exclusively induced by T-cell derived cytokines, T cells powerfully instruct SHM either in an extrafollicular B cell response or more effectively in GCs, which are highly specialized structures in secondary lymphoid organs designed to enable stringent selection of high-affinity B and T cells (192). As GCs are the most efficient site of formation of high-affinity antigen-specific B cells (193), in SLE these GCs give rise to most memory B cells and long-lived plasma cells that persist during conventional immunosuppressive treatments and thus continuously propagate disease (151). The importance of SHM in the generation of lupus autoantibodies is overt, since multiple studies demonstrated that most or all

autoantibodies in murine SLE models or lupus patients harbour point mutations imposing self-antigen recognition on antibodies that were found to be innocuous once reverted to their germline-encoded sequence (147, 194-196). While somatically hypermutated auto-antibodies in SLE can be produced upon T-cell interaction in extrafollicular B cell responses (197-199), they are thought to arise predominantly during dysregulated GC reponses (200).

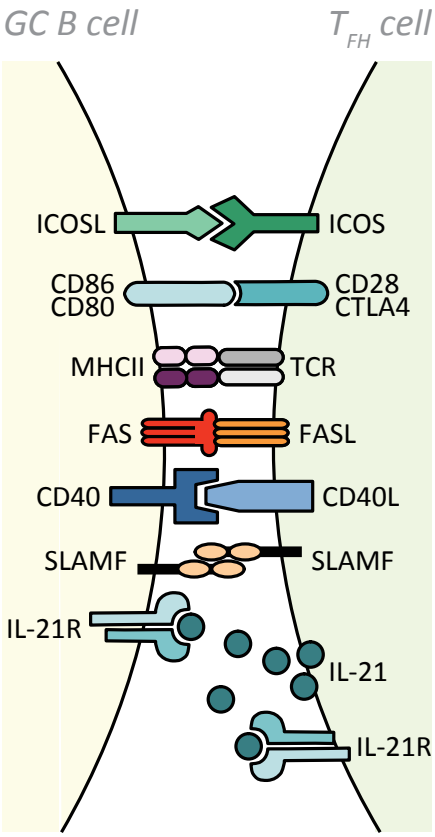
The decisive role of dysfunctional GCs in SLE-like autoimmunity is exemplified by the recurrent observation of spontaneous germinal center formation in multiple murine lupus models (201). Very convincing evidence for dysregulated GC reactions and defective counterselection of autoreactive GC B cells in SLE was provided by the observation of failed GC exclusion of self-reactive V_H4-34 expressing B cells that are inherently self-reactive to glycoproteins including CD45, while in GCs from healthy controls and RA patients such B cells were absent (202). Importantly, other rheumatic diseases as RA and Sjögrens's syndrome (SS) are frequently characterized by the ectopic formation of GCs in inflamed organs as synovium or salivary glands, pointing to a role for dysregulated GCs in driving or sustaining rheumatic diseases in general (203-205).

In GCs signals from the BCR were originally thought to be decisive in the positive selection of antigen-specific GC B cells, but it has now become clear that T cells actively perform the selection of GC B cells (206, 207) and that strikingly BCR signaling is even intermittently shut down in GC B cells (208). Though minimizing a role for BCR signaling in the positive selection of GC B cells, the BCR may still provide essential survival signals for GC B cells that counteract T-cell mediated negative selection of these cells (163, 209, 210). In this context, it was very recently shown that GC B cell selection is based on restriction of antigen access and that GC B cells govern their own fate via antibody feedback (211). For the proper selection of alloreactive GC B cells and counterselection of autoreactive GC B cells the balance of numerous positive and negative selection signals from T cells to B cells is crucial (Figure 4). In this section of the review, we will focus those selection signals which were found to be most relevant in preventing lupus-like disease.

For initiating T-dependent B cell differentiation pathways, the interaction between CD40 and CD40L (CD154) is indispensable (212), but whereas it is only transiently needed during extrafollicular responses, it is incessantly required for the maintenance of GC reactions, as demonstrated by the acute disintegration of GCs upon blocking this interaction (213). It was soon recognized that this interaction may contribute to SLE development, since CD40L was found to be overexpressed in murine SLE models and SLE patients alike (214-216). Indeed, treatment of lupus-developing mice with CD40L-blocking antibodies can establish long-term suppression of symptomatic diseases or could even ameliorate established nephritis (214, 217). This encouraged the testing of the therapeutic potential of CD40L blocking antibodies in SLE patients, and small pilot studies showed that the numbers of anti-DNA B cells, circulating GC-like B cells and GC-derived memory B cells, ANA levels, proteinuria and disease score were drastically diminished (218, 219). Disappointingly however, larger trials could not always reproduce these findings (220), or reported serious adverse thromboembolic events (221).

Other critical factors that govern mutual B-T cell activation during early T-dependent antibody responses include the B7 proteins (CD80/B7-1 and CD86/B7-2) on B cells that bind to the stimulatory TCR co-receptor CD28 on T cells (222). Disrupting CD28 co-stimulation by treatment with a CTLA4-

Figure 4. B-T cell interactions in GC reactions that guide B cell selection.



During T-dependent antibody responses, B cells initiate contact with T cells by presenting antigen-derived peptides in MHCII molecules to the TCR and by providing co-stimulation via the B7 proteins CD86 and CD80. An indispensable reciprocal co-stimulatory signal to initiate T-dependent differentiation of B cells in general comprises CD40L-CD40 binding. For the establishment of GC reactions, other interactions are required that prime the differentiation of TFH cells (ICOS-ICOSL), that stabilize B-T cell interactions in the GC (SLAMF interactions), and that direct the transcriptional program of both GC B cells and TFH cells (IL-21). Apart from these co-stimulatory signals, T cells may control the emergence of autoreactive GC B cells by eliminating these cells by instructing apoptosis through FAS-FASL interactions. ICOS, inducible co-stimulator; MHCII, major histocompatibility complex II; TCR, T cell receptor; SLAMF, signaling lymphocytic activation molecule family member.

immunoglobulin fusion protein that outcompetes CD28 in B7 binding could indeed suppress lupus development in NZB/NZW mice by indirectly correcting B cell activation (223, 224). Trials however investigating treatment of SLE with such a fusion protein (abatacept) showed limited success, which was accompanied by an increase in serious adverse effects (225). Finally, another receptor-ligand pair that is only expressed on activated B and T cells and seems essential for mutual B-T cell activation is OX40-OX40L (226). Not only does OX40-OX40L interaction support memory T cell formation, it boosts B cell

proliferation and activation (227). Importantly, in SLE patients genome-wide association (GWA) studies have associated OX40L (TNSF4) with SLE, the identified polymorphisms correlated with increased OX40L expression levels (228).

It was found that long-term humoral memory poorly developed in SLAM associated protein (SAP) deficient mice due to defective GC formation (229, 230), since SAP proved to be required for stabilizing prolonged B-T interactions that are critical for GC formation and maintenance (231, 232). Interestingly, SAP deficiency (either due to targeted genetic disruption or due to a spontaneous missense mutation) could counteract lupus development in mice (230, 233). The reduction in GCs and autoimmunity in the absence of SAP was also recapitulated in lupus-developing *sanroque* mice (234), where SAP proved to be crucial for inducing T follicular helper cells (T_{FH}) (235), the GC-specialized T_H subset that provides essential help for B cells in GC reactions (236).

SAP may aid T_{FH} formation by up-regulating the expression of inducible T-cell costimulator (ICOS) (235), a co-stimulatory receptor belonging to the CD28/CTLA4 family (237). Although ICOS is critical for T_H cell activation in general, it exerts a critical role in GC formation since deletion or blocking of ICOS specifically hampers the generation of T_{FH} cells in mice and men (238, 239). Several groups have investigated the contribution of activating ICOS signals to the development of GC-driven autoimmune disease. ICOS indeed seems to be a promising target in the treatment of SLE, since lupus manifestations in NZB/NZW mice were reduced upon treatment with ICOS-L blocking antibodies (240). ICOS proved to contribute to auto-antibody responses in MRL/lpr mice through the activation of extrafollicularly located CXCR4+ T_H cells (241). Importantly, ICOS is overexpressed on T_H cells in SLE patients, and ICOS+ T cells cluster with B cells and plasma cells in inflamed kidneys in SLE patients (242).

The exact mechanism however by which ICOS promotes T_{FH} development is still unclear. One study indicates that through activation of transcription factor c-Maf transcription of IL-21 is stimulated (243), a cytokine that is reported to be essential for the proper development of T_{FH} cells (244, 245). Moreover, IL-21 maintains expression of the GC B cell transcription factor Bcl6 and promotes GC B cell proliferation, (late) memory B cell formation and affinity maturation, while the extrafollicular antibody response to exogenous antigen seems to be intact (235, 246). In autoimmunity however, the role of IL-21 (like ICOS) in directing T-dependent antibody responses seems not to be exclusively confined to the GC reaction, as in lupus-prone BXSB-Yaa mice IL-21 is predominantly produced by extrafollicular T_H cells (247), a finding that seems more common in other murine SLE models as well (241). The autoimmunity-promoting role of IL-21 is undisputed since the lupus phenotype of BXSB-Yaa or MRL/lpr mice complete resolves on a IL-21R deficient genetic background (247, 248), and since treatment of MRL/lpr mice with IL-21R.Fc fusion proteins ameliorates disease (249). Importantly, polymorphisms in both IL-21 as well as the IL-21R are associated with SLE in humans (250, 251).

IL-21 may also propel autoimmunity by inducing the production of other pathogenic cytokines. It has been found that IL-21, in concert with TGF-β and IL-23, can induce T_H cells producing IL-17 (T_H17 cells) (252, 253). This cytokine is normally not produced by T_{FH} cells, since Bcl6 in T_{FH} cells was shown to repress the transcription factors T-bet and RORγT that are required for IFN-γ and IL-17 production, respectively (254). Nevertheless, a role for IL-17 in driving lupus-like autoimmunity through corrupting GC's is likely, since in lupus-prone mice IL-17 producing T_{FH} cells can be observed (255), and since in the BXD2 SLE-

susceptible mouse strain IL-17 can drive autoimmunity by desensitizing CXCR4 and CXCR5 signaling and thereby altering GC B cell migration and retention within GCs (256). Apart from corrupting GCs in SLE mouse models, in SLE patients IL-17 was shown to promote plasmablast differentiation, and elevated IL-17 serum levels proved to correlate with disease severity in SLE patients (257).

To keep autoreactive GC B cells in check that continuously arise due to the random introduction of self-reactivity imposing somatic mutations of the BCR, negative feedback signals can halt the differentiation of these cells into memory B cells or plasma cells. One of the main regulatory signals provided by T cells is a death signal conferred by FasL expressed on activated T cells that directly induces apoptosis in Fas expressing lymphocytes through the activation of caspases 8 and 10 (222). The critical role of Fas-FasL interactions in guarding B cell homeostasis was first discovered in MRL/lpr and MRL/gld mice that harbour mutations in Fas or FasL, respectively (258, 259). These mice develop an identical fatal systemic autoimmune disease with striking similarities to human SLE, accompanied with lymphadenopathy. Fas mutations in humans are rare and do not cause a true SLE-like phenotype, but instead lead to autoimmune lymphoproliferative syndrome (ALPS) or Canale-Smith syndrome, a disease characterized by excessive lymphoproliferation and various autoimmune symptoms (260-263).

It was shown that deprivation of BCR signals or continuous antigen binding to the BCR triggers FasL-induced B cell apoptosis (209). Further protection against Fas-mediated apoptosis was demonstrated in other studies as well (210), and we have recently shown that enhanced BCR signaling due to increased Btk protein expression can raise the threshold for Fas-induced B cell apoptosis in mice (163). In this light, it is important to notice that enhanced BCR signaling upon BCR crosslinking is commonly observed in SLE B cells (264). Moreover, polymorphisms in BCR signaling molecules as BLK, BANK-1 and LYN are strongly associated with the risk for developing SLE and are amongst the most frequently found risk genes in SLE GWAS (187, 265, 266).

Another regulatory signal that potently dampens B cell activation comprises a negative feedback signal of secreted Ig that can bind to the inhibitory FcγRIIB expressed on (GC) B cells (267). When immune complexes accumulate during the course of an antibody response, antigen-specific Ig will bind to the FcγRIIB of activated B cells and shut down their activation by counterbalancing activating signals. The protecting role in preventing lupus-like disease was demonstrated by the spontaneous development of SLE in FcγRIIB deficient mice (158). Since the FcγRIIB is not exclusively expressed by B cells but also by myeloid cells, autoimmunity could arise due to simultaneous activation of multiple immune cell types. This is unlikely however since exclusive transgenic overexpression of FcγRIIB in B cells could reduce SLE features in MRL/lpr mice (268). A contribution of FcγRIIB dysfunction in SLE pathogenesis was further supported by dysregulated expression of FcγRIIB in NZW mice and in human SLE B cells (269, 270). In addition, FcγRIIB has been shown to be even downregulated on memory B cells and plasma cells in SLE patients (271). In addition, FcγRIIB polymorphisms have been shown to confer an increased SLE susceptibility, although not in all ethnic groups examined (272-275). Strikingly, GWA studies indicate that polymorphisms in other activating Fc receptors (FcγRIIA, FcγRIIIA) or other genes connected to immune complex processing such as complement factors (C1Q, C4A, C4B, C2) are more strongly associated with SLE (187, 265). Since these receptors are not expressed on B cells themselves, their possible indirect contribution B cell tolerance breakdown in SLE remains elusive.

Innate immune cells promoting self-reactive B cell activation

New findings during the last decade have highlighted the crucial contribution of innate cells to aberrant B cell activation in SLE, and some findings have even demonstrated that these cells can substitute functions originally attributed only to T cells, thereby establishing SLE-like disease in the absence of any T cell help (170, 276).

A variety of myeloid cells can produce crucial B cell survival cytokines that are members of the tumor necrosis factor (TNF) ligand family: B cell activating factor (BAFF, BLyS or TNFSF13B) and a proliferation-inducing ligand (APRIL or TNFSF13). The functions of these cytokines partly overlap in B cell activation but they unequally affect B cells and plasma cells as these cells differentially express three receptors that bind BAFF and APRIL with different affinities (277). BAFF is the sole ligand of the BAFF-R that is upregulated on B cells beyond the immature B cell stage, and BAFF primarily promotes survival rather than proliferation of transitional B cells and several mature B cell subsets (278, 279). A second receptor, TACI, that can bind BAFF and APRIL with comparable affinity, is expressed on all peripheral B cells and seems to exert an additional regulatory role in B cell homeostasis as its signaling restricts peripheral B cell numbers (280). Unlike BAFF-R and BCMA, TACI is strongly upregulated on TLR-activated B cells and B cell subsets like marginal zone (MZ) B cells and B-1 cells that mount T-independent antibody responses, suggesting a critical role for promoting the survival of T-independently generated plasmablasts (281-283). A third receptor, BCMA, can bind BAFF as well but has higher affinity for APRIL (284). Since BCMA expression is only initiated late during GC B cell and plasma cell differentiation, APRIL and to lesser extent BAFF primarily govern plasma cell survival, and BCMA-deficient mice therefore display a selective defect in the survival of long-lived plasma cells (285-287).

BAFF and APRIL are produced by different innate immune cells at different anatomical localizations to aid B cell functioning beyond the mere promotion of survival. $CD11c^{high}$ dendritic cells do not only produce high levels of BAFF and APRIL at extrafollicular sites in secondary lymphoid organs to stimulate early B cell responses, but they also capture and display antigen to B cells (288, 289). In addition, in the absence of T cell help the production of BAFF or APRIL by dendritic cells promotes class switch recombination (CSR), B cell survival, and differentiation of marginal zone (MZ) B cells into IgM plasmablasts (290-292). Comparably, macrophages can support the formation of IgM producing blasts from B1 cells through the production of TACI ligands (291, 293), and neutrophils provide essential help to MZ B cells that induces antibody production, CSR and SMH through co-stimulation factors including BAFF, APRIL and IL-21 (294).

As BAFF and APRIL confer critical survival and differentiation signals to B cells and plasma cells, deregulated expression of these cytokines were postulated to hamper the elimination of emerging autoreactive cells. Whereas APRIL overexpression in mice does invoke B cell hyperplasia but not systemic autoimmunity (295), overexpression of BAFF induces severe autoimmune disease with characteristics of both SLE and Sjögren's syndrome (296). Consistent with the predominant production of BAFF by multiple myeloid cell types, the specific role of BAFF in T-independent antibody responses, and the increased sensitivity to BAFF signals due to upregulation of TACI on TLR-activated plasmablastst, autoimmune disease resulting from excess BAFF can develop in the absence of any T cell help (281). Conversely, when the generation of anti-DNA B cells largely depends on T-dependent GC reactions in the context of the

introduction of a transgenic IgH chain on the NZB/W SLE-susceptible genetic background, blocking BAFF and APRIL could not prevent anti-DNA antibody production or the persistence of anti-DNA B cells in GC's (297).

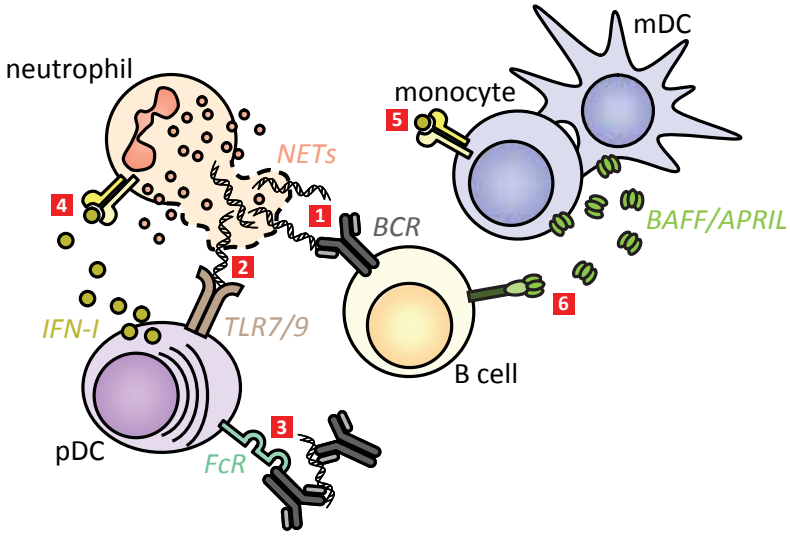
During the last years, an entirely new concept has emerged by which innate immune cells may trigger the development of autoimmune diseases. This concept known as neutrophil extracellular traps (NETs) describes the expulsion of a chromatin meshwork containing antimicrobial peptides by neutrophils in a cell death process (NETosis) designed to ensnare a variety of extracellular pathogens (298) (Figure 5). Since incitation of NETosis by a broad variety of stimulatory signals can quickly halt invading pathogens, these NETS expose a dangerous texture of intracellular self-antigens as DNA and nucleosomes that are normally not abundantly available to self-reactive B cells (299). These NETs may thus provide the ultimate source of auto-antigens that drives the activation and differentiation of self-reactive B cells in SLE, thereby complementing or perhaps even challenging the original hypothesis that nuclear self-antigen exposure to B cells is mainly increased in SLE due to defects in the clearance of apoptotic cells by tinginble body macrophages in GCs (300, 301).

Apart from the risk of B cells reacting to NET self-antigens, these NETs harbor molecules that can potently activate other immune cells that in turn may fuel B cell driven autoimmune disease (302). For example, DNA-containing immune complexes can trigger in plasmacytoid dendritic cells the release of the pro-inflammatory cytokine IFN-α, which in return can trigger NETosis. This vicious circle is further fuelled by self-reactive B cells that produce NET-reactive antibodies that as immune complexes trigger NETosis and stimulate pDC's. This interplay between neutrophils, pDC's and B cells (Figure 5) potently propels high-level IFN-α production (303-305) and may thus provide a mechanism for the widely reported IFN-I signature observed in SLE and related rheumatic diseases (306-308). It is noteworthy that in Sjögren's syndrome a clear connection exists between an IFN-I signature in monocytes and elevated BAFF production by these cells, providing another mechanism by which other innate immune cells can further propagate B cell tolerance breakdown and the pro-inflammatory loop with neutrophils and pDC's (309). In addition, it was found that serum of SLE patients not only instructs monocyte differentiation into DC's in an IFN-I dependent fashion (310), but that additionally these DC's promote the formation of IgG and IgA producing plasmablasts through BAFF and APRIL production (311). In MRL/lpr mice, DC depletion could ameliorate auto-immune disease (312) and a T-independent role for DC's in stimulating B cell activation and differentiation in murine lupus (Figure 5) was demonstrated in mice deficient for the ubiquitin editing enzyme A20 encoded by *Tnfaip3* (181). In co-culture with B cells, these A20-deficient DC's directly enhance B cell proliferation and plasmablast formation. In this light it is important that polymorphisms in the *Tnfaip3* locus are associated with SLE (183).

Aim of the thesis

For the host defense against pathogens B lymphocytes fulfill multiple roles, including production of protective antibodies and presentation of pathogen-derived antigens to T lymphocytes. To exert these functions, the specific recognition of antigens through the B cell receptor (BCR) is indispensable. To ensure infinite diversity in BCRs in the mature B cell repertoire, B cells form and diversify the antigen-recognizing interface of the BCR through multiple stochastic processes in B cell development and B

Figure 5. Pro-inflammatory loops between B cells and innate immune cells in SLE.



Upon activation neutrophils can undergo programmed cell death while extruding NETs (NETosis) composed of chromatin and pro-inflammatory molecules. The nuclear antigens in these NETs may not only activate B cells that are self-reactive to nuclear self-antigens (1) but activate pDCs as well through TLRs (2). The activation of B cells will lead to the production of ANAs that form circulating chromatin-containing immune complexes that can stimulate pDCS through Fc receptors (3). This pDC activation triggers the release of type-I interferons that can in turn promote NETosis (4). In addition, these interferons can prompt myeloid cells as monocytes and mDCs to produce B cell survival cytokines as BAFF and APRIL (5) that further promote autoreactive B cell survival and differentiation (6). NET, neutrophil extracellular trap; BCR, B cell receptor; TLR, toll like receptor; FcR, Fc receptor; IFN-I, type-I interferons; BAFF, B cell activating factor; APRIL, a proliferation-inducing ligand; pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cell.

cell activation. Despite the implementation of BCR checkpoints during B cell differentiation to assess reactivity of the BCR to self-antigens, mature autoreactive B cells still emerge at considerable frequencies and need to be controlled to prevent auto-immune responses. Signals of the BCR are decisive for this silencing of autoreactive B cells, and perturbations in BCR signaling may therefore jeopardize B cell tolerance.

This thesis comprises studies to unravel how altered BCR signaling due to changes in structural BCR components or BCR signaling molecules may determine the predisposition to systemic B cell-driven rheumatic autoimmune diseases. **Chapter 1** introduces how signals from the BCR and other receptors on B cell intrinsically control the aberrant activation of self-reactive B cells, and also discusses how B cell activation may drive pro-inflammatory loops between different immune cell types that may culminate in auto-immune diseases as systemic lupus erythematosus (SLE).

Previously re-expression of the surrogate light chain (SLC), a pre-BCR component that mimics constitutive BCR signals, was reported in mature B cells in arthritic joints in rheumatoid arthritis patients.

Therefore, in **Chapter 2**, we investigated how ectopic expression of the SLC in mature murine B cells may impose or oppose B cell tolerance.

Next to (pre-)BCR components that are normally not expressed in mature B cells, we also investigated the pathogenic potential in autoimmune disease of Bruton's tyrosine kinase (Btk), a critical signaling molecule downstream of the BCR that has not been implicated in autoimmunity before. In **Chapter 3**, we set out to determine in Btk-transgenic mice whether activating point mutations in Btk may interfere with autoreactive B cell silencing during and after completing B cell development. Since introducing activating mutations in Btk may induce exceptionally strong and constitutive BCR signaling not representative of BCR signaling changes in autoimmune patients, we also studied how Btk may propel autoimmunity by transgenically enhancing the expression level of Btk protein in murine B cells. In **Chapter 4**, we describe how overexpression of wildtype Btk protein enhances BCR-instructed activation and survival of B cells, leading to the development of a syndrome in mice closely resembling SLE in human.

While rheumatic autoimmune diseases in patients are normally propelled by pro-inflammatory amplification loops between multiple cell types, the autoimmunity caused by a B cell-restricted modulation of Btk expression prompted us to investigate pro-inflammatory loops in SLE provoked by Btk signaling in B cells. In **Chapter 5**, we studied in mice how Btk signaling in B cells affects the activation and differentiation of pathogenic T cell subsets in SLE and whether the engagement of T cells is essential for the development of B cell-driven autoimmunity. Importantly, not only the BCR but also toll-like receptors (TLRs) and chemokine receptors employ Btk signaling. We therefore in **Chapter 6** used a total mRNA deep sequencing approach to compare BCR-stimulated and Btk-overexpressing B cells to address whether Btk selectively alters the outcome of BCR signaling and how these changes may affect B cell differentiation in the context of autoimmunity. Finally, to determine whether Btk expression in B cells influences SLE development and clinical presentation, in **Chapter 7** we studied BTK expression dynamics in B cells from SLE patients, assessed BTK levels in B cell subsets in SLE patients, and correlated disease severity and phenotype to BTK expression levels.

Aberrant BCR signaling may not only lead to the development of autoimmune disease in humans, but chronic stimulation of the BCR by self-antigens or pathogens is implicated in malignant transformation of B cells in several B cell lymphomas and in particular chronic lymphocytic leukemia (CLL). Importantly, a previous finding of higher BTK expression levels in human CLL B cells implicated a role for BTK signaling in established CLL, but could not address whether BTK protein levels are decisive for CLL development. In **Chapter 9**, we modulated Btk expression levels in a mouse model for CLL to determine whether Btk protein levels determine CLL susceptibility and clonal selection during malignant transformation. In **Chapter 10**, we provide a detailed overview of the current knowledge on the role of BCR signaling in CLL development and progression.

Finally, in **Chapter 11** the results of these studies will be evaluated in the light of recent scientific insights in B cell selection and activation in autoimmunity and leukemia, and we will discuss future research directions and clinical implications of our study results.

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Surrogate light chain expression beyond the pre-B cell stage promotes tolerance in a dose-dependent fashion

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Abstract

While surrogate light chain (SLC) expression is normally terminated in differentiating pre-B cells, co-expression of SLC and conventional light chains has been reported in a small population of autoreactive peripheral human B cells that accumulate in arthritic joints. Despite this association with autoimmunity the contribution of SLC expressing mature B cells to disease development is still unknown. We studied the pathogenicity of SLC^+ B cells in a panel of mice that transgenically express various levels of the SLC components VpreB and λ5 throughout B cell development. Here we report that although VpreB or λ5 expression mildly activates mature B cells, only moderate VpreB expression levels - in the absence of λ5 - can enhance IgG plasma cell formation. However, no autoantibody production was detectable in VpreB or λ5 transgenic mice and on susceptible genetic backgrounds VpreB expression could not accelerate autoimmunity. Instead, moderate VpreB expression partially protected mice from induced autoimmune arthritis. In support of a tolerogenic role of SLC-transgenic B cells, we observed that in a dose-dependent manner SLC expression beyond the pre-B cell stage enhances clonal deletion among immature and transitional B cells and renders mature B cells anergic. Collectively, these findings suggest that SLC expression in human B cells does not propagate autoimmunity but instead imposes tolerance.

Submitted

Introduction

Over a decade ago in arthritic joints of RA patients a new candidate pathogenic B cell subset was identified of which ~68% expressed B cell receptors (BCRs) reactive to nuclear self-antigens (1, 2). These cells that constitute up to 1% of circulating B cells in peripheral blood of healthy individuals (1) remarkably co-express conventional Ig light chains (LC) and pre-B cell receptor (pre-BCR) components VpreB and lambda5 (λ5) at the cell surface (1, 3). Despite the pre-BCR expression these SLC^+LC^+ cells cannot be designated as immature bone marrow emigrants based on their low expression of CD10 and CD38 with concomitant upregulation of activation or memory markers, including CD5 and CD27 (1). The notion that SLC^+LC^+ B cells may represent a recently activated B cell population *in vivo* is further supported by the recapitulation of VpreB re-expression in a fraction of $GL\text{-}7^+$ murine B cells in mice subjected to T-dependent immunizations (3). Despite the recognition of their activated phenotype and predominant expression of autoreactive BCRs (1, 2), it is still unknown whether these SLC^+LC^+ B cells propel or modulate autoreactive B cell responses in rheumatic diseases.

Expression of the SLC at the pre-B cell stage is essential to verify successful rearrangement, stable expression, and possibly the IgL chain pairing capacities of the produced IgH chain (4). In addition, the SLC may also function as a counterselector for autoreactive IgH chains since SLC-deficient mice exhibit spontaneous autoantibody production (5). In large cycling pre-B cells with successfully recombined IgH chains SLC signaling induces a transient proliferative burst, ensures allelic exclusion through terminating gene expression that is required for Ig gene rearrangements, and silences its own transcription (reviewed in (6)). The SLC instantly provides such positive feedback signals to differentiating IgH expressing pre-B cells, since it is equipped with autonomous signaling capacity (7, 8). This capacity is not only conferred by the polyreactive nature of the non-immunoglobulin tail of λ5 that can bind multiple self-antigens such as heparin sulfate and galectin-1 (9, 10) but mainly by self-reactivity of the SLC to an N-glycosylation site of the IgH chain providing strong and instant pre-BCR signaling (11).

The importance of SLC signaling for pre-B cell development is evident from mice deficient for SLC components or downstream pre-BCR signaling molecules, such as Bruton's tyrosine kinase (Btk) or BLNK/Slp65 (6, 12). These mice exhibit defects in expression of pre-B cell maturation markers, Ig lambda (Igλ) LC rearrangements and an enhanced in vitro proliferative capacity in response to IL-7. Conversely, transgenic mice with continuous high-level SLC expression throughout B cell differentiation do not exhibit a delay in pre-B cell maturation or prolonged pre-B cell proliferation (13). Instead, constitutive BCR signaling induced by high-level transgenic SLC expression hampers normal B cell maturation but instead drives spontaneous differentiation of peripheral B cells into IgM plasma cells, thereby providing a first indication that constitutive pre-BCR signaling beyond the pre-B cell stage may jeopardize B cell tolerance. However, high-level SLC expression in mature B cells in these mice did not induce spontaneous IgG autoantibody production or systemic autoimmune disease (13), leaving the possibility that lower doses of SLC signaling could still establish systemic autoimmunity. Importantly, in human SLC+LC+ B cells VpreB levels are lower than in pre-B cells, and VpreB levels in SLC+LC+ B cells markedly exceed those of λ5 (1).

To study whether pre-BCR signaling, as occurring in rare human SLC^+LC^+ B cells, could affect B

cell tolerance, we studied autoimmunity in SLC transgenic (SLC-tg) mice with enforced expression of different doses of both or individual SLC components beyond the pre-B cell stage (13). Here we report that although enforced SLC expression promotes mild B cell activation *in vivo*, no spontaneous or enhanced autoimmunity could be observed on autoimmune-resistant or autoimmune-prone genetic backgrounds. In contrast, prolonged VpreB expression partially protects from experimentally induced arthritis by a dose-dependent induction of clonal deletion and anergy in developing and mature B cells, respectively. These findings imply that SLC re-expression in autoreactive $SLC^{+}LC^{+}$ B cells in humans is most likely not pathogenic but instead functions to silence these cells, mainly through tolerance mechanisms other than receptor editing.

Materials & methods

Mice and genotyping

The CD19-VpreB Tg and CD19-λ5 Tg constructs and the generation of multiple independent mouse strains expressing different copy numbers of these constructs have been described (13). Three lines with increasing CD19-VpreB Tg expression ($Vpre^{low}$, $VpreB^{int}$ and $VpreB^{high}$) and two lines with increasing CD19-λ5 Tg expression ($\lambda5^{low}$ and $\lambda5^{high}$) were all crossed onto the c57bl/6 background (>10 generations) and were analyzed on this genetic background, or on c57bl/6 *FcγRIIB*-deficient (14) or c57bl/6 Eμ-Bcl2 (15) backgrounds, or upon crossing onto the dba/1 background for >10 generations. Mice were genotyped by genomic PCR on tail DNA according to standard protocols using the following primers for the CD19-VpreB Tg and CD19-λ5 Tg constructs: a forward primer aligning with the human genomic CD19-promoter fragment (5'-TGAGAAGGAGTCTATGTGCC-3'), a reverse primer specific for mouse genomic *vpreb1* (5'-GCCATAGGAGGAGCAAAGAA-3') and a reverse primer specific for mouse genomic *Igll1* (λ5; 5'- ACTCTGAGCTTCATTGACCC-3'). All mice were kept at specified pathogen free conditions at the Erasmus MC experimental animal care facility, and all experimental protocols were reviewed and approved by the Erasmus MC committee for animal experiments.

Collagen-induced arthritis (CIA) experiments

Mice on the c57bl/6 background were immunized intradermally at the tail base with 100 μg of chicken collagen type 2 (CII; Chondrex) emulsified in complete Freund's adjuvant (CFA), and a secondary immunization was performed at day 21 by injecting intradermally in dorsal skin 100 μg of chicken CII emulsified in CFA. Assessment of arthritis severity was performed as described in Lubberts et al. (16).

General flow cytometry procedures

Preparation of single-cell suspensions of lymphoid organs and fluorescent labeling of cell surface markers was performed according to standard procedures (17). Used monoclonal antibodies were specific for CD19 (ID3, eBioscience), CD23 (B3B4, eBioscience), CD25 (PC61.5, eBioscience), CD69 (H1.2F3, BD Biosciences), CD86 (GL1, eBioscience), CD93 (PB493/AA4.1, eBioscience), CD95 (Jo2, BD Biosciences), CD138 (281-2, BD Biosciences), B220 (RA3-6B2, eBioscience), IgM (II/41, eBioscience), IgD (11-26c.2a, eBioscience), IgG1 (A85-1, BD Biosciences), IgG2a/b (R19-5, BD Biosciences), IgG3 (R40-82, BD Biosciences), Igκ (187.1, BD Biosciences), Igλ (R26-46, BD Biosciences), VpreB/CD179a (R3/VpreB, BD Biosciences), and

λ5/CD179b (LM34, BD Biosciences). Biotin-conjugated antibodies were subsequently stained with fluorochrome-coupled streptavidin (eBioscience), and staining of Gal-β(1-3)-GalNAc carbohydrates on B cells was performed with biotin-conjugated PNA (peanut agglutinin, Sigma-Aldrich). After completing cell surface marker labelling, intracellular stainings for Ig or SLC components were performed by fixing and permeabilizing/staining in Cytofix/Cytoperm™ and Perm/Wash™ buffers respectively (BD Biosciences) according to the manufacturer's instructions. All flow cytometric measurements were performed on a LSRII™ flow cytometer (BD Biosciences) and data were analysed using FlowJo software (Tree Star Inc.).

Flow cytometric measurement of Ca2+ influx

Splenic cell suspensions were prepared in loading buffer (HBSS/5% FCS/10mM HEPES) and splenocytes were loaded at a concentration of $10{\cdot}10^6$ cells/mL with 5 μM Fluo-3 (Molecular Probes) and 5 μM Fura Red™ (Molecular Probes) in loading buffer for 30 minutes at 37°C. Cells were washed and resuspended in flux buffer (HBSS/5% FCS/10mM HEPES/1mM $CaCl_2$) and during stimulation at 37°C with 20 μg/mL $F(ab'_2)$ goat anti-mouse-IgM (Jackson Immunoresearch) Fluo-3 and Fura Red™ fluorescence was measured in FITC (515-545 nm) and PerCP-Cy5.5 (675-715 nm) detection channels respectively on an LSRII™ flow cytometer (BD Biosciences). The ratio of Fluo-3/Fura Red™ fluorescence was calculated and normalized for maximum fluorescence upon stimulation with 2μg/mL ionomycin (Sigma-Aldrich).

Anti-nucleosome autoantibody ELISA

Measurements of anti-nucleosome autoantibodies in serum by ELISA was performed as described previously (17). Briefly, nucleosome-coated plates of an anti-nucleosome ELISA kit (Orgentec) were incubated with 100-fold diluted serum samples, and subsequently plate-bound antibodies were detected using the protocol and reagents provided by the manufacturer. As secondary reagent polyclonal anti-mouse total IgG antibodies (Southern Biotech) were used.

B cell purification and *in vitro* stimulation

Magnetic-activated cell sorting (MACS) of naïve splenic B cells was performed by indirect labeling of non-B cells, activated B cells, B1 cells and plasma cells using biotinylated anti-CD5 (53-7.3), anti-CD11b (M1-70), anti-CD43 (S7), anti-CD95 (Jo2), anti-CD138 (281-2), anti-Gr-1 (RB6-8C5), and anti-TER-119 (PK136) antibodies (BD Biosciences) and streptavidin-conjugated magnetic beads (Miltenyi Biotec). After magnetic depletion of labeled cells the purity of naïve B2 cells typically exceeded 95% as verified by flow cytometric analysis. Purified naïve B2 cells were cultured for 24 hours and stimulated with $F(ab'_2)$ goat anti-mouse-IgM (Jackson Immunoresearch) or LPS (own production) as described previously (17).

mRNA deep sequencing

mRNA was isolated using the RNeasy Mini Kit (Qiagen) from $F(ab'_2)$ goat anti-mouse-IgM stimulated or unstimulated splenic naïve B cells. Sequencing of total mRNA was performed using a HiSeq 2000 sequencing system (Illumina). Reads were mapped to Ensembl transcripts base on the University of

California at Santa Cruz (UCSC) mouse genome annotation (mm9). Fragments per kilobase of a transcript per million mapped reads (FPKMs) were calculated and assigned per transcript. Sequencing data analysis was performed with MultiExperiment Viewer (MeV) software v 4.8.1 (Dana-Farber Cancer Institute).

Statistical analysis

The Student's t-test was used for calculating levels of significance of differences between groups of continuous data. Levels of significance in the analysis of mRNA deep sequencing data were calculated using a one-way ANOVA with Bonferroni correction.

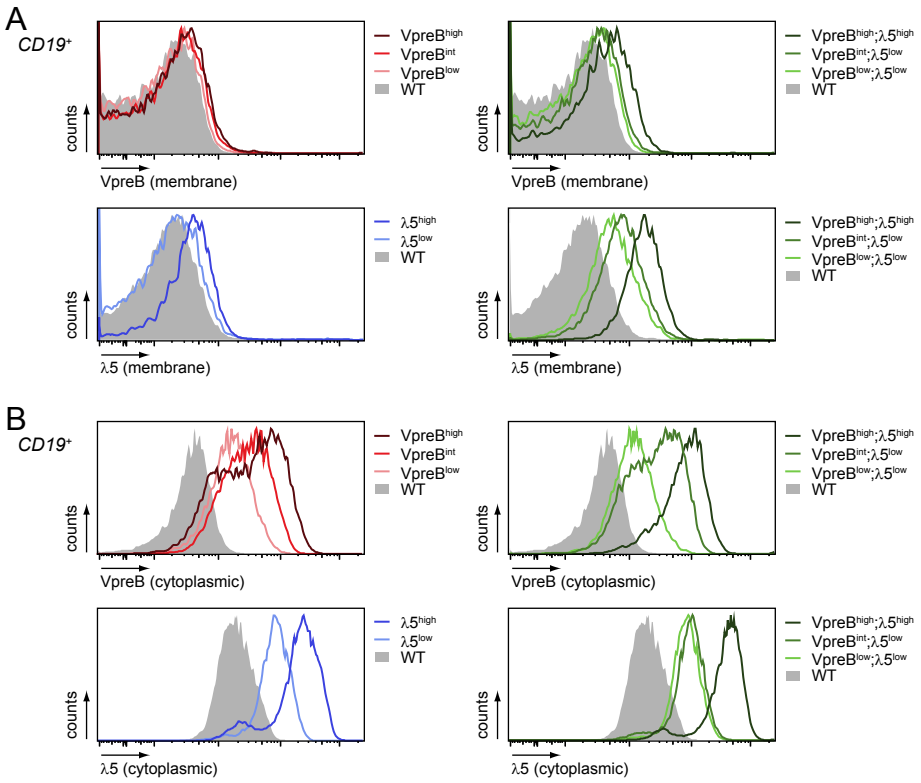
Results

Expression of different doses of VpreB and λ5 in peripheral B cells

Since VpreB re-expression has been reported *in vivo* in germinal center B cells upon T-dependent immunization (3), we examined whether antigenic stimulation of murine mature B cells could induce SLC expression. We used total mRNA deep sequencing analysis to compare gene expression in MACS-purified naïve splenic B cells from wild-type mice that were either unstimulated or stimulated with $F(ab')_2$ anti-IgM for 12 hours. Whereas activation marker CD69 showed a robust and significant upregulation upon anti-IgM stimulation, we could not detect a significant increase in transcription of *Vpreb1* or *Vpreb2* genes (Figure S1). Transcription of Vpreb2 was even reduced after BCR stimulation, but importantly *Vpreb3* transcription was strongly induced (Figure S1). Expression of *Igll* (λ5) was very variable. Since in human SLC^+LC^+ B cells expression of other pro- and pre-B cell genes as *Dntt* (TdT), *Rag1* and *Rag2* was reported (1), we examined whether anti-IgM stimulation could also instruct re-expression of these genes. No significant increase in *Rag1* or *Rag2* transcripts could be observed, while *Dntt* was minimally expressed (Figure S1).

From these findings it appears that antigenic stimulation of wild-type B cells differentially affects transcription of *Vpreb* and *Igll*. Therefore, we decided to employ mice that express individual VpreB or λ5 components or both of them in mature B cells to study the effects of SLC signaling on B cell tolerance. Since it is conceivable that potential effects of SLC expression on mature B cell tolerance are limited to a narrow range of lower SLC expression levels, we used multiple independent CD19-VpreB Tg and CD19-λ5 Tg mouse lines harboring different copy numbers of these transgenic constructs. Most of these CD19-VpreB and CD19-λ5 transgenic mouse lines (expressing high levels of VpreB and λ5, respectively throughout B cell development under the control of the CD19 promoter region) we have previously described on an mixed genetic background (13). After crossing these lines onto the relatively autoimmune-resistant c57bl/6 genetic background for >10 generations, we analyzed three independent CD19-VpreB transgenic mouse strains: Vpreb#1 ($VpreB^{high}$), Vpreb#3 ($VpreB^{int}$) and $VpreB^{low}$, as well as two independent CD19-λ5 transgenic mouse strains: λ5#2 ($λ5^{high}$) and λ5#1 ($λ5^{low}$). Flow cytometric analysis of splenic B cells revealed increasing, but very low membrane expression of VpreB and λ5 in mice with increasing transgene copy numbers (Figure 1A). In mice carrying both a VpreB and λ5 transgene membrane expression of individual SLC components was markedly increased (Figure 1A).

Figure 1. VpreB and λ5 expression in peripheral SLC transgenic B cells.



Flow cytometric analysis of CD19+ gated wildtype (WT), VpreB and λ5 transgenic splenocytes for (A) membrane and (B) cytoplasmic expression of VpreB and λ5. Of each transgenic mouse strain representative histograms of >3 mice (10-14 weeks of age) are shown.

Previous studies have shown that in the absence of VpreB λ5 has very limited binding capacity to the IgH chain, and that the eighth β-strand in λ5 is aiding the pairing of VpreB to the IgH chain (18, 19). To confirm that the increase of VpreB and λ5 membrane expression in double-transgenic B cells was due to mutual VpreB and λ5 stabilization rather than altered transcriptional activity of the individual transgenes, we measured intracellular VpreB and λ5 expression in these cells. We noted that single versus double-transgenic B cells carrying the same transgenic constructs did not exhibit overtly different VpreB or λ5 cytoplasmic expression levels (Figure 1B). Furthermore, the discrepancy between membrane versus cytoplasmic VpreB or λ5 expression was larger in single-transgenic than in double-transgenic B cells, indicating that the low membrane expression of VpreB and λ5 in single-transgenic B cells may not only be resulting from enhanced BCR internalization, but partially from the mutual dependence of VpreB and λ5 for stable membrane expression.

Taken together, these analyses show that our panel of SLC transgenic mice express a range of doses

of VpreB and λ5 in mature peripheral B cells that allows us to analyse whether individual or combined expression of SLC components at the mature B cell stage can induce autoimmunity.

Moderate VpreB expression drives mild B cell activation and germinal center formation

Since the SLC has autonomous signaling capacity that may provide activating BCR signals to mature B cells independent of (allo-)antigens (11), we examined whether enforced SLC expression in mature B cells could induce their activation. To this end, we assessed membrane expression of various activation markers on transgenic splenic B cells. Flow cytometric analysis demonstrated a spontaneous mild upregulation of activation markers CD86 and to lesser extent CD69 on VpreB and/or λ5 transgenic B cells (Figure 2A), whereas CD25 was not differentially expressed. Upregulation of these B cell activation markers correlated with transgene expression levels in VpreB or λ5 transgenic B cells, while co-expression of VpreB and λ5 transgenes could only minimally increase CD86 and CD69 levels.

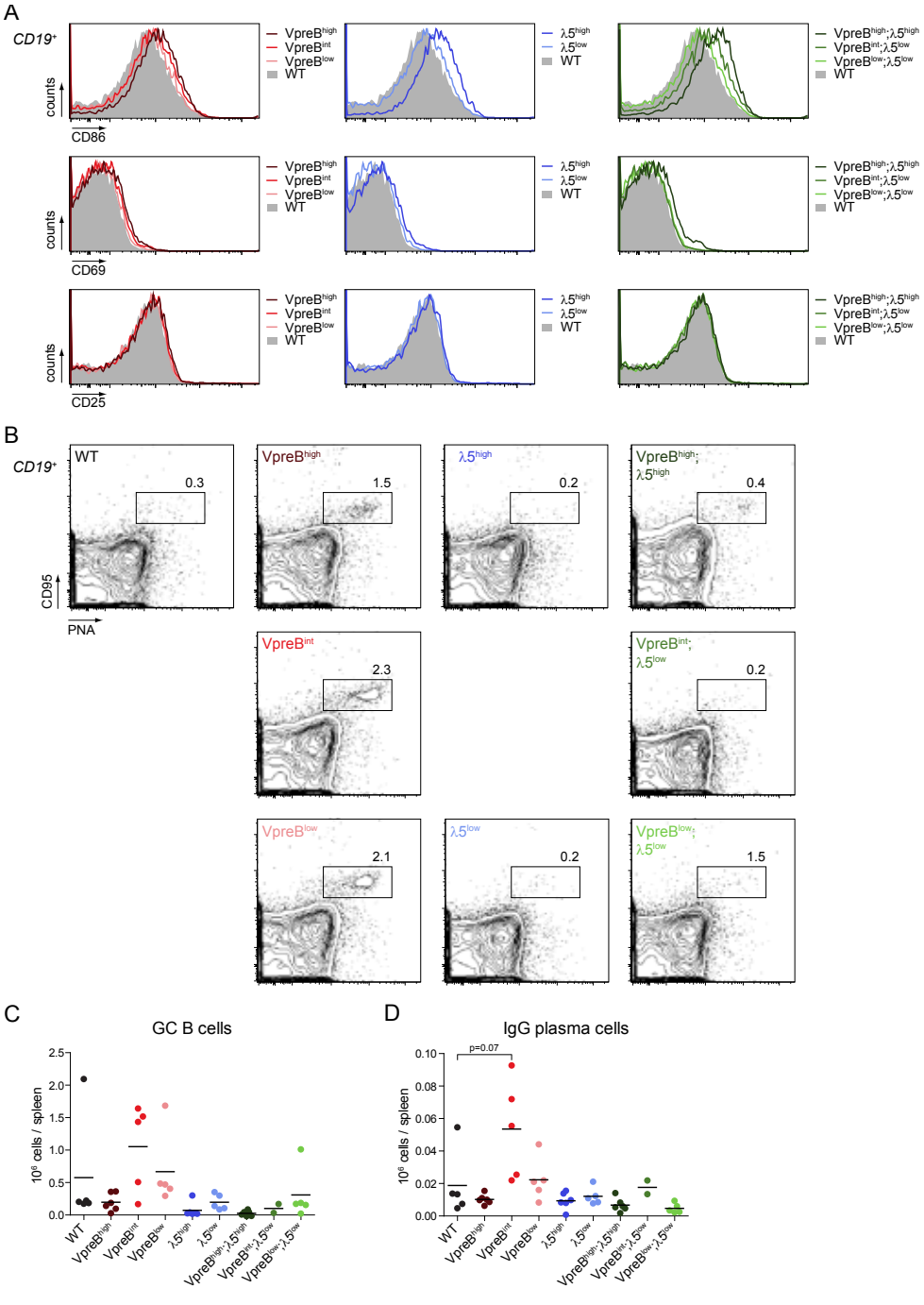
It has previously been published that high-level SLC expression in mature B cells could induce spontaneous differentiation of IgM plasma cells in vivo (13), but it is unclear whether lower levels of SLC expression may also drive activated B cells in SLC transgenic mice into T-cell dependent plasma cell differentiation. In all VpreB transgenic lines, but in particular in VpreB^{int} mice, we observed spontaneous germinal center (GC) formation in the spleen. This was reflected by a relative increase in CD95^{+}PNA^{+} GC B cells among total CD19^{+} gated cells (Figure 2B), whereas λ5 expression alone or in conjunction with VpreB could not induce this increase. Quantification by flow cytometry demonstrated however that the increase in GC B cell numbers was not significant between wild-type and VpreB transgenic mice (Figure 2C). Concomitantly we observed in VpreB^{int} mice a near-significant (p=0.07) increase in splenic IgG plasma cell numbers, using flow cytometry (Figure 2D). These findings demonstrate that VpreB expression in mature B cells may facilitate GC formation and IgG plasma cell production, whereas λ5 expression can even preclude this spontaneous B cell differentiation.

VpreB^{+} B cells do not aggravate autoimmunity and ameliorate arthritis

As VpreB^{int} mice displayed a trend towards autonomous GC formation and enhanced IgG plasma cell formation in the absence of infection or immunization, we explored whether VpreB expression in mature B cells may facilitate the activation of self-reactive B cells. We examined the spontaneous development of autoimmunity in VpreB^{int} transgenic mice on a pure c57bl/6 genetic background by screening serum from aging mice for auto-antibodies. Using ELISA we could not detect SLE-associated auto-antibodies, including IgG anti-nucleosome antibodies, in VpreB^{int} transgenic mice nor in any other SLC transgenic strain (Figure 3A). Further screening for auto-antibody production by HEp2 autoreactivity assays did not reveal any anti-nuclear or anti-cytoplasmatic auto-antibody production in aging SLC transgenic mice (data not shown).

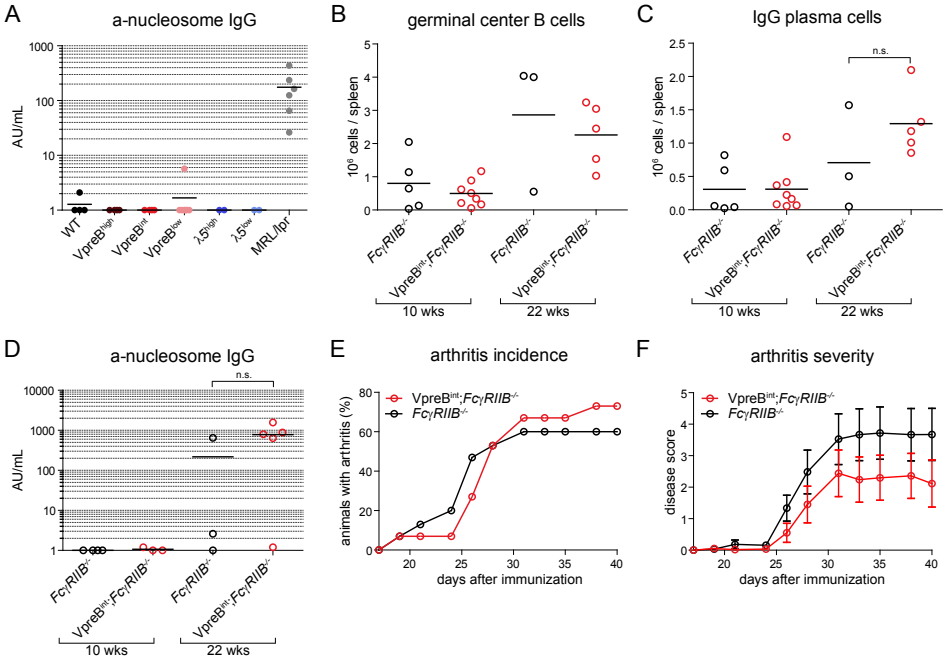
To investigate whether moderate VpreB expression in mature B cells could accelerate auto-immunity in SLE-prone mice, we bred VpreB^{int} mice onto the *FcγRIIB*$^{-/-}$ background, which predisposes c57bl/6 mice to lethal SLE (20). In young (10 weeks) or aging (22 weeks) VpreB^{int};*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice we could not detect enhanced GC formation (Figure 3B) or IgG plasma cell formation (Figure 3C) using flow cytometry. Evaluation of IgG anti-nucleosome auto-antibody levels in serum showed a non-significant

Figure 2. B cell activation and terminal B cell differentiation in SLC transgenic mice.



(A) Expression of activation markers CD86, CD69 and CD25 on splenic CD19+ cells in young (10-14 weeks old) VpreB and λ5 transgenic mice versus wild-type (WT) mice as determined by flow cytometry. Representative data of 3 independent experiments are shown. (B) Flow cytometric identification of CD95+PNA+ germinal center B cells among gated CD19+ splenocytes in wildtype (WT), VpreB and λ5 transgenic mice (10-14 weeks of age). Representative data of at least 2 independent experiments are shown. (C and D) Flow cytometric quantification of germinal center (GC) B cells and IgG plasma cells in spleens of young SLC transgenic (10-14 weeks old). GC B cells were identified as CD19+CD95+PNA+ cells and IgG plasma cells as CD138high cells with high cytoplasmic expression of either IgG1, IgG2 or IgG3. Collective data of more than 2 independent experiments are shown.

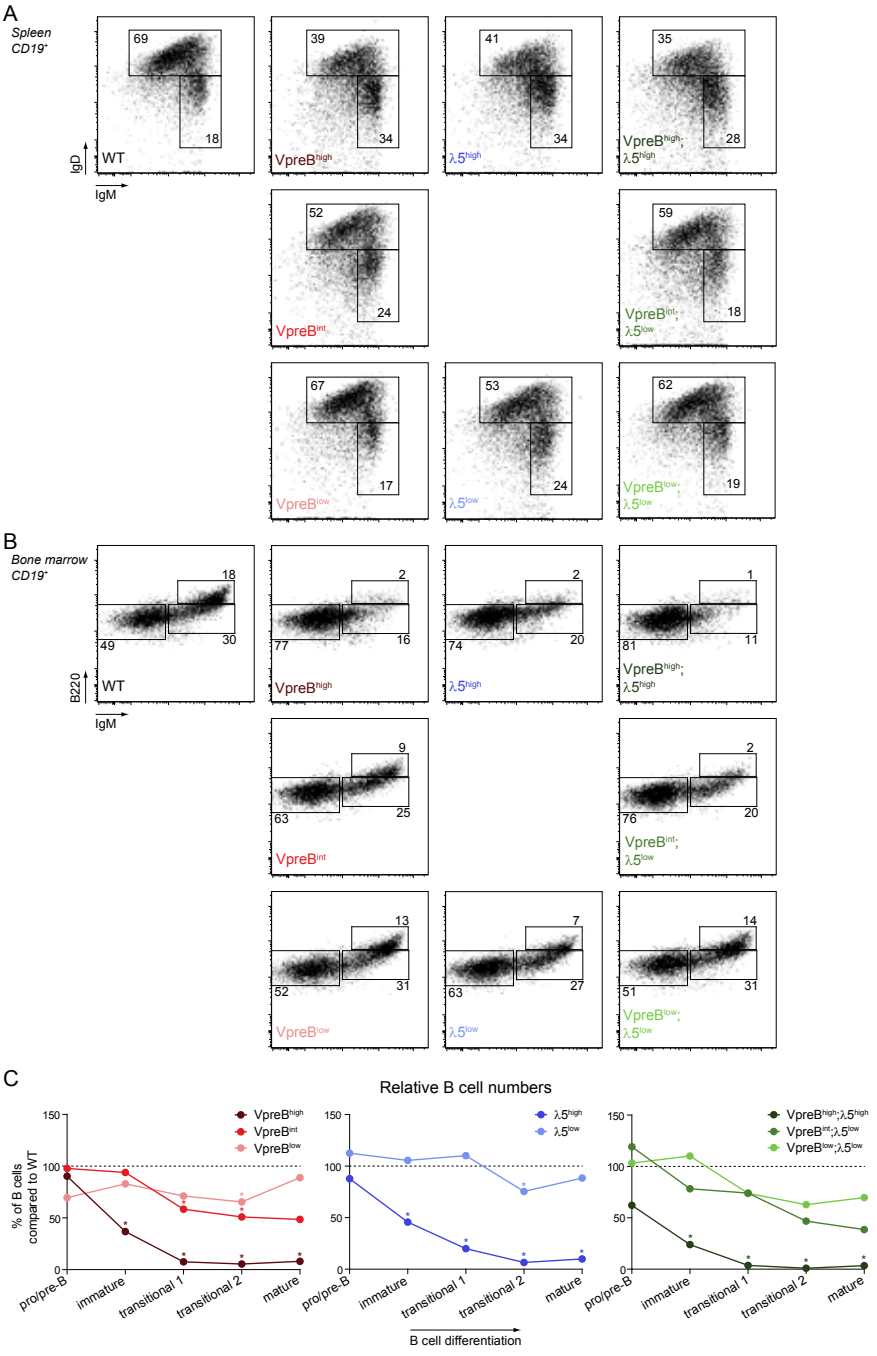
Figure 3. No spontaneous or enhanced autoimmunity in SLC transgenic mice.



(A) Serum IgG anti-nucleosome levels were determined in wildtype (WT), SLC transgenic mice (all 22 weeks of age) and diseased MRL/lpr mice by ELISA. (B and C) Splenic germinal center B cells and IgG plasma cells in young (10 weeks old) and aging (22 weeks old) VpreBint transgenic versus non-transgenic littermates on the FcγRIIB-/- background were quantified as described in Figure 2C and 2D. (D) ELISA measurement of IgG anti-nucleosome antibody levels in serum from young and aging VpreBint transgenic versus non-transgenic littermates on the FcγRIIB-/- background. (E) Proportion of animals with signs of arthritis and (F) arthritis scores in young (10-14 weeks) VpreBint;FcγRIIB-/- and non-transgenic FcγRIIB-/- littermates monitored after immunization against chicken collagen type II (CII) on day0 and day 21. Data from 1 out of 2 independent experiments are shown; n=15 mice per group.

(p=0.18) trend towards higher auto-antibody levels in aging VpreB^{int};*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice (Figure 3D). Likewise, no significant increase in auto-antibody production could be observed in aging c57bl/6 Eμ-Bcl2 mice when a VpreB^{int} or VpreB^{low} transgene was introduced (Figure S2 and data not shown).

Figure 4. SLC dose-dependent clonal deletion of immature and transitional B cells.



➡
(A) B cell maturation in young (10-14 weeks) wildtype (WT) and SLC transenic mice was assessed by flow cytometric characterization of IgM and IgD expression on splenic CD19+ cells. Numbers indicate the percentage of B cells in quadrant gates. Results of 1 representative out of at least 2 independent experiments are shown. (B) Pro- and pre-B cells (B220lowIgMneg), immature B cells (B220lowIgMpos) and mature recirculating B cells (B220highIgMhigh) were identified by flow cytometry in bone marrow from young (10-14 weeks old) wildtype (WT) mice and SLC transgenic littermates. Numbers correspond to B cell percentages within the gates. Data from 1 out of at least 2 independent experiments are shown. (C) Relative numbers of pro/pre-B cells (bone marrow CD19+B220lowIgMneg cells), immature B cells (bone marrow CD19+B220lowIgMpos cells), transitional 1 (T1) B cells (splenic CD19+CD93+IgMhighCD23low cells), transitional 2 (T2) B cells (splenic CD19+CD93+IgMhighCD23high cells) and mature B cells (splenic CD19+IgDhigh cells) were quantified using flow cytometry in young (10-14 weeks old) SLC transgenic versus non-transgenic littermates. Asterisks indicate significant reductions ($p<0.05$) in B cell numbers, compared with wild-type mice. Collective data from 3 independent experiments are shown.

While SLC expression in mature B cells did not induce spontaneous arthritis in SLC transgenic strains, the enrichment of SLC^+LC^+ cells in inflamed joints of RA patients (1) prompted us to examine arthritis development in SLC transgenic mice. Since $VpreB^{int}$ mice exhibited a propensity to spontaneous GC and IgG plasma cell formation (Figure 2C and 2D) and a trend towards more auto-antibody production on the auto-immune prone *FcγRIIB*$^{-/-}$ background (Figure 3D), we provoked auto-immune arthritis in $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice using a collagen-induced arthritis (CIA) protocol (16). After the secondary immunization with chicken collagen type II (CII) on day 21, we observed a similar rise in arthritis incidence in both groups culminating in ~73% of $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus ~60% of *FcγRIIB*$^{-/-}$ mice with arthritis symptoms at day 40 (Figure 3E). Importantly however the severity of arthritis in $VpreB^{int}$;*FcγRIIB*$^{-/-}$ mice mas markedly reduced compared to *FcγRIIB*$^{-/-}$ mice (Figure 3F). At day 40 the overall arthritis disease in groups of $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice was 3.7 versus 2.1 respectively ($p=0.17$, Figure 3F) while the score of diseased $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice was 6.2 versus 4.0 respectively ($p=0.08$).

Taken together, these findings indicate that ectopic expression of SLC components in mature B cells cannot provoke or enhance systemic auto-immunity but rather alleviates autoimmune symptoms in auto-immune prone mice.

SLC expression induces clonal deletion in developing B cells

The SLC chain has the unique capacity to signal autonomously through the non-immunoglobulin tail of λ5 (7, 8, 11). It may therefore lower BCR membrane expression on mature B cells by inducing enhanced BCR internalization and thereby preclude the development of strong autoimmunity. We therefore examined IgD and IgM expression levels on the surface of SLC transgenic splenic B cells, but failed to detect a clear SLC-induced decrease in IgM or IgD expression (Figure 4A). However, correlating with increasing levels of transgenic VpreB and/or λ5 expression a relative decrease in IgD^{high} mature splenic B cells was observed in SLC transgenic mice (Figure 4A), suggestive of a loss of B cells during final B cell maturation stages.

A loss of B cells at the immature B cell stage has been previously described in high-level SLC-transgenic mice and may reflect aspecific toxicity effects of high-level SLC expression at this developmental stage (13). It is, however, conceivable that the loss of B cells could reflect a more stringent clonal deletion of

developing auto- or polyreactive peripheral B cells. We therefore investigated whether such elimination of B cells would occur in a dose-dependent fashion at known autoreactivity checkpoints during B cell development. Flow cytometric analysis of bone marrow B cells confirmed a SLC dose-dependent relative reduction of immature ($B220^{low}IgM^{pos}$) and recirculating mature ($B220^{high}IgM^{high}$) B cells (Figure 4B). Quantification of B cell numbers throughout B cell differentiation in the SLC transgenic strains showed that no loss of B cells occurred before the immature B cell stage in the bone marrow. We noted a SLC dose-dependent reduction of absolute B cell numbers at the immature B cell stage in the bone marrow and at transitional B cell stages in the spleen (Figure 4C). While in transgenic mice expressing high levels of VpreB and/or λ5 a strong and significant reduction of B cell numbers already occurred at the immature B cell stage, this B cell loss was only noticeable during splenic transitional B cell stages in transgenic mice expressing intermediate or low VpreB and/or λ5 levels. In these analyses, the B cell reduction in most SLC transgenic strains was less prominent at the mature B cell stage compared to transitional B cell stages, possibly reflecting an compensatory enhanced survival of fully mature B cells to maintain peripheral B cell numbers.

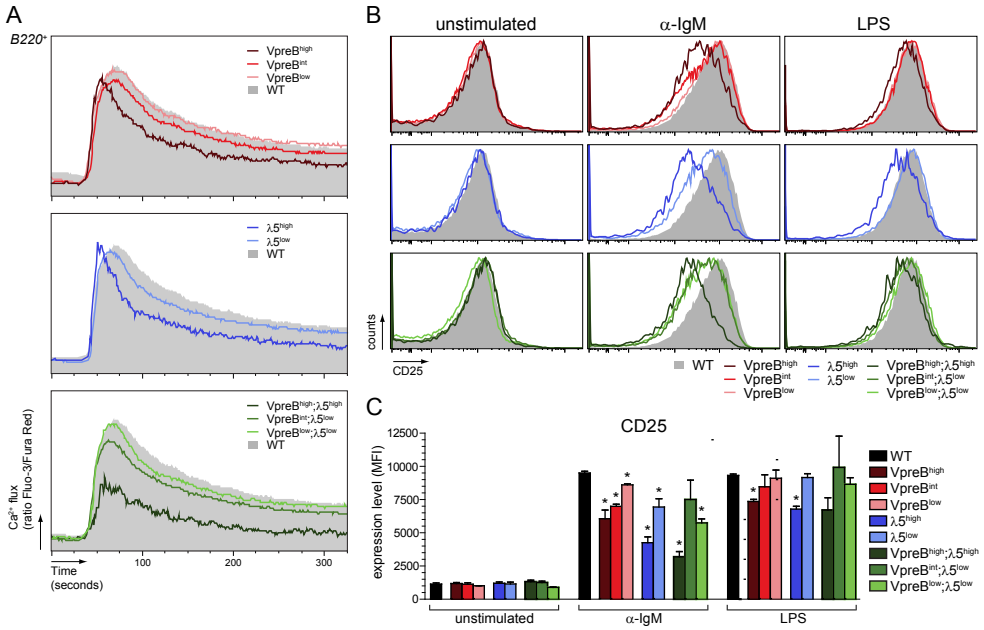
To test whether next to clonal deletion SLC signaling in immature B cells could also enforce receptor editing in the bone marrow (21), we explored Igλ usage as a read-out of receptor editing. Using flow cytometry we could not detect a significant increase in Igλ usage of by peripheral B cells in any of the SLC transgenic lines (Figure S3A and S3B), arguing against enhanced receptor editing instructed by SLC signaling.

SLC expression in mature B cells instructs BCR unresponsiveness in a dose-dependent fashion

The deletion of auto- and polyreactive immature and transitional B cells strongly depends on activating BCR signals (22, 23). The gradual deletion of immature and transitional B cells in SLC transgenic mice prompted us to investigate the BCR signaling quality in SLC transgenic B cells. Using a Ca^{2+} mobilization assay we determined the BCR signaling strength of SLC transgenic B cells upon α-IgM stimulation. We noted a reduction in Ca^{2+} influx in SLC transgenic splenic B cells tightly correlating to the dose of VpreB and/or λ5 expression in these cells (Figure 5A). Although single-transgenic B cells exhibited an apparently normal initial Ca^{2+} influx upon BCR stimulation, the decay in cytoplasmic Ca^{2+} levels was faster than in wild-type B cells. High-level expression of both VpreB and λ5 invoked both a reduced initial Ca^{2+} influx and a quick loss of cytoplasmic Ca^{2+}.

This reduced Ca^{2+} mobilization in SLC transgenic B cells strongly resembles the BCR signaling characteristics of anergic B cells. First we tried using flow cytometry to identify anergic B cell populations in SLC transgenic mice *in vivo* by the expression of markers as CD93, CD23 and CD138, but we were unable to detect an increase in previously described anergic B cell fractions (data not shown) (24, 25). We next tested whether the expression of SLC on mature B cells renders them selectively unresponsive to BCR signals by comparing the activation of SLC transgenic B cells after BCR versus TLR stimulation. While enforced SLC expression could not induce spontaneous upregulation of activation marker CD25 on unstimulated B cells *in vitro*, CD25 upregulation was significantly reduced on α-IgM stimulated SLC transgenic B cells compared to wildtype B cells (Figure5B and 5C). Again, VpreB and/or λ5 expression levels inversely correlated with B cell activation. Importantly, the reduced upregulation of CD25 in

Figure 5. Dose-dependent induction of anergy by SLC expression.



(A) Measurement of Ca2+ mobilization in B220+ gated splenocytes loaded with Fluo-3 and Fura Red ™ from wildtype (WT) versus SLC transgenic mice after stimulation with 20 µg/mL F(ab')2 anti-IgM. Measurements were normalized for maximum Ca2+ influx upon ionomycin stimulation (2 µg/mL). (B) Flow cytometric evaluation of CD25 membrane expression after F(ab')2 anti-IgM (10 µg/mL) or LPS (5 µg/mL) stimulation of MACS-purified splenic B cells from SLC transgenic mice and non-transgenic littermates. (C) Quantification by mean fluorescence intensity (MFI) of CD25 membrane expression levels as measured in the experiment of Figure 5B. Per group at least 3 mice were analyzed. Error bars represent standard deviations; asterisks indicate MFI levels significanty different from wildtype MFI levels ($p<0.05$).

response to BCR stimulation was not resulting from a general unresponsiveness to B cell activating signals since B cells expressing low or intermediate levels of VpreB and/or λ5 showed normal CD25 upregulation (Figure 5B and 5C). A small but significant reduction in CD25 upregulation was observed in VpreB^{high} and λ5^{high} B cells upon LPS stimulation, but this reduction was not as large as observed in these cells upon α-IgM stimulation.

Taken together, these results demonstrate that continued SLC expression beyond the pre-B cell stage in a dose-dependent mode instructs deletion of developing immature and transitional B cells and anergy of surviving mature B cells.

Discussion

Over a decade ago, human SLC expressing mature B cells were described and readily associated with

autoimmunity due to their abundance in arthritic joints and their frequent expression of autoreactive BCRs (1, 2). Since their discovery no studies have been performed that address whether SLC^+ mature B cells may actively drive autoimmune disease. Here we report that in mice transgenically expressing SLC in mature B cells no enhanced or spontaneous autoimmunity could be observed on multiple genetic backgrounds despite mild spontaneous activation of SLC^+ B cells. Instead, we found that SLC expression in mature B cells partially protects mice from autoimmune arthritis. This protective role of SLC re-expression can be attributed to a more stringent clonal deletion of immature and transitional B cells as well as induction of anergy in mature B cells.

Although SLC re-expression has been reported in splenic murine germinal center B cells upon their activation during a T-dependent antibody response (3), the role of SLC^+ mature B cells in autoimmunity has not been previously studied in murine models. This may be attributed to the very low frequencies of naturally occurring SLC^+ mature B cells. Despite our finding in mRNA deep sequencing experiments that *Vpreb3* transcription is upregulated upon BCR activation of splenic B cells *in vitro*, we were unable to detect considerable frequencies of $VpreB^+$ wildtype B cells in lymphoid organs that typically contain germinal centers, including Peyer's patches or mesenteric lymph nodes (data not shown). This discrepancy between our and previous studies (3) in the identification of SLC^+ mature B cells *in vivo* in wild-type mice may be explained by differences in (i) antigen dose and thus BCR stimulation, in (ii) the use of alum adjuvant, or (iii) in anatomical localization of antigen-activated B cells, leading to altered B cell engagement and therefore SLC re-expression in these different T-dependent responses.

Given the selective conditions that permit SLC re-expression in only a limited number of B cells in wildtype mice, we employed transgenic mice that express various doses of the SLC components VpreB and λ5 in B cells beyond the pre-B cell stage to study a possible contribution of the SLC to the development of autoimmunity. Our studies in SLC transgenic mice show enforced SLC expression on mature B cells induces mild B cell activation *in vivo* that in a dose-dependent fashion, whereas GC formation and IgG plasma cell differentiation and are not enhanced. Only in mice expressing moderate levels of VpreB in mature B cells ($VpreB^{int}$) we observed a discernible, but non-significant, increase in GC B cells and IgG plasma cells. The absence of spontaneous GCs in mice co-expressing VpreB and λ5 indicates that λ5 signaling prohibits a GC differentiation program in activated $VpreB^{int}$ B cells. Differences in B cell fate between activated mature B cells expressing either VpreB or λ5 may be explained by differences in the BCR signals provided to these cells, and indeed an extensive body of literature exists that links BCR signaling strength to B cell differentiation fate (26-29). However, in our models the absence of clear differences between VpreB or λ5 transgenic B cells in Ca^{2+} influx and activation marker upregulation in response to in vitro α-IgM stimulation contradicts a difference in BCR signaling quality as the primary cause of differences in GC formation.

To explain these differences in B cell fate between VpreB versus λ5 transgenic mice alternative causes must be considered, including the possibility that not λ5 but VpreB may alter terminal B cell differentiation by manipulating antigen-based B cell selection through allelic inclusion, a phenomenon that may rescue B cells with irrelevant BCR specificities. (30, 31). A prerequisite for allelic exclusion would be the stable membrane expression of either SLC component. Although our studies show that neither VpreB nor λ5 can be individually expressed at the cell membrane at high levels, previous

reports demonstrated that VpreB had a better IgH chain pairing capacity (19) and thus possibly a greater capacity to establish allelic inclusion. In naturally occurring dual light chain expressing B cells, the failing allelic exclusion of one of the two light chains is associated with very similar expression levels of the two light chains, thereby effectively reducing the signaling strength of either BCR by twofold.

If transgenic expression of SLC components would mimic allelic exclusion, one would expect allelic exclusion to be effective only if comparable membrane expression levels of SLC components and IgL are being achieved. This is not the case, based on the low membrane VpreB and λ5 levels on SLC transgenic cells (Figure 1A) while normal Igκ and Igλ membrane levels are being observed on these cells (Figure S3). However, the intrinsic autonomous signaling capacity of SLC components may compensate for low membrane expression levels, establishing a state of BCR signaling compatible with allelic inclusion while SLC components and LCs are unequally expressed.

Despite the spontaneous formation of germinal centers in VpreB^{int} mice in the absence of any challenge with exogenous antigen, we could not detect any spontaneous autoimmunity on the relatively autoimmune-resistant c57bl/6 background nor any aggravation of autoimmunity when moderate VpreB expression was introduced on autoimmune-susceptible genetic backgrounds (Figure 3A-D). In contrast, autoimmune arthritis proved to be less severe in VpreB^{int} transgenic *FcγRIIB*$^{-/-}$ mice compared with non-transgenic littermates, demonstrating a protective role of VpreB re-expression in autoimmune arthritis. It may be argued that this protection could be solely conferred by the approximate two-fold reduction in total IgD^{+} B cell numbers in VpreB^{int} mice. The deletion would then not have been random, but rather specifically affecting developing B cells with a polyreactive or self-reactive BCR in SLC transgenic mice, thereby establishing a more robustly purged mature B cell repertoire. The elimination of collagen type II-reactive B cells in VpreB^{int} mice is probably incomplete, since equal proportion of these mice and non-transgenic littermates develop arthritis symptoms. An additional mechanism that suppresses the pathogenicity of such cells is provided by our finding that SLC-component expressing B cells are anergic as evident from reduced Ca^{2+} mobilization and CD25 upregulation upon α-IgM but not LPS stimulation. Since anergy can only be induced by chronic BCR occupation (32), the anergic response of mature SLC transgenic B cells demonstrates the continuous signals provided by SLC components to these cells. Although our analyses on BCR-induced Ca^{2+} influx only include peripheral B cells, the SLC signals autonomously and therefore this chronic BCR signaling most likely is the driving force behind the enhanced deletion of immature and transitional B cells.

The finding that especially VpreB^{int} transgenic mice seem predisposed to spontaneous GC and IgG plasma cell formation is striking, since these cells most closely resemble human peripheral SLC^{+}LC^{+} B cells that are reported to express hardly any λ5 and VpreB levels that are lower than those in pro- and pre-B cells (1). In addition to moderate VpreB expression, human SLC^{+}LC^{+} B cells also re-express early B cell genes as TdT and RAG at low levels, suggesting that SLC signaling may launch a transcriptional program to revise their (autoreactive) BCRs (33). In accordance with this view, in human the IgH and IgL chains of SLC^{+}LC^{+} B cells frequently display characteristics of extended receptor editing, such as more distal J gene usage and D-D fusions (33). Such receptor revisions could abolish the pathogenic potential of autoreactive SLC^{+}LC^{+} B cells, suggesting that SLC re-expression could be a mechanism to induce tolerance in mature B cells, either through the previously suggested induction of receptor editing,

or by anergy and clonal deletion. Although our results in murine transgenic models may be difficult to directly extrapolate to the pathogenesis of human rheumatic diseases, our finding that moderate VpreB expression ameliorates rather than aggravates autoimmunity supports the idea that the largely autoreactive pool of $SLC^{+}LC^{+}$ B cells found in arthritic joints does not actively contribute to autoimmunity and therefore is not an eligible target in the treatment for autoimmune disease.

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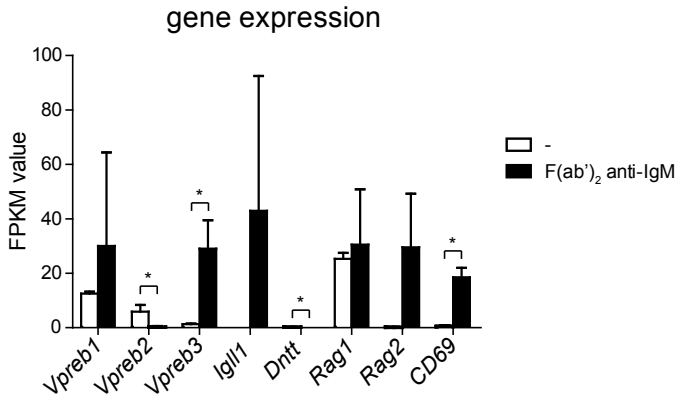
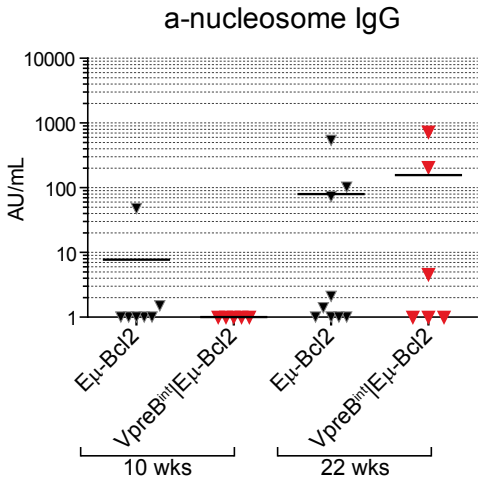
Figure S1. Expression of SLC components in BCR stimulated splenic B cells.



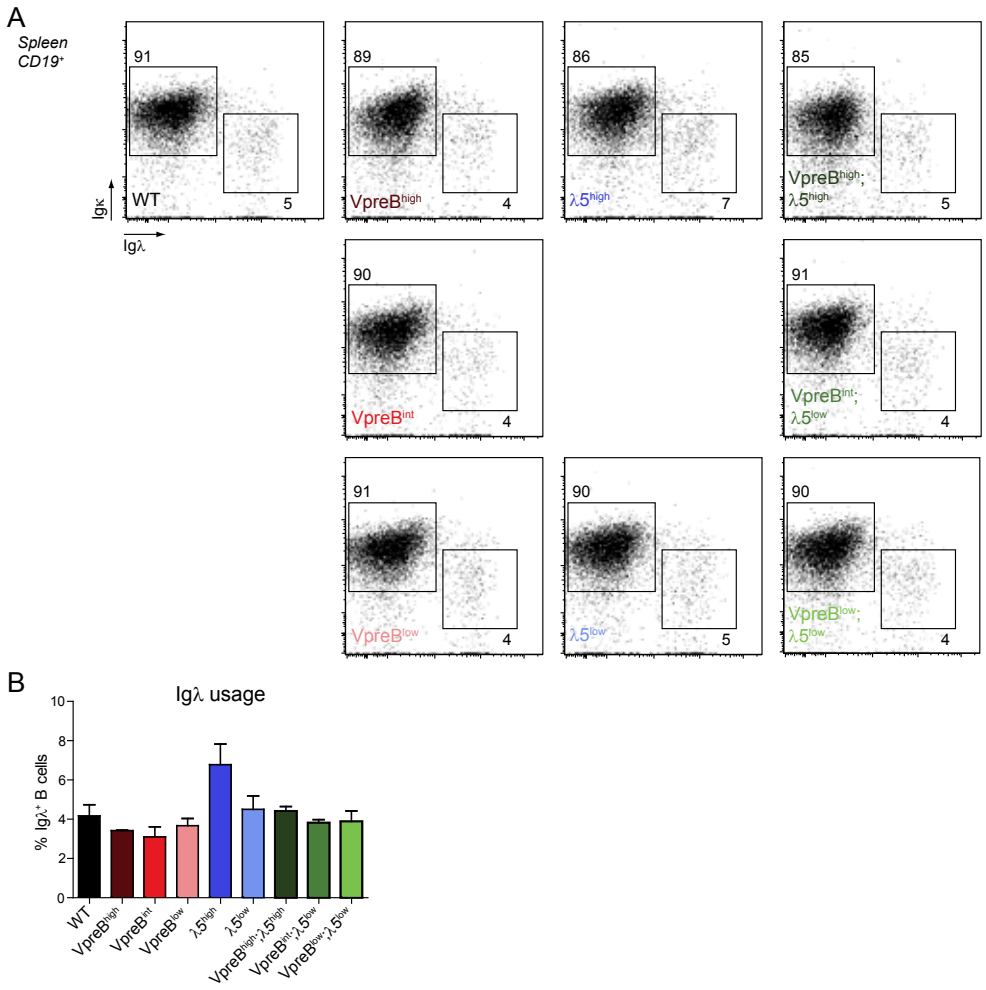
MACS-purified naïve splenic B cells ($CD19^+CD43^-CD5^-CD138^-$) from 4 wild-type mice were cultured for 12 hours with 10 µg/mL $F(ab')_2$ anti-IgM (black bars) or left unstimulated (white bars). Transcript levels in these cultured cells are shown for the indicated genes as determined by total mRNA deep sequencing analysis; error bars represent standard errors of the mean. Asterisks indicate significant differences ($p<0.05$) in gene expression. FPKM: fragments per kilobase of a transcript per million mapped reads.

Figure S2. No enhanced autoantibody production in aging $VpreB^{int}$;Eµ-Bcl2 mice.



Quantification by ELISA of serum IgG anti-nucleosome autoantibodies in young (10 weeks old) and aging (22 weeks old) $VpreB^{int}$ versus non-transgenic littermates on the Eµ-Bcl2 genetic background.

Figure S3. No increased Igλ usage by peripheral SLC transgenic B cells.



(A) Relative frequencies of Igκ+ and Igλ+ cells among CD19+ gated splenic B cells as determined by flow cytometry. Numbers indicate the percentage of cells within the indicated gates. (B) Summarized data of the flow cytometric analysis in (A). Error bars indicate standard deviations; >3 mice per group were analyzed.

3

Constitutive Activation of Bruton's Tyrosine Kinase Induces the Formation of Autoreactive IgM Plasma Cells

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Abstract

B cell receptor (BCR)-mediated signals provide the basis for B-cell differentiation in the bone marrow and subsequently into follicular, marginal zone (MZ) or B-1 B-cell subsets. We have previously shown that B-cell specific expression of the constitutive active E41K mutant of the BCR-associated molecule Bruton's tyrosine kinase (Btk) leads to an almost complete deletion of immature B cells in the bone marrow. Here, we report that low-level expression of the E41K or E41K-Y223F Btk mutants was associated with reduced follicular B cell numbers and significantly increased proportions of B-1 cells in the spleen. Crosses with 3-83md and VH81X BCR transgenic mice showed that constitutive active Btk expression did not change follicular, MZ or B-1 B cell fate choice, but resulted in selective expansion or survival of B-1 cells. Residual B cells were hyperresponsive and manifested sustained Ca^{2+} mobilization. They were spontaneously driven into germinal center-independent plasma cell differentiation, as evidenced by increased numbers of IgM^+ plasma cells in spleen and bone marrow and significantly elevated serum IgM. Because anti-nucleosome autoantibodies and glomerular IgM deposition were present, we conclude that constitutive Btk activation causes defective B cell tolerance, emphasizing that Btk signals are essential for appropriate regulation of B cell activation.

Published in Eur J Immunol. 2010 Sep;40(9):2643-54

Introduction

Signals transmitted by the B cell receptor (BCR) control the response of B cells to cognate antigen and are also essential regulators of survival, tolerance and differentiation (For review: (1, 2)). Inducible and stage-specific targeting experiments demonstrated that mature B cells undergo apoptosis upon in vivo BCR ablation or mutation of one of its signaling units, Ig-a, and consequently disappear from the circulation (3, 4). A critical survival signal is provided by phosphoinositide 3-kinase (PI3K) signals (5), but how this signaling is initiated in resting mature B cells in not fully understood. BCR signal strength is also a key factor in deciding between the three functionally distinct mature B cell compartments of follicular, marginal zone (MZ) and B-1 B cells. Increases in BCR signaling strength, induced by low-dose self-antigen, directs maturation of naive immature B cells from the follicular into the B-1 or MZ B cell fate (6, 7).

In mature B cells BCR engagement by antigen induces phosphorylation of Ig-a and Ig-b and the formation of a lipid raft associated calcium-signaling module. This complex consists of multiple signaling proteins including the protein tyrosine kinases Lyn, Syk and Btk (Bruton's tyrosine kinase), the adapter molecule Slp65 and the enzymes PI3K and phospholipase Cg2 (Plcg2). In this complex Syk phosphorylates Slp65, thereby providing docking sites for Btk and Plcg2. Activation of Plcg2 by Btk results in the generation of the Ca^{2+}-releasing factors inositol-1,4,5-trisphosphate and diacylglycerol (for review: (8, 9)). During these events various co-receptors modulate BCR signaling either positively or negatively (10).

Deficiencies of BCR signaling molecules, such as Btk, Slp65, or Plcg2 or the excitatory co-receptor CD19 result in a hyporesponsive phenotype, mainly characterized by defects in the maturation of splenic follicular B cells, impaired MZ B cell survival, absence of $CD5^+$ B-1 B cells and impaired T cell independent antibody responses (11). Conversely, a complex B cell phenotype characterized by reduced numbers of follicular B cells, elevated numbers of B-1 B cells and to some extent MZ B cells, B cell hyper-responsiveness and auto-antibody formation is found in various genetic changes that increase BCR signaling. These include gain-of-function mutants of Lyn or Plcg2, deficiency for PTEN, a lipid phosphatase that antagonizes PI3K activity, overexpression of CD19, or mutations that disable inhibitory signaling of membrane receptors such as CD22, Pir-B, Siglec-G and FcgRIIB or their downstream signaling molecules Shp1 or Ship (12-21).

Btk is a member of the Tec protein tyrosine kinase family that mediates many aspects of B cell development, survival and function (8, 22). Whereas in humans mutations in Btk cause a severe arrest of B cell development at the pre-B cell stage leading to X-linked agammaglobulinemia, in the mouse there is only a mild pre-B cell defect, differentiation of transitional into mature peripheral B cells is impaired and B-1 cells are lacking (23-25). The pleckstrin homology domain mutant E41K-Btk displayed robust transformation potential in a soft-agar assay, increased membrane localization and phosphorylation in quiescent cells, independent of PI3K activity (26). This capacity was augmented by Y223F mutation of the main autophosphorylation site in the SH3 domain, although the role of Y223 phosphorylation for Btk function in vivo remains unclear (22, 27). We have previously reported that expression of transgenic E41K-Btk throughout the B cell lineage resulted in an almost complete deletion of immature B cells in

the bone marrow (BM), irrespective of the presence of the endogenous intact Btk gene (28). Immature B cells were arrested at the progression from IgM^{low} into IgM^{high} cells, reflecting the first immune tolerance checkpoint at which autoreactive B cells become susceptible to apoptosis, and as a result peripheral mature B cell pool was reduced to <1% of the normal size. This phenotype is in marked contrast with that of other mouse models with increased BCR signaling (12-19), which were mainly characterized by B cell hyperresponsiveness, enhanced B-1 cell differentiation and autoimmunity. In our transgenic mice the expression levels of mutated E41K-Btk were in the same range as the endogenous, unmutated Btk. As it is expected that even small amounts of activated Btk will affect B cell development, we decided to study the effects of lower levels of constitutive active Btk expression.

Here we report the phenotype of mice harboring low copy numbers of E41K-Btk (E-Btk) and E41K-Y223F-Btk (EY-Btk) transgenes, the expression of which was driven by the B-cell specific CD19 promoter. We found that low-level expression of these constitutive active Btk mutants was associated with a reduction of follicular B cells and an increase in the proportions of B-1 cells. Residual B cells were hyperresponsive, resulting in their efficient differentiation into autoreactive IgM plasma cells. Our findings show that expression of constitutive active Btk did not change B cell fate choice, but rather resulted in selective expansion or survival of B-1 B cells.

Materials & methods

Mice and Genotyping

Btk-deficient, Slp65-deficient, VH81X or 3-83md transgenic mice have been described (8, 24, 29, 30). We previously reported the generation of CD19-driven E41K-Btk and E41K-Y223F-Btk transgenic mice (28, 31). Additional low-copy number transgenic mice on the FVB background were generated using the same constructs and crossed onto the Btk-deficient background (28, 31). In single experiments transgenic mice were analyzed together with non-transgenic littermates at the age of 8-16 weeks. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen free conditions. Experimental protocols were reviewed and approved by the Erasmus MC Committee of animal experiments.

Flow cytometric analyses

Preparations of single-cell suspensions, flow cytometry and Ca^{2+} measurements upon anti-IgM F(ab)2 stimulation have been described previously (25, 32). The 35-1 anti-idiotype antibody was kindly provided by J.F. Kearney (University of Alabama, Birmingham, USA) and the 54-1 anti-3-83md hybridoma by D. Nemazee (The Scripps Research Institute, La Jolla, USA). 1-5x10^5 Events were scored using a FACSCalibur flow cytometer and analyzed using CellQuest (BD Biosciences, Mountain View, USA) or FlowJo (Tree Star, Ashland, OR) software. To calculate relative Btk expression median fluorescence intensities (MFI) values of $Btk^{-/-}$ cells were used as background and those in wild-type cells were set to one, as previously described (27).

Immunohistochemistry

Tissues were embedded in OCT compound; frozen 6 mm cryostat sections were fixed in acetone and processed as previously described (28).

In vivo immunizations and Ig detection

Primary and secondary T-cell dependent (TD) immune responses were measured both after primary i.p. immunization (100 mg TNP-KLH in alum) and after i.p. booster immunization (100 mg TNP-KLH in PBS). Booster doses were given after 4-5 weeks. T-cell independent type II (TI-II) responses were analyzed after i.p. injection of 50 mg TNP-Ficoll. TNP-specific and total Ig subclass ELISA assays were performed as described (32).

Levels of anti-nucleosome antibodies in serum were measured by ELISA using coated oligonucleosomes as antigen and peroxidase-coupled anti-mouse Ig isotype-specific antibodies for subsequent detection. Serially diluted sera were incubated at r.t. for 2 h, and azino-bis-ethylbenzthiazoline sulfonic acid (Sigma) was used as a substrate.

ELISPOT assays

96-well Multiscreen plates with a mixed cellulose membrane (MAHAN4550; Millipore) were coated overnight at 4°C with 1 mg/ml unlabeled anti-Ig subclass antibodies (BD Pharmingen) and subsequently blocked in PBS / 1% BSA at r.t. for 1 hr. Serial dilutions of splenic cell suspensions were incubated at 37°C for 3h. Production was detected with corresponding biotin-labeled anti-Ig isotype-specific antibodies, streptavidin-peroxidase (BD Pharmingen) and 3-amino-9-ethylcarbazole. Antibody secreting cells (ASC) were counted under the microscope and calculated as number of ASC/100.000 cells.

Statistical Analysis

Statistical significance was calculated using the Mann-Whitney U test.

Results

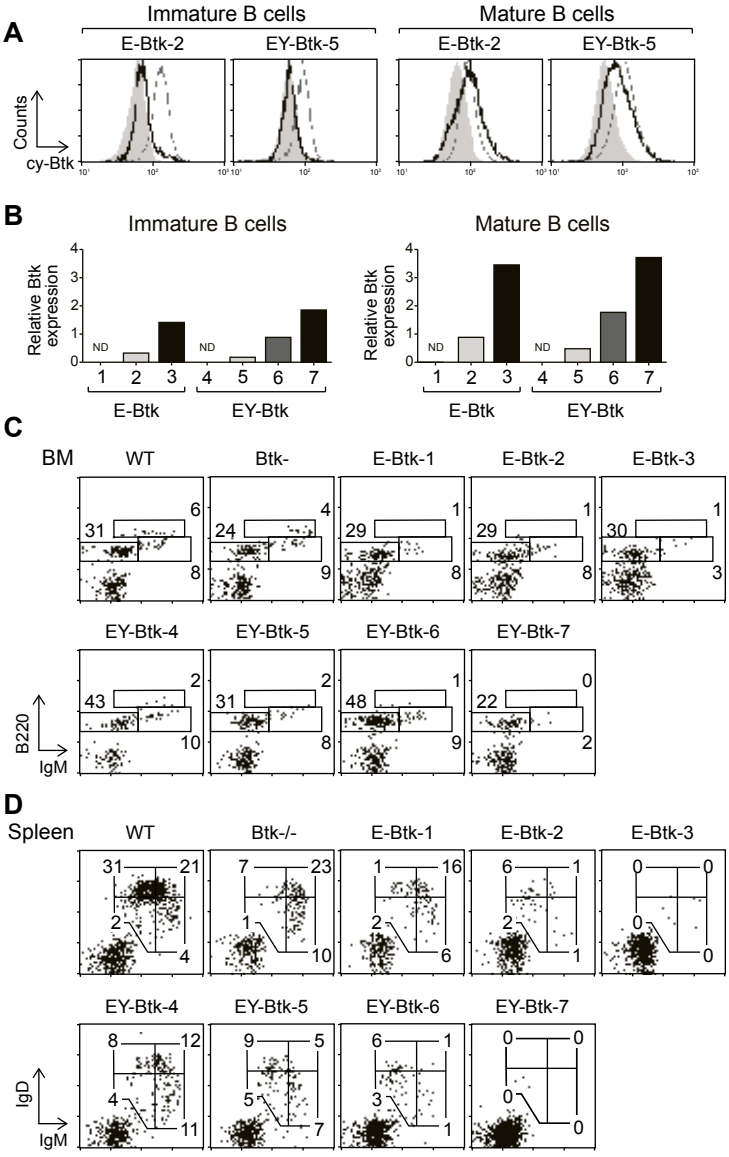
Dose-dependent phenotypes of E-Btk and EY-Btk transgenic mice

To investigate dose-dependent effects constitutive Btk activation, independent transgenic E-Btk single mutant (n=3) and the EY-Btk double mutant (n=4) mouse lines were generated and crossed onto the Btk-deficient background (24).

Expression levels of transgenic Btk were evaluated by intracellular flow cytometry (Figure 1A). Median fluorescence intensities from transgenic mice, wild-type mice and Btk deficient mice were used to calculate the relative Btk expression in the immature and mature B cells in the BM (Figure 1B). Btk expression of the appropriate molecular weight was confirmed by Western blot of B cell-enriched splenic or BM cell suspensions (data not shown). The mouse lines exhibited a wide range of Btk protein expression levels that correlated with the transgene copy numbers, as estimated by Southern blotting analysis of tail DNA (data not shown). Overall, Btk expression increased during B cell development (Figure 1B).

To examine the dose-dependent effects of various E-Btk and EY-Btk expression levels on B cell development, BM and spleen from 8-week old transgenic mice were analyzed by flow cytometry and compared to wild-type and Btk-deficient littermates (Figure 1C). As previously described (23, 24), Btk-deficient mice had a specific defect in $B220^{high}$ mature recirculating cells in the BM and exhibited

Figure 1. Dose-dependent phenotypes of E-Btk and EY-Btk transgenic mice.



(A) Intracellular Btk expression in gated populations of immature B cells ($IgM^+IgD^-B220^+$) and mature B cells ($IgM^+IgD^+B220^{high}$) in BM, displayed as histogram overlays of wild-type (*thin lines*), Btk-deficient (*gray shaded area*) and Btk transgenic mice (*bold line*). (B) Quantification of transgenic Btk expression, using MFI values. MFI values in wild-type mice were set to one. (C) Flow cytometric IgM/B220 profiles of BM lymphoid cells from the indicated mice. Percentages of cells within the indicated gates, pro-pre-B cells (IgM^-B220^+), immature B cells (IgM^+B220^+) and mature B cells (IgM^+B220^{high}), are given. (D0. IgM/IgD profiles of splenic lymphoid cells from the indicated mice. Percentages of cells within the indicated gates are given. Data shown are representative of 4-14 animals within each group.

relatively increased IgM^{high}IgD^{low} transitional B cell fractions with impaired maturation into IgM^{low}IgD^{high} mature follicular B cells in the spleen. We have previously reported that high expression of E41K-Btk (E-Btk-3) resulted in an almost complete arrest of B cell development at the B220^{low}IgM^{low} immature B cell stage in the BM (28). In transgenic lines expressing a lower dose of the E41K-Btk mutant (E-Btk-1 and E-Btk-2) the B220^{low}IgM^{low} immature B cell fractions were less affected, but the fractions of recirculating B220^{high} B cells were still severely reduced (Figure 1C). Accordingly, in the spleen of E-Btk transgenic mice a dose-dependent reduction in the proportions of B cells was observed, whereby residual B cells in E-Btk-2 mice were more mature in terms of IgM/IgD profile than those found in Btk-deficient mice (Figure 1D). For the EY-Btk double mutant transgenic mice a similar dose-dependent phenotype was found.

The severe block of B cell development at the immature B cell stage in the BM of E-Btk-3 transgenic mice was suggestive of clonal deletion. This was confirmed by an *in vivo* kinetic study using the thymidine analogue BrdU, which showed that the absolute numbers of Ig m+ immature B cells generated in the BM were limited and decreased after 24 h (data not shown), indicating a short life span of immature E-Btk-3 Tg B cells.

Taken together, these findings show that low-level expression of the E41K-Btk single or the E41K-Y223F-Btk double mutant resulted in an arrest of B cell development at the immature B cell stage in the BM and subsequently a dose-dependent reduction of peripheral B cells.

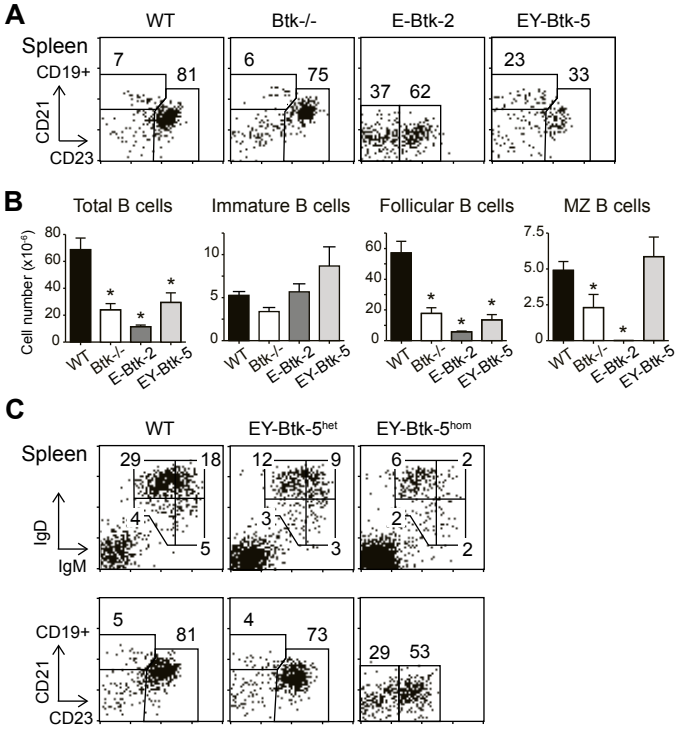
Characterization of the peripheral B cell compartment in E-Btk-2 and EY-Btk-5 transgenic mice

For the remainder of our study we focused on the mouse lines E-Btk-2 and EY-Btk-5, because these lines expressed detectable levels of transgenic Btk, while deletion in the BM was limited (Figure 1C), resulting in splenic B cell numbers that were in the range of Btk-deficient mice (~30 x 10^6 for EY-Btk-5 mice) or markedly lower (~12 x 10^6 for E-Btk-2; compare wild-type mice: ~70 x 10^6 and Btk-deficient mice: ~24 x 10^6; Figure 2B).

Next, we determined the B cell subset composition of spleen, peritoneal cavity and mesenteric lymph nodes (MLN) in E-Btk-2 and EY-Btk-5 transgenic mice, using wild-type and Btk-deficient mice as controls. Analysis of CD21/CD23 profiles of CD19$^+$ B cells demonstrated that Btk-deficient mice had reduced numbers CD21^{int}CD23^{high} follicular and CD21^{high}CD23^{low} MZ B cells in the spleen, when compared with wild-type mice (Figure 2A and 2B). Analysis of the CD21/CD23 profile of E-Btk-2 Tg splenic B cells revealed an apparently normal population of CD21$^-$CD23$^-$ immature B cells, but the follicular B cells were significantly reduced in number and manifested low surface expression of both CD21 and CD23 (Figure 2A and 2B). CD21^{high}CD23^{low} MZ B cells were completely lacking in E-Btk-2 mice. In contrast, EY-Btk-5 Tg mice had reduced numbers of follicular B cells and apparently normal numbers of immature and MZ B cells.

The milder phenotype in EY-Btk-5 transgenic mice, as compared to E-Btk-2 transgenic mice might originate from differential effects of the E41K single and the E41K-Y223F double mutation, or alternatively from the ~2 times higher expression levels of the E-Btk-2 mutant, as compared with EY-Btk-5. To investigate this, we generated mice homozygous for the EY-Btk-5 transgene and analyzed the B cell compartment by flow cytometry. Strikingly, homozygous EY-Btk-5 mice manifested a phenotype

Figure 2. Constitutively active Btk affects B cell subset composition.



(A) Flow cytometric CD23/CD21 profiles of splenic CD19$^+$ cells from the indicated mouse groups. Percentages of cells within the gates for transitional (CD21$^-$CD23$^-$), follicular (CD21$^+$CD23$^+$) and MZ B cells (CD21^{high}CD23^{low}) are given. (B) Quantification of splenic B cell subsets, as identified in (A). Bars represent average values and SEM of 8-14 animals per genotype; significant differences ($p<0,001$) are indicated by asterisks. (C) Homozygous EY-Btk-5 mice have a phenotype resembling that of E-Btk-2 mice. Total lymphoid cells from the spleen of wild-type, heterozygous (het) and homozygous (hom) EY-Btk-5 were analyzed for IgM/IgD expression (*upper part*) and gated CD19$^+$ cells were analyzed for surface CD23/CD21 expression (*lower part*).

reminiscent of that found in E-Btk-2 mice, with severely reduced numbers of B cells, a complete lack of CD21^{high}CD23^{low} MZ B cells and a significant reduction in the numbers of follicular B cells, whereby residual B cells were CD21^{low}CD23^{low} (Figure 2C).

Taken together, these findings show that expression of constitutive active Btk significantly affected B cell differentiation beyond the transitional B cell stage, resulting in reduced numbers of follicular B cells and the absence of MZ B cells in E-Btk-2 transgenic mice and in homozygous EY-Btk-5 transgenic mice.

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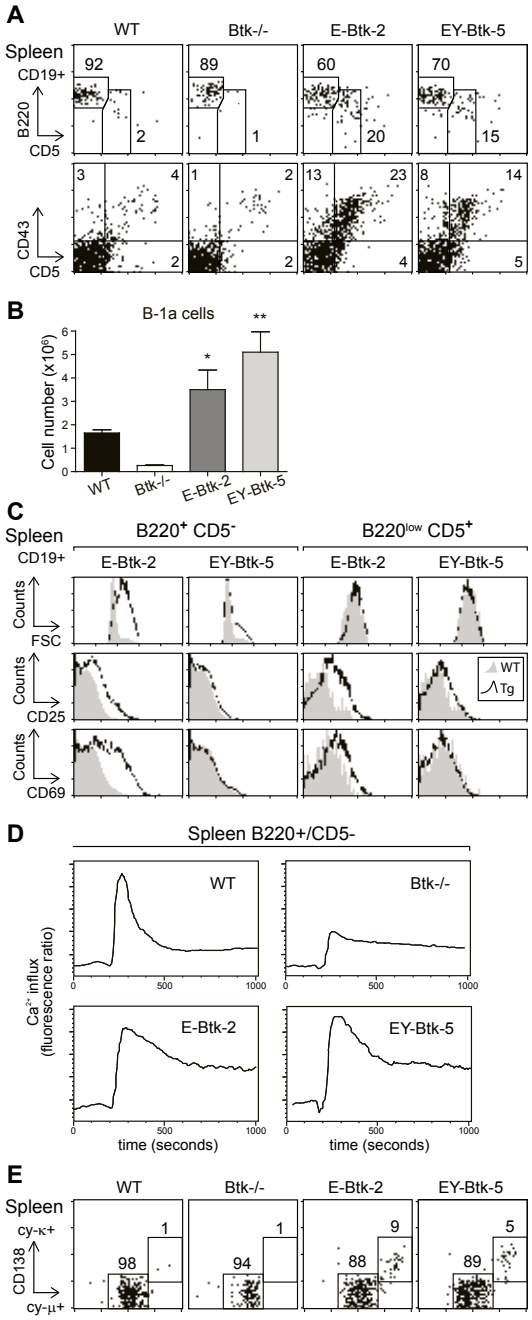
Figure 3. Constitutive active Btk mutant mice have increased proportions of B-1 cells and hyperresponsive B cells.



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(A) Flow cytometric profiles of gated splenic $CD19^+$ cells from the indicated mouse groups. Percentages of cells within the gates are given. (B) Quantification of splenic $CD5^+$ B-1 B cell subsets (average values and SEM), as identified in (A). Significant differences ($p<0,001$) are indicated by asterisks. (C) FSC, CD25 and CD69 expression profiles are displayed as histogram overlays for $B220^+/CD5^-$ cells or $B220^{low}/CD5^+$ cells from transgenic (*bold line*) and wild-type (*shaded area*) mice. (D) The effect of constitutively active mutant Btk on BCR induced Ca^{2+} mobilization. The plots are representative for 3-4 mice of each genotype. (E) CD138/cytoplasmic (cy) Ig m H chain flow cytometric analysis of splenic cytoplasmic k+ cells from the indicated mouse groups. Data are representative for 8-14 (A, B, E) or 3-5 (C,D) animals of each genotype.

-Btk-2 and EY-Btk-5 mice have increased proportions of B-1 cells and hyperresponsive B cells

Because mutant mice with enhanced BCR signaling often show increased numbers of B-1 B cells (12-19), we evaluated the expression of the B-1 associated surface markers CD5 and CD43 in spleen, mesenteric lymph node (MLN) and peritoneal cavity. We identified significant proportions of $B220^{low}CD5^+CD43^+$ B-1 B cells in the spleens of E-Btk-2 and EY-Btk-5 mice, in contrast to spleens of wild-type and Btk-deficient mice which contained only small fractions of B-1 cells or completely lacked B-1 cells, respectively (Figure 3A and 3B).

In MLN of both E-Btk-2 and EY-Btk-5 mice, the proportions of B cells were significantly reduced, whereby $B220^{low}CD5^+CD43^+$ B-1 B cells, which are normally not present in MLN (Suppl. Figure S1A), were prominent. One of the hallmarks of Btk-deficiency is the complete absence of $CD5^+$ B-1 cells in the peritoneal cavity (23, 24) (Suppl. Figure S1B). The proportions of total $CD19^+$ B cells in the peritoneal cavities of the transgenic mice were reduced (E-Btk-2) or normal (EY-Btk-5), but consisted almost exclusively of $CD5^+CD43^+$ B-1 cells (Suppl. Figure S1B), which were $B220^{low}$ and $CD11b^+$ (not shown).

Next, we evaluated cell size and the expression of activation markers on both $B220^+CD5^-$ and $B220^{low}CD5^+$ splenic B cells. B220+$CD5^-$ B cells from E-Btk-2 transgenic mice but not from EY-Btk-5 transgenic mice exhibited significantly higher forward scatter (FSC) values, and elevated expression of the CD25 and CD69 activation markers than those from wild-type mice (Figure 3C). Likewise, $B220^{low}CD5^+$ B-1 B cells from E-Btk-2 mice but not from EY-Btk-5 mice had increased surface CD25 and CD69, when compared to splenic $B220^{low}CD5^+$ B-1a B cells from wild-type mice (Figure 3C).

The hyperresponsive phenotype of Btk transgenic B cells was substantiated by our finding of analyses of sustained Ca^{2+} elevation in response to BCR engagement, when compared with wild-type B cells (Figure 3D). Moreover, increased expression of various activation markers was found when E-Btk-2 and EY-Btk-5 transgenic B cells were cultured in vitro, both in medium and stimulated by anti-IgM or LPS (shown for CD86 in Suppl. Figure S2).

Finally, significant proportions of cytoplasmic Ig L chain positive cells in the spleens of E-Btk-2 and EY-Btk-5 mice were $CD138^+$ and expressed high levels of intracellular Ig m heavy chain, consistent with a plasmablast or plasma cell phenotype (Figure 3E). This was confirmed by immunohistochemistry, which revealed strong IgM staining in the red pulp of E-Btk-2 and EY-Btk-5 transgenic spleens, indicative of IgM^+ plasmablasts or plasma cells (Figure 5B, *left panels*). Double labelings with anti-IgM and MOMA-1 (specific for MZ methallophilic macrophages) revealed in wild-type, Btk-deficient and EY-Btk-5 mice a typical pattern with IgM^+ follicular B cells, surrounded by a rim of MOMA-1^+ cells and outside this

rim MZ B cells (Figure 5B, *left panels*). By contrast, spleens of E-Btk-2 mice contained few methallophilic macrophages with weak MOMA-1 staining and MZ B cells were lacking, consistent with the flow cytometry data (Figure 5B, *left panels*).

In summary, these findings show that residual B cells in E-Btk-2 and EY-Btk-5 mice appeared hyperresponsive, whereby proportions of B-1 B cells and IgM^+ plasmablasts or plasma cells were increased.

E-Btk-2 and EY-Btk-5-mediated effects on B cell subsets are dependent on Slp65

Crosses of E-Btk-2 and EY-Btk-5 mice onto the Btk-Slp65 double-deficient background showed that in the absence of Slp65 the effects of constitutive Btk activation were diminished, as the spleens no longer contained large proportions of $CD5^+$ B-1 lineage cells (Suppl. Figure S2A), therefore the effects of constitutive active Btk expression on the follicular, MZ and B-1 B cell subsets were dependent on Slp65.

Constitutive activation of Btk does not change the follicular versus B-1 B cell fate choice

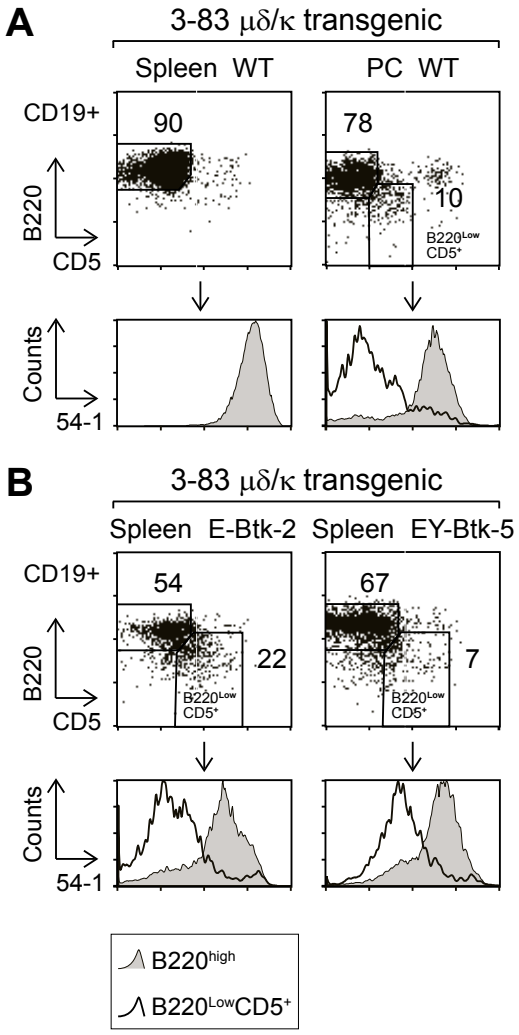
The increased proportions of $CD5^+$ B cells in E-Btk-2 and EY-Btk-5 transgenic mice might reflect a change in follicular versus B-1 B cell fate choice, or alternatively reflect differential effects on expansion or survival of already committed cells. To distinguish between these two possibilities, we directly investigated whether constitutive activation of Btk had the capacity to change the B cell fate in the 3-83md transgenic system. The 3-83md transgene encodes an antibody specific for MHC class I of the $H\text{-}2K^{k,b}$ haplotype (30). On a non-autoreactive background, the expression of the 3-83md BCR commits B cells to the follicular or MZ subsets in the spleen. In these 3-83md BCR transgenic mice only B cells that have edited their BCR are able to differentiate into $CD5^+$ B-1 B cells: all peritoneal $B220^{low}CD5^+$ B-1 B cells have lost the 3-83md BCR specificity detected by the 54-1 anti-idiotypic antibody (Figure 4A). We generated 3-83md transgenic E-Btk-2 and EY-Btk-5 mice on the non-autoreactive $H2\text{-}K^d$ background. As expected, in the spleen of these mice all conventional $B220^{high}CD5^-$ B cells had high 54.1 reactivity, but $B220^{low}CD5^+$ B cells had lost their 54.1 reactivity (Figure 4B), while surface Ig m and k expression levels were similar (not shown). These results indicate that 3-83md transgenic E-Btk-2 and EY-Btk-5 $B220^{low}CD5^+$ B-1 B cells in the spleen had undergone receptor editing.

From these findings we conclude that the presence of the E-Btk-2 and EY-Btk-5 transgene did not change the follicular versus B-1 B cell subset choice. Rather, we conclude that the increased proportions of splenic $CD5^+$ B cells in E-Btk-2 and EY-Btk-5 transgenic mice resulted from increased expansion or survival of B-1 B cells.

Constitutive activation of Btk does not change the MZ B cell subset choice in VH81X transgenic mice

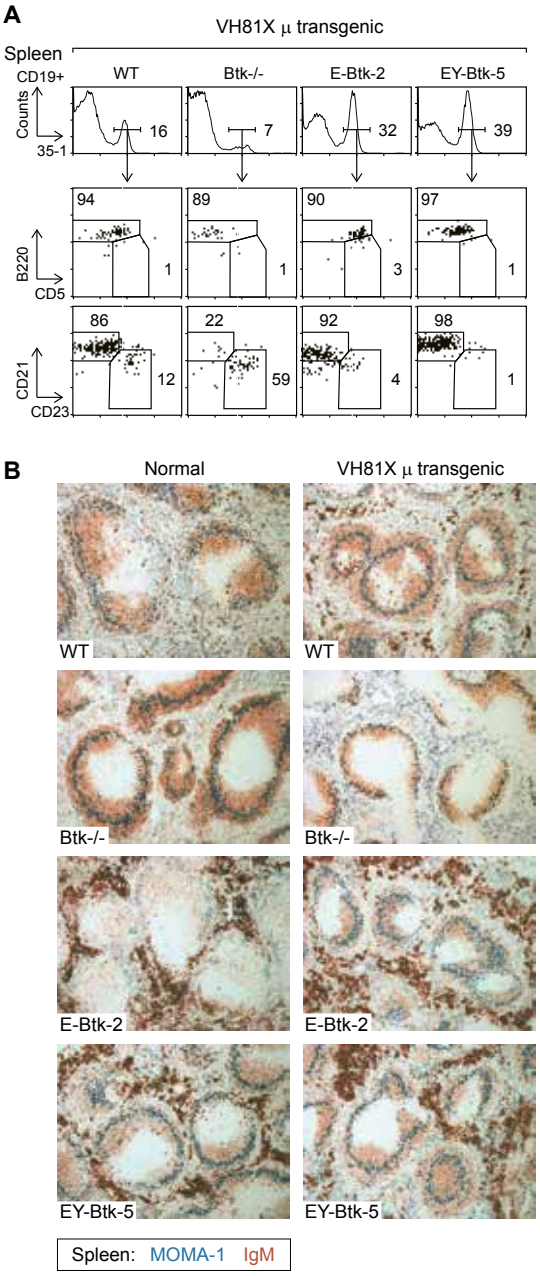
The presence of the E-Btk-2 and EY-Btk-5 transgenes also did not change the MZ B cell subset choice in VH81X transgenic mice, which carried a VH81X transgene encoding an Ig heavy chain favoring MZ B cell development (29). As shown in Figure 5A, B-cells recognized by the 35-1 anti-idiotypic antibody are efficiently selected into the MZ B cell compartment in VH81X wild-type but not in VH81X Tg Btk-deficient spleens (Figure 5B). Splenic $35\text{-}1^+$ $CD19^+$ B cells from VH81X E-Btk-2 transgenic mice expressed similar CD5 levels as those from VH81X wild-type mice, lacked the $B220^{low}$ phenotype characteristic for $CD5^+$ B cells and,

Figure 4. Constitutive activation of Btk does not change the follicular versus B-1 B cell fate choice in 3-83md Tg mice.



Flow cytometric analysis of CD19$^+$ cells from spleen and peritoneal cavity (PC) from 3-83md mice (WT, A) or from spleen from 3-83md mice in the presence of a E-Btk-2 or EY-Btk-5 transgene (B). CD19$^+$ cells were analyzed for their CD5/B220 profile and results are shown as dot plots, whereby the proportions of cells in the indicated gates are given (*upper part of A and B*). In the lower part of A and B histogram plots are shown as overlays of gated CD5$^-$B220$^+$ (*gray shaded area*) and CD5$^+$B220^{low} B cells (*bold line*). Data are representative for 3 independent experiments.

Figure 5. Lack of MZ B cells and disrupted splenic architecture in E-Btk-2 mice is corrected by expression of the VH81X BCR transgene.



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(A) Flow cytometric analysis of splenic CD19$^+$ cells, from the indicated mouse groups on a VH81X transgenic background. Data are displayed as histograms for the anti-idiotype marker 35-1. CD19$^+$35-1$^+$ B cells were gated (*upper part*) and analyzed for their CD5/B220 or CD23/CD21 profiles (*lower part*). The proportions of cells in the indicated gates are given. Data are representative for 3 independent experiments. (B) Immunohistochemical analysis of 6 mm splenic frozen sections from the indicated genotypes in the absence (*left*) or the presence (*right*) of the VH81X transgene. Sections were stained with anti-IgM (*brown*) and the metallophilic macrophage marker MOMA-1 (*blue*).

importantly, had a CD21^{high}/CD23^{low} MZ phenotype similar to those of VH81X wild-type mice (Figure 5A).

Moreover, in contrast to E-Btk-2 mice (which had few MOMA-1$^+$ macrophages and no MZ B cells in the spleen), in E-Btk-2 mice that carried a VH81X transgene splenic architecture was corrected: EY-Btk-2 VH81X double Tg spleens contained IgM$^+$ B cells within and outside rims of brightly staining MOMA-1$^+$ macrophages (Figure 5A; *right panels*).

Collectively, these findings show that MZ cell fate was maintained in the presence of constitutive active Btk, indicating that the VH81X BCR specificity is dominant over the increased BCR signal strength generated by the E-Btk-2 transgene.

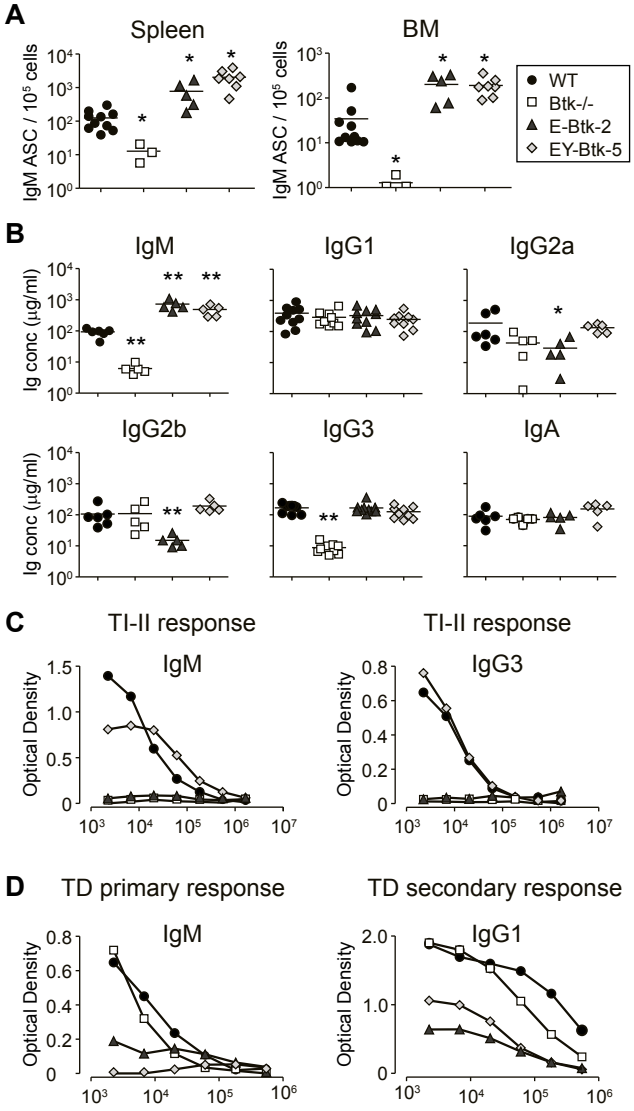
Enhanced generation of IgM$^+$ plasma cells in E-Btk-2 and EY-Btk-5 mutants

To determine whether the large fractions of CD138$^+$IgM^{high} cells observed in E-Btk-2 and EY-Btk-5 mice (Figure 3E) were antibody secreting plasma cells (ASC), we performed ELISPOT assays. Both in spleen and in BM, we found that Btk-deficiency resulted in reduced numbers of IgM ASC and that the presence of constitutive active Btk resulted in a ~6-10 fold increase in IgM ASC (Figure 6A). In E-Btk-2 and EY-Btk-5 mice IgM serum levels were increased, as determined by ELISA (Figure 6B), consistent with the presence of increased numbers of IgM ASC in the long-lived compartment in the BM. The E-Btk-2 and EY-Btk-5 mice did not show an increase in ASC of other subclasses (not shown), and accordingly serum levels were either decreased or in the wild-type ranges (Figure 6B).

To investigate mature B cell functionality, we analyzed the immune response to the T-cell independent type II (TI-II) antigen, TNP-Ficoll and the T-cell dependent (TD) antigen, TNP-KLH. Consistent with previous reports (8, 23) Btk-deficient mice did not show TI-II IgM or IgG3 responses (Figure 6). Likewise, E-Btk-2 mice were unresponsive to TNP-Ficoll immunization, whereas EY-Btk-5 mice responded similarly to wild-type mice (Figure 6C). The response to the TD antigen TNP-KLH was assessed one week after immunization (IgM) and one week after booster immunization (IgG). Wild-type and Btk-deficient mice had similar primary IgM and secondary IgG1 responses (Figure 6D), but these were severely reduced in E-Btk-2 and EY-Btk-5 mice. Consistent with defective TD responses, clusters of PNA$^+$ germinal center B cells were not detectable (E-Btk-2) or severely reduced (EY-Btk-5) in immunohistochemical analyses (data not shown).

In summary, these findings demonstrate that both E-Btk2 and EY-Btk5 transgenes efficiently drive IgM$^+$ plasma cell differentiation, but this is not associated with increased functional TI-II responses. Moreover, TD responses and germinal center formation were significantly affected by the presence of constitutive active Btk.

Figure 6. Constitutive activation of Btk increases IgM⁺ plasma cell formation.



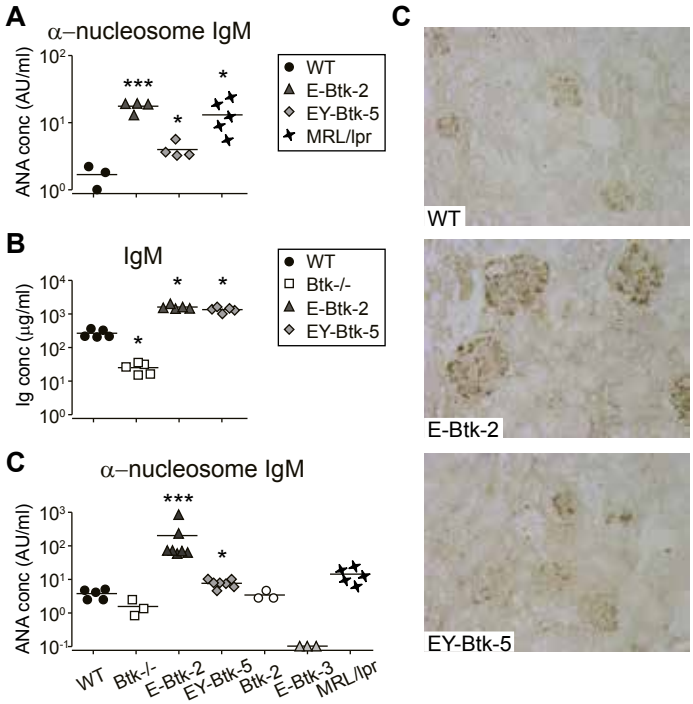
(A) Numbers of IgM-producing cells per 10^5 cells in spleen and BM of the indicated mouse lines, determined by ELISPOT assay. (B) Serum Ig concentrations within the different subclasses in 8-10 week-old mice from the indicated groups, determined by ELISA. Each symbol repreents an individual animal. Significant differences with wild-type are indicated: *= $p<0.05$; **= $p<0.01$. (C) TNP-specific IgM and IgG3 of TI-II immune responses 7 days after injection with TNP-Ficoll, determined by ELISA. Mean optical densities are from of 4-10 mice per group. (D) TNP-specific IgM 7 days after primary injection with TNP-KLH and TNP-specific IgG1 7 days after booster injection with TNP-KLH, determined by ELISA. Mean optical densities are from 3-8 mice per group.

Constitutive activation of Btk in E-Btk-2 mice results in spontaneous IgM autoantibody production

As the E-Btk-2 and EY-Btk-5 transgenic B cells were spontaneously driven into germinal center-independent plasma cell differentiation, the Btk transgenic mice may have lost self-tolerance. In serum of 9-12 week old animals we found that anti-nucleosome-specific IgM was increased in E-Btk-2, but not in EY-Btk-5 mice, to levels that were similar to those found in autoimmune MRL/lpr control mice (Figure 7A). No anti-nucleosome IgG was detectable in wild-type or Btk transgenic mice.

Serum analysis of ~35 week-old mice revealed that total IgM levels increased with age and that E-Btk-2 and EY-Btk-5 IgM serum levels remained dramatically elevated compared to those found in wild-type mice (Figure 7B). Strikingly, E-Btk-2 mice had developed dramatically increased anti-nucleosome IgM which was at east >15 times the level found in MRL/lpr mice (Figure 7C). Also in YE-Btk-5 mice anti-nucleosome activity was detectable, but the concentrations were lower. Overexpression of unmutated human Btk or high-

Figure 7. E-Btk-2 transgenic mice have IgM autoantibodies.



(A) Serum anti-nucleosome IgM antibody (ANA) concentrations in the indicated mouse groups, determined by ELISA. Mice were 8-10 weeks of age. (B) Serum total IgM and (C) anti-nucleosome IgM concentrations at 35-40 weeks of age, determined by ELISA. (D) Immunohistochemical analysis of kidneys from 35-40 week-old mice. 6 mm renal frozen sections were incubated with antibodies to IgM (brown). AU= arbitrary units.

level expression of E41K-Btk (in E-Btk-3 mice) did not induce anti-nucleosome IgM (Figure 7C). Although immunohistochemical stainings of kidneys revealed the presence of enlarged glomeruli containing IgM deposition in E-Btk-2 transgenic mice (Figure 7D), we did not find evidence for autoimmune disease, such as proteinuria or glomerular inflammation. We therefore conclude that E-Btk-2 mice produced anti-nucleosome IgM that was associated with mild pathological changes without overt clinical disease.

Discussion

The E41K Btk mutant displays increased, PI3K-independent membrane localization (26) and therefore we expected that even low-level expression of E41K-Btk would affect B cell development. In this report, we describe the effect of expression of lower levels of E41K-Btk and E41K-Y223F-Btk transgenes. Firstly, expression of constitutive activated Btk resulted in a copy-number dependent deletion of peripheral B cells beyond the transitional B cell stage. Deletion of B-1 B cell was limited and in general the absolute numbers of B-1 cells in the spleen were increased. Secondly, residual B cells were hyperresponsive, as evidenced by increased FSC and expression of CD25 and CD69 and sustained Ca^{2+} mobilization upon BCR stimulation. Thirdly, residual B cells were efficiently driven into plasma cell differentiation, resulting in increased numbers of plasma cells in spleen and BM and increased serum IgM. Finally, we found anti-nucleosome autoantibodies and glomerular IgM deposition in aging mice.

When comparing the phenotypes of E-Btk-2 and EY-Btk-5 transgenic mice it is clear that expression of E-Btk-2 more profoundly affected B cell differentiation than EY-Btk-5 did. The observed differences may originate from differential effects of the two mutants or from expression level differences between the two transgenes. The latter is most likely, because when EY-Btk-5 transgenic mice were bred to homozygosity, we observed a more severe phenotype that was quite similar to that of E-Btk-2 mice, e.g. in terms of surface CD21/CD23 profiles of B cells (Figure 2C) and micro-architecture of the spleen (not shown). Moreover, we have previously found that Y223 phosphorylation is not essential for Btk function in vivo: Y223F-Btk can fully correct the features of the Btk-deficient phenotype, including pre-B cell B-1 cell development, serum IgM levels and TI-II responses (27).

The complex phenotype of mice with constitutively activated Btk largely resembles that of other transgenic or knock-out mice with increased BCR signaling, including gain-of-function mutants of Lyn or PLCg2, overexpression of CD19, and deficiency for PTEN, inhibitory co-receptors such as CD22, PIR-B and FcgRIIB or their downstream signaling molecules SHP1 or SHIP (12-19). These mice also contain fewer follicular B cells, increased numbers of B-1 B cells, together with B cell hyperresponsiveness and autoimmunity. However, from the observed phenotypes it is not clear whether altered BCR signaling directly affects B cell fate or affects selection, survival or differentiation of cells that are committed to a specific B cell subset. Our crosses with 3-83md and VH81X BCR transgenic mice clearly showed that constitutive active Btk expression did not change the follicular, MZ or B-1 B cell fate, but resulted in selective expansion or survival. In this regard, the effects of constitutively activated Btk may be different from other genetic changes that enhance BCR signaling, because it was consistently associated with a profound reduction of total numbers of mature B cells and only a modest increase in the proportion of B-1 cells. By contrast, mice with constitutive active Lyn or PLCg2 manifested a substantial increase in

the absolute size of the total B-1 B cell population (13, 14). Nevertheless, our finding that constitutive active Btk does not change B cell subset choice, but only affects selection or survival of cells that are committed may be in apparent conflict with previous conclusions that BCR signaling strength rather than BCR specificity is the major determining factor in cell subset differentiation decisions. Studies using transgenic mice expressing the Epstein Barr virus encoded protein, LMP2A, which mimics a constitutive-active BCR, showed that mice carrying a targeted replacement of Ig H chain by LMP2A leading to high or low expression of the LMP2A protein developed B-1 or follicular/MZ B cells, respectively (33, 34). LMP2A expression allows the generation of BCR-negative B cells, and therefore provides a model where BCR signaling strength could be evaluated independently of BCR specificity. Likewise, it has been demonstrated that a natural serum autoantibody specific for the Thy-1 glycoprotein was produced in mice by B-1 cells that are positively selected by self-antigen (6). Whereas lack of Thy-1 engagement in Thy-1$^{-/-}$ mice permitted B cells specific for the Thy-1 glycoprotein to proceed to the follicular B cell subset (35), increases in BCR signaling strength, induced by low-dose self-antigen, directed naive immature B cells to mature instead into the marginal-zone B cell subset (7). It is therefore conceivable that LMP2A or Thy-1 antigen-mediated signals direct differentiation into B cell subsets, whereas isolated Btk-mediated signals primarily affect cellular survival.

The molecular mechanisms involved in the failure of self-tolerance in mice that express the E-Btk-2 transgene are presently unknown. The efficient deletion of immature B cells in mice expressing high-levels of E41K-mutated Btk (Figure 1C and Ref. (28)) indicates that E-Btk transgenic immature B cells are subject to efficient clonal deletion. We did not detect any defects in receptor editing when 3-83md, E-Btk-2 double transgenic B cells were crossed on a central deleting C57BL/6 background (R.K., unpublished results). Because a significant fraction of circulating mature B cells is thought to be auto- or poly-reactive (36), such B cells may become activated because constitutive active Btk suppresses inhibitory effects of FcgRIIb or SHIP (as was previously shown for a membrane-associated Btk chimera which led to sustained elevation of intracellular calcium (37)). In this context, it is conceivable that the E-Btk-2 transgene can also counteract inhibitory signals generated by FcgRIIb crosslinking that were recently found to induce apoptosis and thereby govern differentiation and maintenance of plasma cells (38). Persistence of plasma cells in E-Btk-2 transgenic mice would be supported by our finding of increased numbers of these cells in the BM.

In summary, we have found that expression of constitutive activation of Btk results in efficient deletion of peripheral B cells beyond the transitional B cell stage and reduced numbers of follicular B cells. In contrast, the proportions of B-1 cells were significantly increased in the spleen. Crosses with BCR transgenic mice revealed that Btk activation did not change B cell subset fate choice, but rather resulted in selective expansion or survival of B-1 cells. Residual B cells were efficiently driven into plasma cell differentiation, as evidenced by increased numbers of plasma cells in spleen and BM, high serum IgM and the presence of anti-nucleosome autoantibodies. This phenotype shows that Btk signals are essential for appropriate regulation of B cell activation. Since successful treatment of patients with autoimmune disorders such as lupus and rheumatoid arthritis have demonstrated the importance of B cells in disease pathology (39), it should be worthwhile developing treatment strategies for autoimmune diseases based on Btk-specific small molecule inhibitors.

Acknowledgements

We thank the people from the Erasmus MC Animal Care facility for their assistance. This work was supported by the Netherlands Organization for Scientific Research, the Dutch Cancer Society and the Dutch Arthritis Association.

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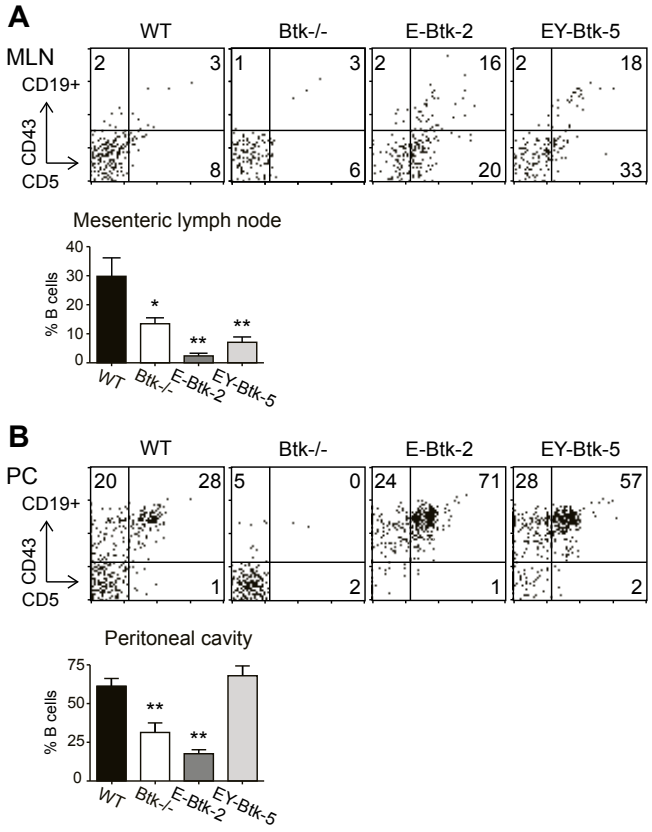
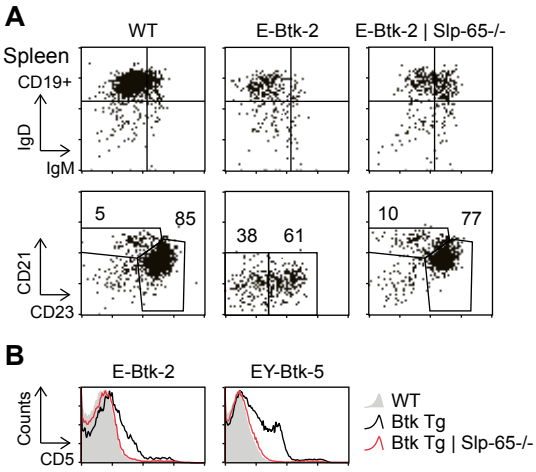
Figure S1. Numbers of CD1dhigh marginal zone B cells in mutant Btk transgenic mice.



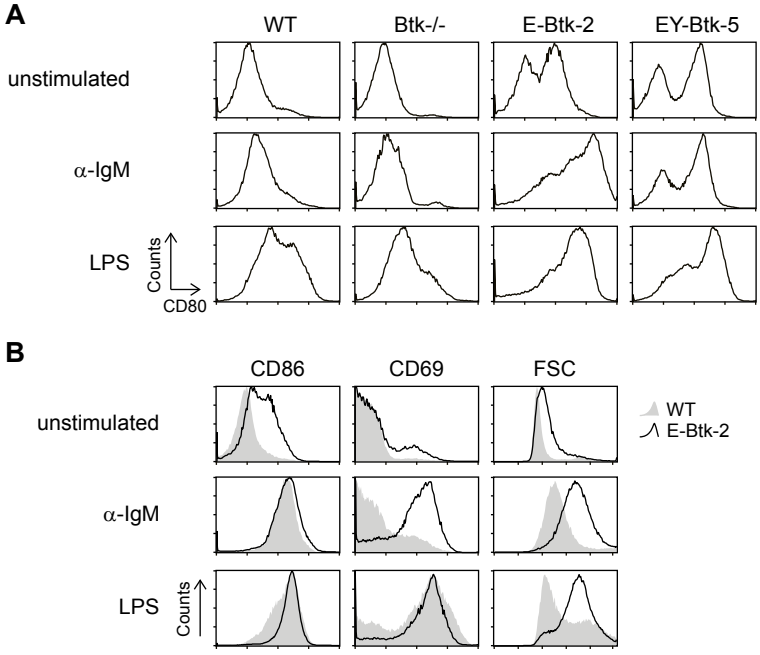
A) Identification of CD1dhighCD23low marginal zone B cells in spleens of wildtype (WT) and the indicated mutant Btk transgenic mice, using flow cytometry. For analysis, CD19+B220+CD43- B-2 cells were gated from live cells within the lymphocyte FSC/SSC gate. B) Relative numbers (average values and SEM, per 100.000 live CD19+B220+CD43- B-2 cells) of marginal zone B cells in spleens of wildtype and mutant Btk transgenic mice. Marginal zone B cells were defined by low expression of CD23 and either high CD1d (left) or high CD21 (right) expression.

Figure S2. Low-level expression of E-Btk and EY-Btk severely affects peripheral B cell populations.



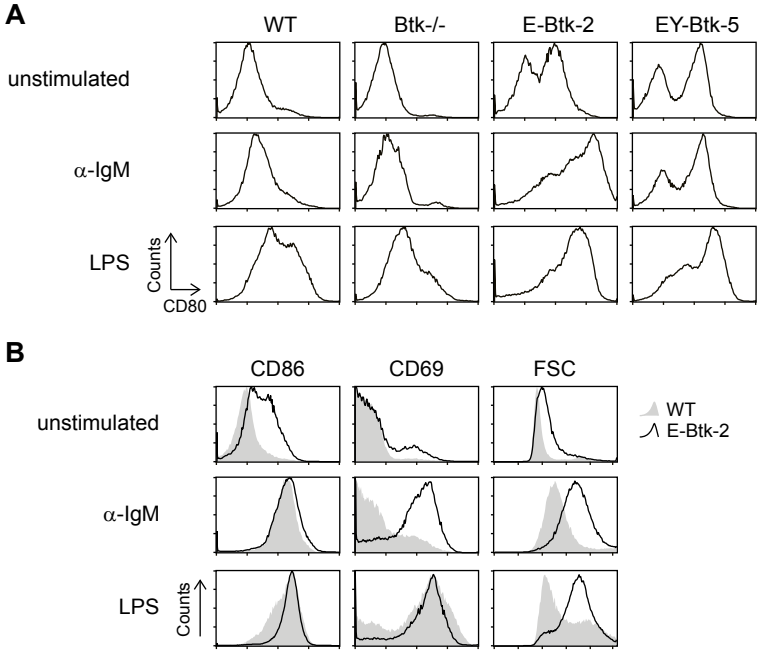
Flow cytometric CD5/CD43 profiles of gated CD19+ cells from mesenteric lymph nodes (MLN, panel A) or peritoneal cavities (PC, panel B) from the indicated mice at 8-12 wks of age. Data are shown as dot plots and numbers indicate the proportions of cells in each quadrant. The lower parts of the figures show the quantifications of the proportions of B220+CD19+ B cells in the MLN or PC from the indicated mouse groups. Data shown are representative of 5-9 animals per group and bars show average values and SEM. Significant differences with wild-type are indicated: *= p<0.03; **= p<0.01, Mann-Whitney U test.

Figure S3. Enhanced activation in vitro of B lymphocytes expressing constitutively active Btk mutants.



(A) Flow cytometric analysis of CD80 expression on wildtype, Btk deficient and mutant Btk transgenic B cells after 2 days of in vitro culture, either in the absence of stimulation, with 10 µg/ml F(ab')2 anti-IgM (Jackson Laboratories), or 10 µg/ml LPS (serotype 026:B6, Difco Laboratories, Detroit, MI). (B) Flow cytometric comparisons of the activation markers CD69 and CD86 and FSC (as a measure of cell size) in wildtype and E-Btk-2 transgenic B cells, in the same cultures. For EY-Btk-5 B cells the profiles for CD86, CD69 or FSC were closer to wildtype and were more variable between mice (not shown). Data are representative of 3-5 independent experiments. Total spleen cell suspensions were depleted of erythrocytes by standard NH4Cl lysis and enriched for B cells by magnetic cell sorting. Cells were cultured in 24-wells plates at a concentration of 2 x 106 cells/well.

Figure S4. The effect of constitutive activation of Btk in E-Btk-2 and EY-Btk-5 transgenic mice is dependent on Slp-65.



To explore whether the effects of E-Btk-2 and EY-Btk-5 on the mature B cell compartment were dependent on Slp65, transgenic mice were crossed onto the Btk-Slp65 doubledeficient background. Flow cytometric analyses show that in the absence of Slp65 the effects of constitutive Btk activation were diminished. The CD21+CD23+ follicular and CD21highCD23low MZ compartments were restored and the spleens no longer contained large proportions of CD5+ B-1 lineage cells. Therefore, we conclude that the effects of constitutive active Btk expression on the follicular, MZ and B-1 B cell subsets were dependent on the presence of Slp65. (A) Flow cytometric analysis of splenic CD19+ cells. CD19+ cells from the indicated mouse lines were gated and analyzed for their IgM/IgD and CD21/CD23 profiles. (B) CD19+ cells were gated and analyzed for CD5 expression. Results are displayed as histograms overlays of CD19+ cells from wild-type mice (black lines), E-Btk-2 or EY-Btk-5 mice (dark gray line) and E-Btk-2 or EY-Btk-5 mice on a Slp-65 deficient background (red line).

4

Btk levels set the threshold for B cell activation and negative selection of autoreactive B cells in mice

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Abstract

Upon antigen binding by the B cell receptor (BCR), B cells upregulate protein expression of the key downstream signaling molecule Bruton's tyrosine kinase (Btk), but the effects of Btk upregulation on B cell function are unknown. Here, we show that transgenic mice overexpressing Btk specifically in B cells spontaneously formed germinal centers and manifested increased plasma cell numbers, leading to antinuclear autoantibody production and SLE-like autoimmune pathology affecting kidneys, lungs and salivary glands. Autoimmunity was fully dependent on Btk kinase activity, since Btk inhibitor treatment (PCI-32765) could normalize B cell activation and differentiation, and since autoantibodies were absent in Btk transgenic mice overexpressing a kinase inactive Btk mutant. B cells overexpressing wildtype Btk were selectively hyperresponsive to BCR stimulation and showed enhanced Ca^{2+} influx, NF-κB activation, resistance to Fas-mediated apoptosis, and defective elimination of selfreactive B cells *in vivo*. These findings unravel a crucial role for Btk in setting the threshold for B cell activation and counterselection of autoreactive B cells, making Btk an attractive therapeutic target in systemic autoimmune disease like SLE. The finding of *in vivo* pathology associated with Btk overexpression may have important implications for the development of gene therapy strategies for X-linked agammaglobulinemia, the immunodeficiency associated with mutations in BTK.

Published in Blood. 2012 Apr 19;119(16):3744-56

Introduction

B cells are of crucial importance for the host defense against pathogens. Their full activation does not solely depend on antigen recognition through the B cell receptor (BCR), but usually requires a second signal delivered by other immune cells or by the pathogen itself[1-2]. Although the integration of two activating signals should pose a strong barrier to the activation of autoreactive B cells, dysregulated BCR signaling alone can greatly contribute to the development of systemic auto-immune diseases[3]. Genome-wide association studies point to a role of BCR-associated molecules such as BLK and BANK1 in the pathogenesis of various systemic auto-immune diseases, including SLE, systemic sclerosis, rheumatoid arthritis and Sjögren syndrome[4]. A hallmark of SLE is the loss of B cell tolerance to nuclear self-antigens, leading to production of antinuclear autoantibodies that elicit inflammation upon deposition in various organs. The crucial role of BCR signaling in development of SLE-like immune diseases is further supported by data from genetically engineered mouse models[5]. In addition, defective B cell apoptosis can induce aggressive auto-immune disease in mouse models[5], and enhanced B cell survival has been implicated as a causative factor in Sjögren's syndrome[6].

Bruton's tyrosine kinase (Btk) is a BCR signaling molecule that directly links BCR signals to B cell survival, through activation of the transcription factor NF-κB[7-9]. Although Btk is widely expressed in the hematopoeietic system, only B cells greatly rely on Btk signaling. Btk deficiency in men or mice results in the B cell specific immunodeficiencies X-linked agammaglobulinemia (XLA) or x-linked immuno-deficiency (*xid*) respectively[10]. Whereas XLA is characterized by an almost complete arrest of pre-B cell differentiation, in *xid* the pre-B cell block is limited, but differentiation and survival of mature B cells is severely impaired[11-13]. The defects in murine Btk-deficient B cells range from reduced responsiveness to BCR and TLR stimulation to decreased signaling of chemokine receptors[14].

Several lines of evidence indicate that Btk expression levels may present the rate-limiting step in BCR signaling and that tight regulation of Btk expression and kinase activity is essential for normal B cell development and activation. Firstly, expression of sub-physiological levels of transgenic Btk only partially corrects the phenotype of Btk-deficient B cells[15], while physiological levels fully rescue this phenotype[16]. Secondly, upon BCR stimulation mature B cells increase Btk levels within 4 hours via a posttranscriptional mechanism[17]. Thirdly, Btk uses a proteasome-dependent positive autoregulatory feedback mechanism to stimulate transcription from its own promoter via NF-κB[18]. Fourthly, many factors can negatively regulate Btk, either by downregulating Btk kinase activity, such as PKC-β[19], iBtk[20] and caveolin-1[21], or by reducing Btk expression, such as Pin1[22] or miR185[23]. Finally, detrimental effects of dysregulated Btk kinase activity are evident from the Btk-dependency of B cell malignancies, including diffuse large B cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL). Treatment of DLBCL or CLL B cells with Btk-inhibitors markedly reduces cell survival[24-25].

Given the stringent regulation of Btk expression and activation in B cells, we addressed the question whether Btk expression levels are critical for mature B cell activation and selection. To study the effects of dysregulated Btk expression in B cells *in vivo*, we used mice that transgenically overexpress human Btk under the control of the MHC class II Ea locus control region[16] or driven by the CD19 promoter region[26]. Previous analyses of Btk transgenic mice on a mixed genetic background showed that all

features of the *xid* phenotype were corrected by these Btk transgenes, while adverse affects on B cell development were limited to elevated serum IgM levels and a mild increase in B cell proliferation upon BCR stimulation[26-27]. In contrast to these limited effects of overexpression of wildtype (wt) Btk on B cell functioning, expression of the constitutively active E41K-Btk mutant induced an almost complete deletion of developing immature B cells in the bone marrow (BM)[26]. Even low levels of E41K-Btk hampered B cell differentiation, characterized by reduced numbers of follicular B cells and selective survival or expansion of B-1 B cells[28].

Here we show that overexpression of wt Btk restricted to the B cell lineage induced spontaneous germinal center (GC) and plasma cell formation, and eventually antinuclear autoantibody production and SLE-like auto-immune disease. This auto-immune phenotype was fully dependent on Btk kinase activity. Our findings provide evidence that expression levels of Btk set the threshold for B cell activation and selection of autoreactive B cells.

Materials & methods

Mice and genotyping

CD19-hBtk[26], CD19-hBtk^{K430R}, CD19-hBtk^{Y223F} [29], and MHCII-hBtk mice[27] on a mixed background (Fvb x 129/Sv x C57BL/6) were backcrossed on C57BL/6 for >10 generations. Mice deficient for Btk[12] were on a C57BL/6 background. Genotyping was performed by PCR, as previously described[29]. Mice were analyzed at 10-14 weeks, or at ~40 weeks to monitor autoimmune pathology. Mice were bred and kept at specified pathogen free conditions in the Erasmus MC experimental animal facility. All experimental protocols have been reviewed and approved by the Erasmus MC Committee of animal experiments.

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

Preparations of single-cell suspensions, flow cytometry and Ca^{2+} measurements were performed using standard procedures. Monoclonal antibodies are listed in Table S3. For intracellular isotype-specific staining of Ig components, cells were fixed in Cytofix/Cytoperm™ and permeabilized and stained in Perm/Wash™ buffer (BD biosciences). For intracellular Btk staining, cells were fixed in PBS/2% PFA, permeabilized and stained with PE-anti-BTK in the presence of 0.5% saponin (Sigma-Aldrich). Flow cytometric detection of Akt and ERK phosphorylation was performed essentially as described in[30]. Cell cycle analysis using propidium iodide has been described previously[13]. Measurement of Ca^{2+} mobilization was performed according to the protocol published in[29]. All measurements were performed on a LSRII™ flow cytometer (BD biosciences), and results were analyzed using FlowJo software (Tree Star).

MACS purification and cultures of mature splenic B cells

Splenic cell suspensions were prepared in MACS buffer (PBS / 2mM EDTA / 0.5% BSA). NonB cells, activated B cells, B1 cells and (pre)plasma cells were depleted by incubating splenic cell suspensions with biotinylated antibodies against CD5, CD43, CD138, CD11b, Gr-1 ad TER-119 (see Table S3), followed by incubation with streptavidin-coupled magnetic beads (Miltenyi Biotec). Unlabeled cells (naive B2 lymphocytes) were collected by magnetic depletion of labeled cells (unlabeled fraction contained >95% $CD19^+CD138^-$ cells). Purified B cells were cultured at 1.2 x 10^6 cells/ml for 1-3 days in culture medium

(RPMI 1640 / 10% FCS / 50 µg/ml gentamycin / 0.05 mM ß-mercapto-ethanol). Cells were stimulated in vitro with 10 µg/ml F(ab')$_2$ α-IgM (Jackson ImmunoResearch), 5 µg/ml LPS (own production), 20 µg/ml α-CD40 (clone 3/23; BD Biosciences), 1 mM CpG (Invitrogen), or 50 µg/ml Poly I:C (Invivogen).

Immunohistochemistry

Immunohistochemical analyses and periodic acid-Schiff (PAS) staining were performed according to standard procedures[31]. Used antibodies are listed in Table S3. After staining, tissue sections were embedded in Kaiser's glycerol gelatin (Merck). Micrographs were made using a DM LB light microscope (Leica, Germany), a DFC500 camera (Leica), and Imaging for Windows software (Kodak, Rochester, New York).

ELISAs for Ig isotypes, autoantibodies and albuminuria

Isotype-specific serum Ig concentrations were measured by sandwich ELISA[27]. For IgG antinucleosome antibody levels, we used nucleosome-coated 96 wells plates (Orgentec) and biotinylated anti-mouse total IgG antibodies (SouthernBiotech) as secondary reagent. IgG anti-dsDNA antibody levels were determined using a mouse anti-dsDNA IgG-specific ELISA (Gentaur). Levels of albuminuria in urine samples were determined by ELISA[32] and corrected for levels of creatinin, which were measured using an autoanalyzer (Hitachi 717, Roche).

HEp2 reactivity assay

Serum samples (diluted 1/100 in PBS) or BALf samples (undiluted) were incubated for 1 hour on Kallestad HEp-2 slides (Bio-Rad Laboratories). As secondary antibody AlexaFluor®488-conjugated donkey anti-mouse IgG (Invitrogen) was applied to the HEp2 slides, and after embedding the slides in glycerol (Sigma-Aldrich, St. Louis, Missouri) the fluorescence intensity of HEp2 slides was evaluated using a LSM 510 META confocal fluorescence microscope (Zeiss, Germany) and LSM 510 software (Zeiss).

Btk inhibitor treatment

Btk inhibitor PCI-32765[33] or placebo was orally administered (25 mg/kg/day in water/5% mannitol/0.5% gelatin) to the mice once or for 8 consecutive days.

Influenza infection and hemagglutinin inhibition assay (HAI)

Mice were infected intranasally with 10^5 H3N2 virus particles (X-31; Medical Research Council, London, UK) in 50 µl PBS. At day 18 dpi animals were sacrificed for analysis of BALf and serum. To determine anti-hemagglutinin (HA) antibody titers in serum of influenza infected animals, a HA inhibition assay was performed[31].

Western blotting

For analyses of c-Rel nuclear translocation, nuclear protein extracts were prepared from MACS sorted splenic B2 cells that were stimulated *in vitro* for 4 hours with 10 µg/ml F(ab')$_2$ α-IgM (Jackson ImmunoResearch). Preparation of nuclear protein extracts and Western blotting have previously been

described[29]. Levels of c-Rel were analyzed using ImageJ software (Wayne Rasband) and normalized to nucleophosmin levels, detected with mouse-antinucleophosmin (clone B23; Santa Cruz Biotechnology) and peroxidase-conjugated rabbit-anti-mouse Ig (Dako).

Statistical analysis

For calculating the level of significance of differences between groups we used the Student's T-Test.

Results

Btk expression levels increase upon B cell stimulation

It has been shown that Btk expression levels are upregulated upon BCR engagement in mature B cells[17-18]. To test which stimuli can induce Btk upregulation, we isolated naive splenic $CD43^-CD5^-CD138^-$ B lymphocytes and stimulated them *in vitro* through the BCR ($F(ab')_2$ α-IgM), CD40 (α-CD40), TLR4 (LPS), TLR9 (CpG), or TLR3 (Poly(I:C)) for 3 days. When we evaluated Btk expression levels by intracellular flow cytometry, we found that all stimuli tested evoked Btk upregulation, whereby the increase was highest upon BCR engagement (~2.7-fold; Figure 1A, see also Supplementary Figure S1). Modest but significant Btk upregulation (~1.2-fold) was already detected after 6 hours of α-IgM stimulation (data not shown). Therefore, various signals can increase Btk expression levels in mature B lymphocytes, indicating that Btk upregulation by engagement of one receptor could sensitize B cells to signaling through a second pathway.

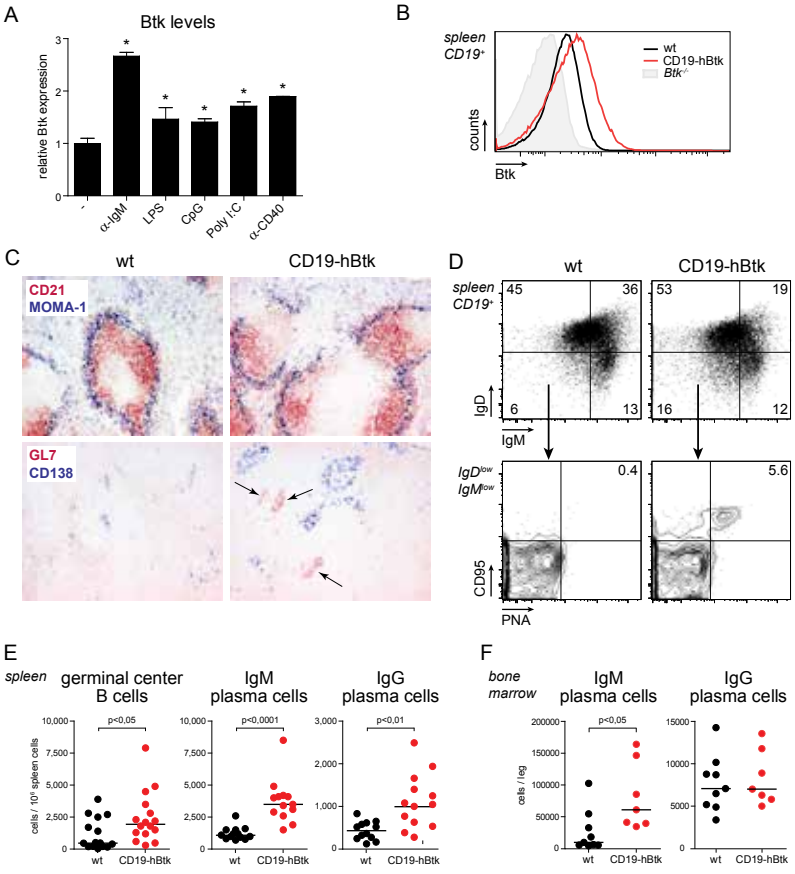
Btk overexpression *in vivo* promotes GC and plasma cell formation

To test the effects of Btk overexpression *in vivo*, we studied CD19-hBtk transgenic mice on a pure C57BL/6 background, in which the human CD19-promoter establishes overexpression of transgenic human BTK throughout B cell differentiation[26]. Using flow cytometry, we observed a rather constant level of Btk overexpression during B cell development in the BM (~1.5 to 3-fold; see Figure S2A). Importantly, CD19-hBtk transgenic mature splenic B cells expressed ~3-fold higher Btk levels, compared with wt B cells (Figure 1B and S2A). These increased Btk levels in CD19-hBtk B cells were comparable to those found in wt B cells after 3 days of α-IgM stimulation *in vitro*. Btk expression could be further increased upon BCR stimulation (Figure S2B), possibly by the same (post)transcriptional mechanisms acting in stimulated wt B cells.

In young CD19-hBtk mice, Btk overexpression did not affect the overall splenic microarchitecture (revealed by immunohistochemistry using CD21 for B cells and MOMA-1 for metallophilic marginal zone macrophages; Figure 1C). However, spleens of CD19-hBtk mice frequently contained $GL7^+$ GC, which are rarely found in wt mice kept under specified pathogen free conditions, and displayed a marked increase in extrafollicularly located $CD138^+$ plasma cells (Figure 1C). When we used flow cytometry to quantify these differences, we found that in CD19-hBtk mice the numbers of $IgD^{low}IgM^{low}CD95^+PNA^+$ GC B cells as well as IgM^+ or IgG^+ plasma cells in the spleen were significantly increased (Figure 1D and 1E). In the BM an increase was only found for IgM^+ plasma cells (Figure 1F), suggesting that most IgG^+ plasma cells formed in the spleens of CD19-hBtk mice were short-lived.

Flow cytometric analysis of the entire B cell compartment in CD19-hBtk mice revealed that B cell

Figure 1. Btk overexpression *in vivo* enhances GC and plasma cell formation.



(A) Quantification of intracellular flow cytometric detection of Btk protein in wt splenic B cells after 3 days of *in vitro* culture in the presence of the indicated stimuli (see Supplementary Figure S1). Levels were calculated relative to average Btk expression levels (MFI) in unstimulated B cells (set to 1) and background signals in unstimulated *Btk*$^{-/-}$ B cells (set to 0). Data are mean values ± SD (n=3) from one representative experiment out of three, asterisks indicate significant increases in Btk expression ($p<0.05$). (B) Intracellular flow cytometric Btk detection in splenic CD19^{+} B cells, shown as histogram overlays from *Btk*$^{-/-}$ (shaded histogram), wildtype (wt; black line) and CD19-hBtk (red line) mice. (C) Immunohistochemical analysis of spleen tissue sections of CD19-hBtk and wiltype mice. Sections were stained for CD21 (red) and metallophilic macrophage marker MOMA-1 (blue), or for GL7 (red) and CD138 (blue); arrows indicate clusters of GL7^{+} GC B cells (objective 10x). (D) Flow cytometric analysis of GC cells in the spleens of the indicated mice. CD19^{+} B cells were gated and analyzed for IgM/IgD profiles. IgD^{low}IgMI^{ow} cells were subsequently gated and analyzed for PNA and CD95. Data are displayed as dot plots and the percentages of cells in the indicated quadrants are given. (E,F) Flow cytometric quantification of GC B cells (PNA^{+}CD95^{+}IgD^{low}IgMI^{ow}CD19^{+}) and IgM^{+}CD138^{high} or IgG^{+}CD138^{high} plasma cells in spleen (E) and BM (F). Horizontal lines indicate median per group; each symbol represents and individual mouse. Mice were 10-14 weeks old.

development was largely normal, except for a small but significant increase in immature B cells and a reduction in recirculating B cells in the BM, compared with wt littermates (Table S1). In the spleen, a reduction in follicular and marginal zone B cell numbers was observed, whereas $CD5^+$ B-1 cells were more numerous.

Taken together, these data demonstrate that B cell-restricted overexpression of Btk is sufficient to induce spontaneous GC and plasma cell formation in the spleen.

Btk overexpression selectively enhances BCR-mediated B cell activation

Next, we investigated whether elevated levels of Btk could amplify BCR signaling upon BCR triggering with α-IgM. Splenic B cells from CD19-hBtk mice displayed a significantly stronger Ca^{2+} influx than wt B cells (Figure 2A). CD19-hBtk B cells also displayed *in vivo* higher expression of activation markers, particularly surface molecules required for T cell costimulation including CD80, CD86 and MHCII (Figure 2B).

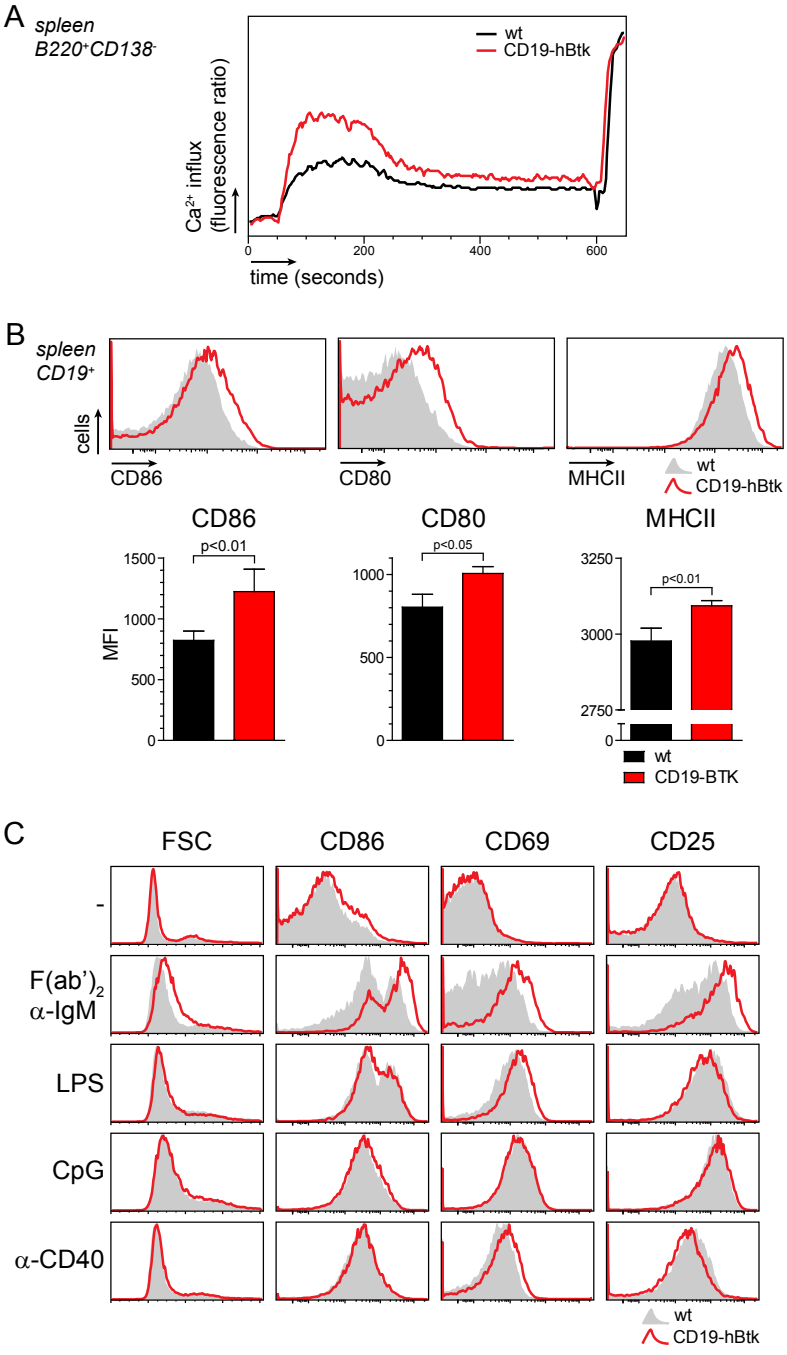
To dissect which Btk-mediated signaling pathways might contribute to the phenotype of enhanced B cell activation in CD19-hBtk mice *in vivo*, we stimulated purified splenic B cells with α-IgM, LPS, CpG and α-CD40 *in vitro*. In agreement with the increased Ca^{2+} influx upon BCR engagement, we found that after 24 hours of α-IgM stimulation CD19-hBtk B cells manifested markedly increased surface expression of CD86, CD69 and CD25, as well as a clear cell size increase, when compared to nontransgenic B cells (Figure 2C; see for quantification Figure S2C). Activation markers continued to be expressed at significantly higher levels throughout 3 days of *in vitro* stimulation (data not shown). In contrast, in the absence of stimulation or in the presence of LPS, CpG, or α-CD40, we did not detect increased upregulation of any of the activation makers tested after 1 or 3 days of culture (Figure 2C, S2C and data not shown).

These findings indicate that Btk overexpression selectively enhances BCR-mediated B cell activation. Although multiple activating signals are capable of inducing upregulation of Btk protein expression (Figure 1A), this upregulation exclusively sensitizes B cells to BCR signaling.

B cell specific Btk overexpression *in vivo* results in systemic autoimmune disease

Young CD19-hBtk mice displayed increased IgM but normal IgG and IgA levels in the serum (Figure 3A), consistent with the observed increased numbers of IgM^+ but not IgG^+ plasma cells in the BM of these mice. Because enhanced GC and plasma cell formation in nonimmunized CD19-hBtk mice might reflect a response to self-antigens, we screened for autoantibody production in CD19-hBtk mice, using a HEp2 reactivity assay. In the serum of ~25% of young CD19-hBtk mice we detected low levels of IgG autoantibodies, which were exclusively directed against nuclear epitopes (Figure 3B). In aging mice (>40 weeks), however, high autoantibody levels were present in all animals (Figure 3B). Quantification of IgG antinucleosome and anti-dsDNA antibodies by ELISA demonstrated high levels (Figure 3C). The levels of anti-nucleosome antibodies (but not of anti-dsDNA antibodies) approximated those observed in diseased MRL/lpr mice, a classical mouse model for spontaneous development of lethal SLE[5]. Antinuclear autoantibodies are a hallmark of SLE and can be detected as deposits in glomeruli in the kidney[34]. Immunohistochemistry performed on kidneys from aged CD19-hBtk mice showed glomerular

Figure 2. Btk overexpression specifically enhances BCR-mediated B cell activation.



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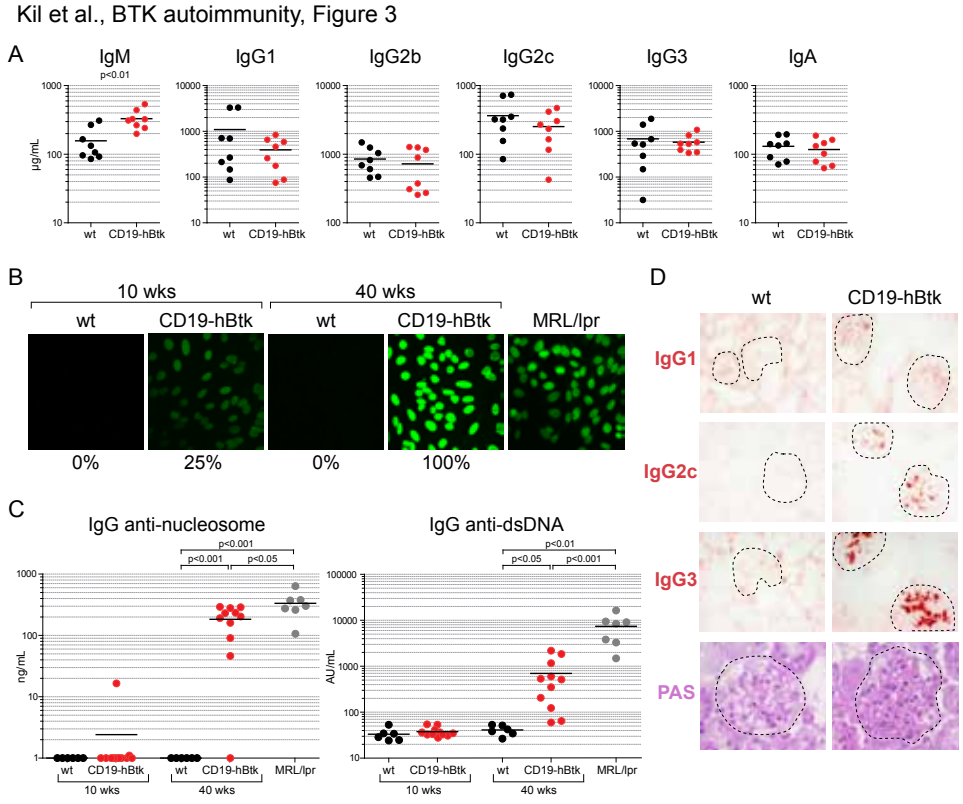
(A) Wild-type (wt; black line) and CD19-hBtk (red line) B cells were stimulated with α-IgM $F(ab')_2$ fragments and Ca^{2+} mobilization was monitored and normalized for maximum influx upon stimulation with ionomycin. Plots are representative for 4 mice of each genotype. (B) Flow cytometric analysis of *in vivo* expression of CD86, CD80 and MHCII on wildtype (gray) and CD19-hBtk (red line) $CD19^+$ B cells. Graphs below summarize the MFI levels of these expression markers on wt (black bars) and CD19-hBtk cells (red bars) (n=4 per group). (C) Flow cytometric analysis of MACS-purified wildtype (gray) and CD19-hBtk (red line) naive B cells that were cultured in the presence of the indicated stimuli for 24 hours. Data are displayed as histogram overlays of the indicated markers; Mice were 10-14 weeks old.

Figure 3. Aging CD19-hBtk mice develop systemic auto-immune disease.



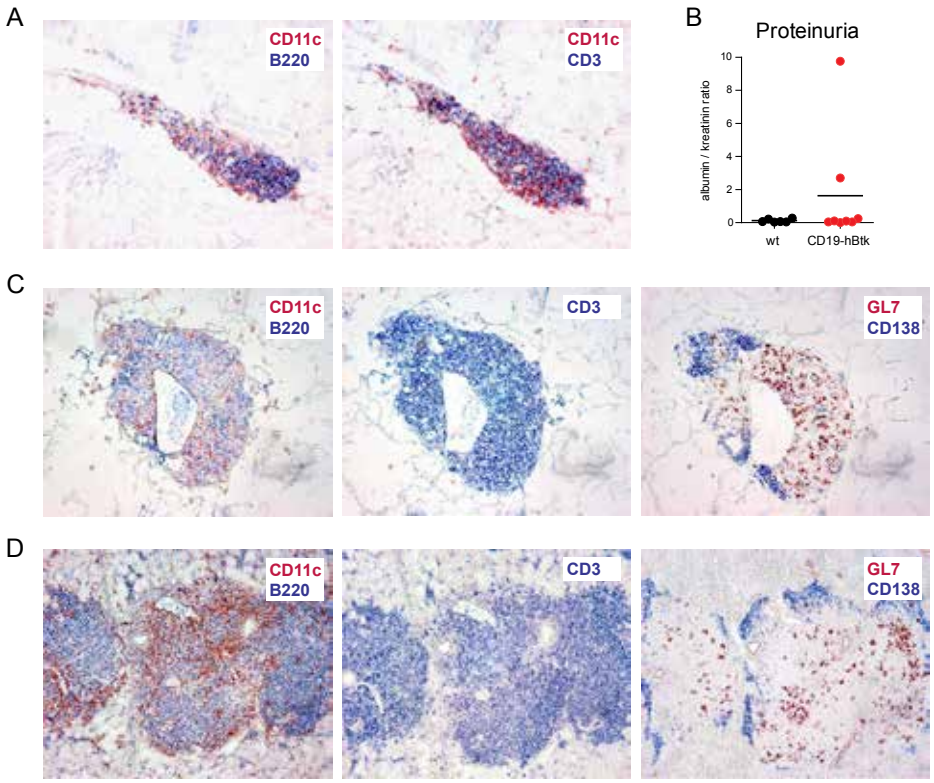
(A) Serum Ig concentrations of the indicated isotypes in 10-14-week-old CD19-hBtk mice and nontransgenic littermates, as determined by ELISA. (B) Antinuclear IgG autoantibodies, detected by HEp2 reactivity, in serum samples from CD19-hBtk mice and nontransgenic littermates, as well as serum from diseased MRL/lpr mice as reference (objective 40x). Percentages indicate proportion of animals (n=5-8 per group) with antinuclear IgG autoantibodies. (C) Serum IgG antinucleosome and anti-dsDNA autoantibody levels in the indicated mice quantified by ELISA. Each symbol represents an individual mouse. D) Glomerular deposition of immune complexes (red) of the indicated IgG isotypes, detected by immunohistochemistry on kidney tissue sections from old (>40 weeks) CD19-hBtk and wildtype (wt) littermates. Lower panels show a periodic acid-Schiff (PAS) staining on kidneys from these mice. Dashed lines encircle individual glomeruli (objective 40x). Data are representative of 5-8 CD19-hBtk mice.

immunoglobulin deposition of all IgG subclasses, and periodic acid-Schiff staining revealed glomerular hypercellularity and mesangial matrix expansion (Figure 3D).

Next, we searched in kidneys of old CD19-hBtk mice for immune cell infiltrates. We were able to identify infiltrates composed of dendritic cells, T and B cells, and occasionally plasma cells in a fraction of old CD19-hBtk mice (Figure 4A and data not shown). In congruence, some mice (~25%) developed detectable proteinuria (Figure 4B).

All CD19-hBtk mice older than 40 weeks manifested large perivascular infiltrates in the lungs, exclusively located adjacent to large veins (Figure 4C). In these infiltrates we detected dendritic cells, T and B cells, plasma cells, and frequently even GC B cells, indicative of tertiary lymphoid structure

Figure 4. Aging CD19-hBtk mice have large perivascular infiltrates in various organs.



A) Immunohistochemical analysis for dendritic cells (CD11c$^+$, red) and B cells (B220$^+$, blue) (*left panel*) or T cells (CD3$^+$, blue) (*right panel*) of kidney tissue sections from old CD19-hBtk mice (objective 10x). (B) Proteinuria in two out of eight (25%) CD19-hBtk mice; albumin levels in urine samples were measured by ELISA. (C,D) Immunohistochemical analysis for dendritic cells (CD11c$^+$, red) and B cells (B220$^+$, blue) (*left panel*), T cells (CD3$^+$, blue) (*middle panel*) or GC B cells (GL7$^+$, red) and plasma cells (CD138$^+$, blue) (*right panel*) of lungs (C) and salivary glands (D) from old CD19-hBtk mice (objective 10x). Data are representative of 5-8 CD19-hBtk mice.

formation, although T cell and B cell zones were not clearly segregated (Figure 4C). Large perivascular infiltrates with similar organization were present in salivary glands of all old CD19-hBtk mice (Figure 4D). Taken together, these data show that CD19-hBtk mice develop all hallmarks of an SLE-like autoimmune disorder, characterized by autoantibody formation including anti-dsDNA, kidney damage and perivascular infiltration of lymphocytes.

Autoimmunity when Btk overexpression is limited to mature B cells and myeloid cells

In CD19-hBtk mice, Btk is overexpressed throughout B cell differentiation (Figure S2A) and therefore enhanced BCR signaling in developing B cells might affect BCR selection in the BM. It is conceivable that an altered BCR repertoire, possibly enriched in autoreactive specificities, may contribute to the autoimmune disorder in these mice.

To analyze the effects of Btk overexpression *in vivo*, independent of possible effects on BCR repertoire selection in the BM, we took advantage of MHCII-hBtk transgenic mice. In these mice Btk overexpression is driven by the mouse MHC class II Ea gene locus control region, targeting Btk overexpression specifically to mature, peripheral B cells and myeloid cells[26-27]. In these mice, only mature IgD^+ B cells overexpressed Btk (~7 fold compared with wt) whereas developing B cells in the BM or transitional B cells in the spleen do not express transgenic Btk (Figure 5A and S3A).

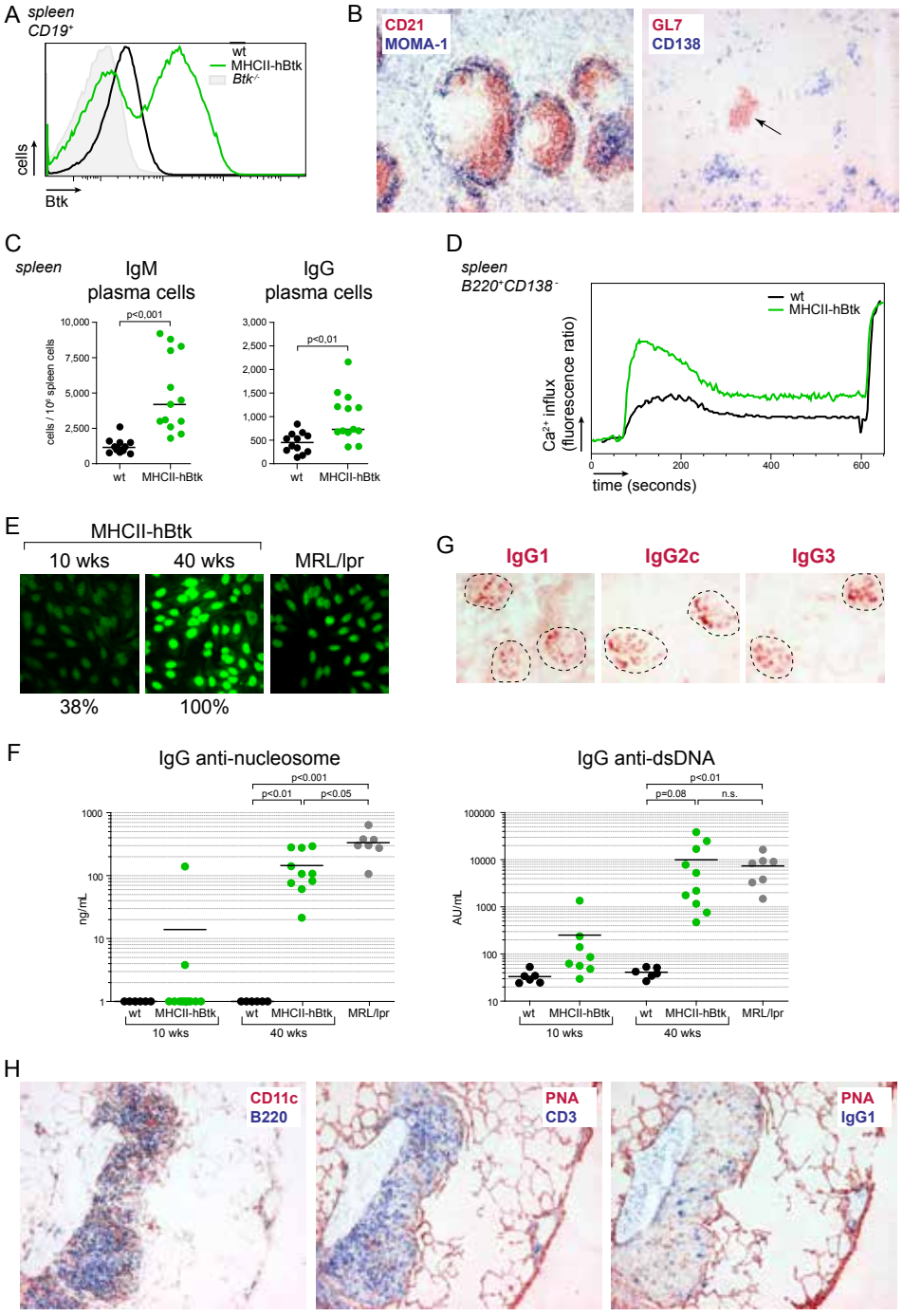
MHCII-hBtk mice (on a C57BL/6 background) manifested a phenotype that essentially resembled that of CD19-hBtk mice. Spleens of these mice contained increased numbers of GC and IgM^+ and IgG^+ plasma cells (Figure 5B and 5C). Splenic MHCII-hBtk transgenic B cells had increased surface CD86 expression (Figure S3B), displayed enhanced Ca^{2+} influx (Figure 5D) and upregulation of surface CD86, CD69 and CD25 (Figure S3C) upon BCR triggering, compared with wt B cells. Using HEp2 and ELISA assays, we detected low levels of antinuclear IgG autoantibodies in the serum of a fraction of young MHCII-hBtk mice (Figure 5E). The levels of serum IgG antinucleosome and especially anti-dsDNA autoantibodies in aging MHCII-hBtk mice were comparable to levels measured in diseased MRL/lpr mice (Figure 5F). Autoantibody reactivity in MHCII-hBtk mice was highly similar to the autoreactivity observed in CD19-hBtk mice, and was mostly restricted to histones and ribonucleoproteins, as demonstrated by line immunoassay (LIA; see Table S2)[35]. Deposits of IgG immune complexes and glomerular damage in the kidney, and perivascular inflammation in lungs and salivary glands were detected in all aging MHCII-hBtk mice (Figure 5G and data not shown).

In summary, Btk overexpression throughout B cell development in CD19-hBtk mice and in mature B cells and myeloid cells in MHCII-hBtk results in a similar phenotype. We therefore conclude that overexpression of Btk selectively in mature B cells is sufficient to induce autoimmunity.

Btk kinase activity is essential for the induction of autoimmunity by Btk overexpression

Btk can serve as a binding partner to many proteins[14] and kinase-independent functions of Btk have been described[29,36-37]. To determine whether the *in vivo* effects of Btk overexpression were dependent on Btk kinase activity, we treated CD19-hBtk mice with PCI-32765, a specific Btk-inhibitor, that irreversibly binds to C481 in the Btk kinase domain and blocks phosphorylation at Y551[33]. Three hours after administering a single dose of PCI-32765 (25 µg/g) to CD19-hBtk and wt mice, splenic B cells were

Figure 5. Btk overexpression in mature B cells is sufficient to induce autoimmunity.



➡

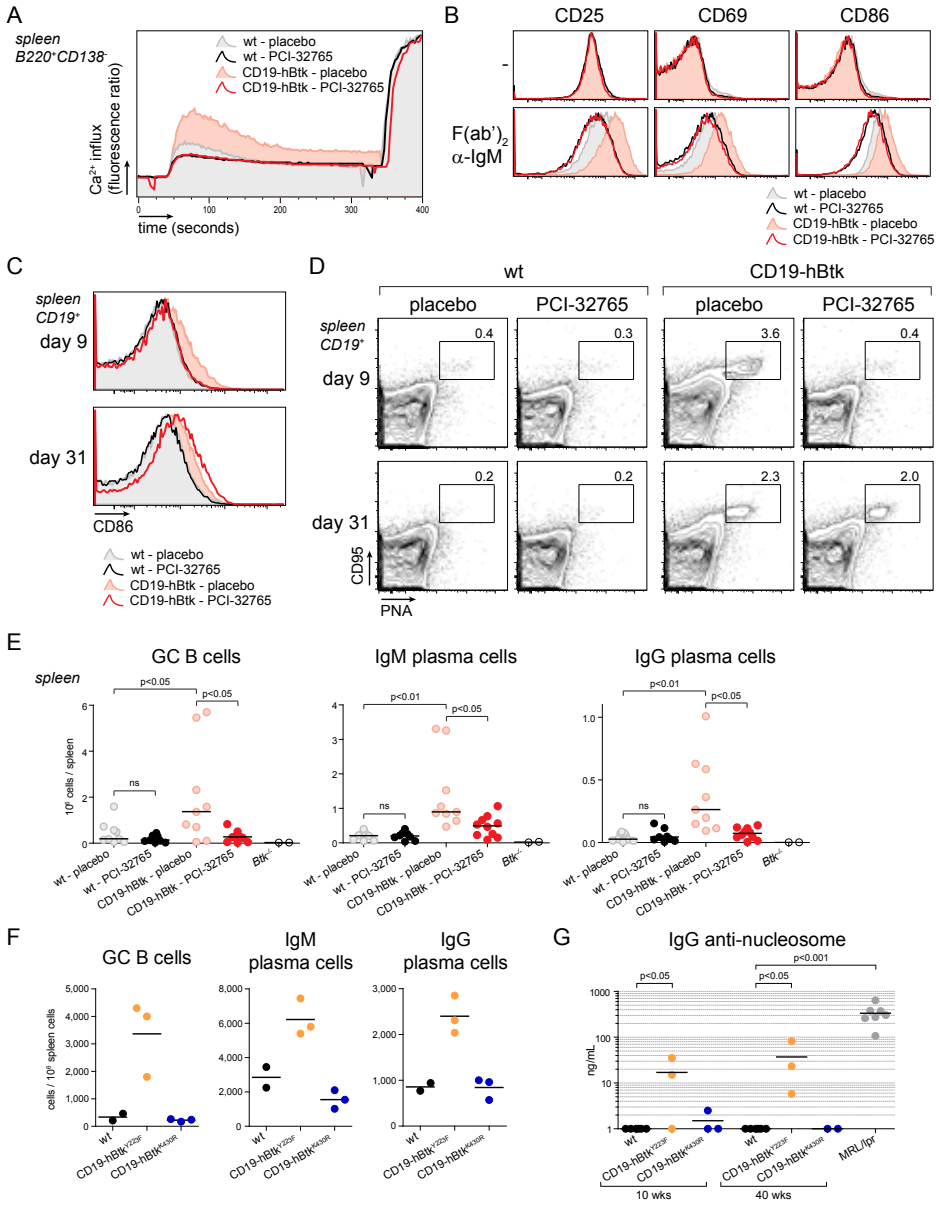
(A) Intracellular flow cytometric Btk detection in splenic CD19$^+$ B cells, shown as histogram overlays from *Btk*$^{-/-}$ (shaded histogram), wildtype (black line) and MHCII-hBtk (green line) mice. (B) Immunohistochemical analysis of CD21$^+$ B cells (red) MOMA-1$^+$ metallophilic macrophages (blue) (*left panel*) and GL7$^+$ GC B cells (red) and CD138$^+$ plasma cells (blue) (*right panel*) in the spleens of young (10-14 weeks) MHCII-hBtk mice. (C) Flow cytometric quantification of splenic IgM$^+$ and IgG$^+$ plasma cell numbers in young MHCII-hBtk mice and nontransgenic littermates (wt). (D) Wt (black line) and MHCII-hBtk (green line) B cells were stimulated with α-IgM F(ab')$_2$ fragments and Ca^{2+} mobilization was monitored and normalized for maximum influx upon stimulation with ionomycin. Plots are representative for 4 mice of each genotype. (E) HEp2 reactivity of serum IgG antibodies in aging MHCII-hBtk mice. Percentages indicate proportion of HEp2 reactive serum samples per group (objective 40x). (F) Quantification of IgG antinucleosome and anti-dsDNA autoantibodies in the indicated mice, as determined by ELISA. (G) IgG immune complex deposition in glomeruli, detected by immunohistochemistry on kidney tissue sections from old (>40 weeks) MHCII-hBtk mice. (H) Immunohistochemical analysis for dendritic cells (CD11c$^+$, red) and B cells (B220$^+$, blue) (*left panel*), GC B cells (PNA$^+$, red) and T cells (CD3$^+$, blue) (*middle panel*) or GC B cells (PNA$^+$, red) and IgG1$^+$ plasma cells (IgG1$^+$, blue)(*right panel*) in lungs from old MHCII-hBtk mice (objective 10x). Data are representative of 6 MHCII-hBtk mice.

isolated and stimulated with α-IgM *in vitro*. PCI-32765 treatment strongly reduced Ca^{2+} influx upon BCR triggering in B cells from CD19-hBtk and wt mice (Figure 6A). It also abolished hyperreactivity of CD19-hBtk transgenic B cells to BCR stimulation: α-IgM stimulated B cells from PCI-32765-treated CD19-hBtk or wt mice expressed equally low levels of CD25, CD69 and CD86 (Figure 6B).

To investigate whether prolonged PCI-32765 treatment could correct enhanced GC and plasma cell formation *in vivo*, we treated CD19-hBtk and nontransgenic mice with PCI-32765 (25 μg/g/day) for 8 consecutive days. Flow cytometric analysis revealed full normalization of CD86, CD80 and MHCII expression levels on B cells in CD19-hBtk mice at day 9 (Figure 6C, and data not shown). This effect was reversible, since B cells from PCI-32765 treated CD19-hBtk mice re-expressed high levels of CD86 at day 31 (23 days after treatment termination; Figure 6C). After prolonged PCI-32765 treatment, CD19-hBtk mice lost enhanced GC formation (Figure 6D, day 9), while termination of the treatment resulted in renewed GC formation within 23 days (Figure 6D, day 31). Likewise, IgM and IgG plasma cells numbers in the spleen were reduced at day 9 (Figure 6E) and reappeared concurrently with GC in CD19-hBtk mice after termination of PCI-32765 treatment (Figure S4A). This finding demonstrates that the enhanced GC and plasma cell formation in Btk overexpressing mice is dependent on Btk kinase function and that most plasma cells in the spleens of CD19-hBtk mice are short-lived. The 8-day treatment with PCI-32765 did not appear to affect B cell development, as the sizes of the B-lineage subpopulations in spleen and BM were not different between PCI-32765-treated versus placebo-treated CD19-hBtk mice or wt littermates (Figure S4B and S4C).

To finally show that also autoantibody formation in Btk overexpressing mice is kinase-dependent, we analyzed CD19-hBtk^{K430R} mice, with ~15-20-fold increased expression of the kinase-inactive Btk^{K430R} mutant in mature B cells[29], and CD19-hBtk^{Y223F} mice, with ~3-fold overexpression of the autophosphorylation site Y223F Btk mutant[29], both analyzed on the C57BL/6 background. Flow cytometric analysis of spleens revealed that enhanced formation of GC B cells, IgM and IgG plasma cells was not dependent on Btk autophosphorylation but did require Btk kinase activity (Figure 6F). Aging CD19-hBtk^{Y223F} but not CD19-hBtk^{K430R} mice produced antinuclear autoantibodies (Figure 6G and S4D). Consistent with these findings, old (>40 weeks) CD19-hBtk^{K430R} mice did not develop signs of autoimmune pathology in lungs, kidneys or salivary glands (data not shown).

Figure 6. BCR hyperresponsiveness of CD19-hBtk B cells is kinase-dependent.



(A) Ca^{2+} influx upon F(ab')$_2$ α-IgM stimulation of splenic naive B cells from the indicated mouse groups, 3 hours after oral administration of PCI-32765 or placebo. Signals were normalized for maximum influx upon stimulation with ionomycin. Plots are representative for 2-4 mice of each genotype. (B) Flow cytometric analysis of expression of activation markers of MACS-purified B cells from spleens of the indicated mouse groups after culture for 24 hours

with or without F(ab')$_2$ α-IgM. (C) Flow cytometric analysis of CD86 on splenic CD19$^+$ B cells of the indicated mice, after 8 days of treatment with PCI-32765 or placebo. B cells were evaluated 1 day or 23 days after termination of treatment (day 9 and 31 respectively). (D) Flow cytometric analysis of splenic CD95$^+$PNA$^+$ GC B cells, gated from CD19$^+$ B cells. Data are shown as dot plots and the percentages GC B cells are given. (E) Numbers of IgM$^+$ and IgG$^+$ splenic plasma cells 9 and 31 days after start of treatment of the indicated mouse groups. Collective data from 3 independent experiments are shown. (F) Autoimmunity in Btk overexpressing mice is dependent on Btk kinase activity, but not on Btk Y223 autophosphorylation. Flow cytometric quantification of splenic GC B cells (CD95$^+$PNA$^+$), IgM$^+$ and IgG$^+$ plasma in 10-14-week-old mice that overexpress autophosphorylation-defective Btk (CD19-hBtk^{Y223F}) or kinase-dead Btk (CD19-hBtk^{K430R}). (G) Quantification of serum IgG anti-nucleosome auto-antibodies, determined by ELISA, whereby sera of diseased MRL/lpr mice were used as a reference. Each symbol represents and individual mouse.

In summary, these data show that the induction of autoimmunity by Btk overexpression is not caused by an increase in Btk protein levels *per se*, but requires Btk kinase activity.

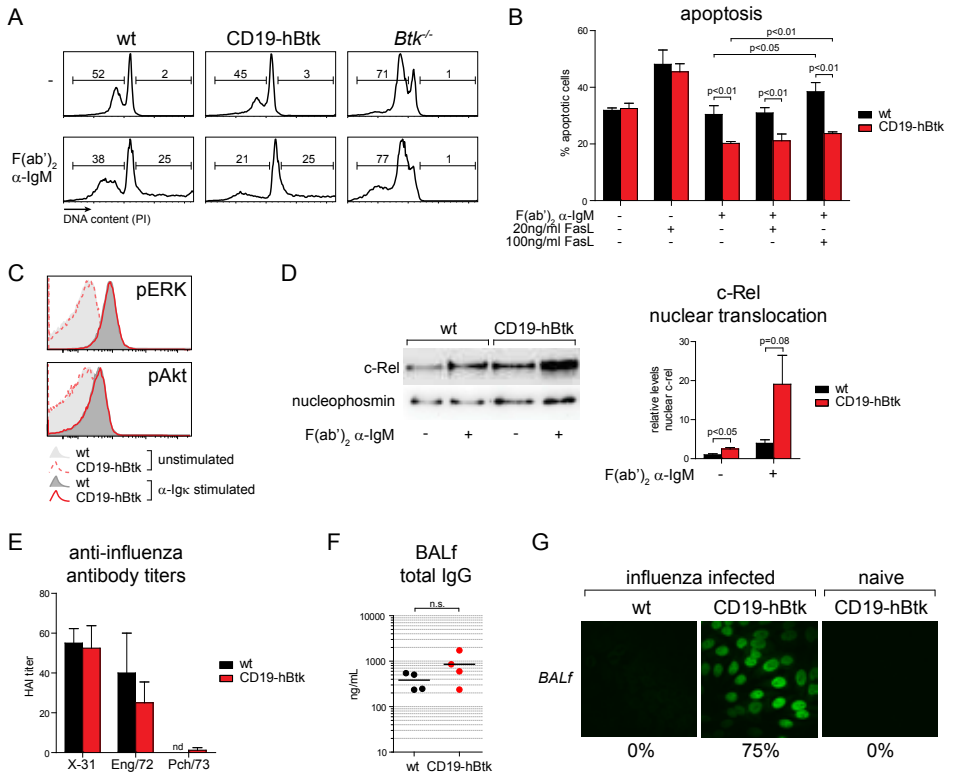
Btk overexpression impairs negative selection of autoreactive B cells

Upon activation, B cells become susceptible to Fas-mediated negative selection. As Btk is known to provide NF-κB-dependent survival signals for B cells, we studied apoptosis induction in purified Btk-overexpressing and wt B cells upon *in vitro* stimulation of BCR, CD40 and TLRs. Whereas apoptosis rates of unstimulated, α-CD40, LPS or CpG stimulated B cells were very similar in the two groups, Btk overexpression induced a clear decrease in apoptosis upon α-IgM stimulation (Figure 7A, Figure S5A, and data not shown). As a control, we included *Btk*$^{-/-}$ B cells, which showed increased susceptibility to apoptosis. To test whether this apoptosis resistance could counteract T-cell-derived pro-apoptotic signals that instruct clonal deletion of B cells, we cultured splenic CD19-hBtk and wt B cells in the presence or absence of α-IgM with increasing FasL concentrations (Figure 7B). Btk overexpression did not directly affect Fas signaling, since in the absence of BCR stimulation the apoptosis rates of FasL-stimulated CD19-hBtk cells and wt B cells were indistinguishable (Figure 7B). However, in the presence of α-IgM stimulation a dose-dependent apoptotic effect of FasL was observed in wild-type B cells, but not in CD19-hBtk B cells (Figure 7B).

Next, we analyzed signaling pathways downstream of Btk that transmit anti-apoptotic BCR signals. In BCR-stimulated CD19-hBtk or MHCII-hBtk B cells phosphorylation of ERK or Akt was not increased (Figure 7C and Figure S5B). However, nuclear translocation of the NF-κB factor c-Rel, as determined by western blotting of nuclear extracts, was more prominent in α-IgM stimulated CD19-hBtk or MHCII-hBtk transgenic than in wt B cells (Figure 7D and Figure S5C).

To study B cell selection in CD19-hBtk mice *in vivo*, we studied the T cell-dependent antibody response to influenza virus. Young CD19-hBtk and wt littermates were infected intranasally with a sublethal dose of the influenza A/HKX-31 (H3N2) strain that induces robust GC formation in lungs and draining lymph nodes[31]. The positive selection of X-31 specific B cells in CD19-hBtk mice proved to be normal, since the levels of X-31 specific antibodies in the serum 18 days post infection (dpi) were equal in CD19-hBtk and wt mice (Figure 7E). Furthermore, the production of antibodies crossreacting with X-31 related influenza strains Eng/72 and Pch/73 was similar between CD19-hBtk and wt mice (Figure 7E). Btk overexpression did not significantly affect GC B cell numbers in lungs or draining lymph nodes (data not shown), and

Figure 7. Phenotype of Btk overexpression is associated with enhanced NF-κB signaling and B cell survival.



(A) Propidium iodide (PI) DNA content analysis of MACS-sorted splenic B cells of the indicated mice after 36 hours of culture in the absence (-) or presence of F(ab')$_2$ α-IgM. Numbers indicate the proportions of apoptotic cells (sub-G1, *left gate*) or cycling cells (S/M/G2, *right gate*). (B) Frequencies of apoptotic cells, as identified by DNA content analysis of MACS sorted naive B cells from the indicated mice, cultured in the absence (-) or presence (+) of F(ab')$_2$ α-IgM and/or α-CD95/FasL antibodies. (C) MACS-purified naive splenic B cells from wildtype (wt) and CD19-hBtk mice were stimulated with α-Igκ (20 µg/ml), and phosphorylation of ERK and Akt (pERK and pAkt) was detected by intracellular flow cytometry. (D) Nuclear translocation of c-Rel in unstimulated (-) or F(ab')$_2$ α-IgM-stimulated (+) B cells from the indicated mice, analyzed by western blotting with nucleophosmin as loading control. Data were quantified after normalization for nucleophosmin levels (n=5 per group); c-Rel levels in unstimulated wt B cells were set to 1. (E) Using ELISA total IgG levels were determined in BALf collected from X-31 infected wildtype (wt) and CD19-hBtk mice at 18 dpi. (F) Serum antibody titers specific for X-31 and related virus strains Eng/72 and Pch/73 in CD19-hBtk and wt mice at 18 dpi, as determined by hemagglutinin inhibition assay (HAI). Mean values ± SEM are shown (n=8 mice/group). (G) Autoreactive IgG antibodies, detected by HEp2 reactivity of BALf collected 18 dpi from X-31 infected or naive mice, as indicated (4 mice/group; objective 40x used).

total IgG levels in bronchoalveolar lavage fluid (BALf) of infected CD19-hBtk mice were modestly, but not significantly, increased at 18 dpi (Figure 7F). However, screening of bronchoalveolar lavage fluid (BALf) for local production of autoantibodies in the lungs revealed IgG antinuclear autoantibodies in 75% of the BALf samples obtained at 18 dpi from influenza infected CD19-hBtk mice (Figure 7G). These autoantibodies were not detected in BALf from infected wt littermates or non-infected CD19-hBtk littermates (Figure 7G), demonstrating defective elimination of locally emerging autoreactive IgG plasma cells in lungs of X-31 infected CD19-hBtk mice.

Collectively, these data show that Btk overexpression induces apoptosis resistance and enhanced NF-κB activation *in vitro* upon BCR stimulation, which interferes *in vivo* with negative selection of B cells with acquired affinity for self-antigens during GC driven antibody responses.

Discussion

In this study, we used B cell-specific transgenic overexpression of wildtype Btk driven by the CD19 promoter region to unravel a crucial function of this tyrosine kinase signaling protein in setting the threshold for B cell activation and negative selection of autoreactive B cells. B lymphocytes overexpressing Btk developed normally, but showed enhanced GC and plasma cell formation. Aging Btk transgenic mice developed systemic autoimmune disease, characterized by significant levels of circulating IgG anti-nuclear auto-antibodies and inflammation of lungs, salivary glands, and kidneys. Autoimmunity was fully dependent on Btk kinase activity, because the phenotype was corrected by Btk inhibitor treatment and was not present in mice overexpressing kinase-inactive Btk. B lymphocytes overexpressing Btk demonstrated a selective hyperresponsiveness to BCR stimulation, associated with increased Ca^{2+}-influx and enhanced upregulation of cell surface activation markers. Moreover, BCR stimulation of these cells induced increased NF-κB activation and resistance to Fas-mediated apoptosis. Finally, we show defective negative selection of autoreactive B cells arising in an *in vivo* influenza infection model.

Many previous observations, including reduced survival of Btk-deficient B cells, the identification of several inhibitory factors of Btk, and the multiple (post)transcriptional mechanisms by which Btk expression is increased upon BCR stimulation[11,19-23 17-18], prompted us to investigate whether the expression level of Btk is a critical factor for correct B cell functioning. In this study, we have shown that Btk upregulation does not only occur upon BCR signaling through Btk, but seems to be inherent to B cell activation, since CD40 or TLR stimulation also instruct Btk upregulation. Using transgenic mice with B cell-specific overexpression of wt human Btk, we were able to demonstrate that high Btk levels exclusively enhance BCR signaling. BCR stimulation of CD19-hBtk B cells evoked stronger Ca^{2+} mobilization and upregulation of activation markers, while increased activation was not observed upon stimulation of CD40 or TLR's, even while TLR4 and TLR9 also employ Btk[14]. We therefore propose that Btk upregulation in B cells upon activation by BCR, CD40 or TLR signals primarily serves to sensitize the B cell for antigen recognition through the BCR. Dynamic regulation of Btk levels of activated B cells may thus aid the proper activation and selection of antigen-activated B cells.

Using CD19-hBtk mice, we demonstrated that continuous Btk overexpression induced spontaneous

B cell activation *in vivo*, characterized by upregulation of surface molecules specifically involved in B-T cell interactions, leading to spontaneous differentiation into GC B cells and IgG plasma cells. Clearly, the selection of activated Btk overexpressing B cells was defective, since IgG plasma cells in aging CD19-hBtk mice produced high levels of antinuclear autoantibodies that elicited systemic autoimmune disease. From our data, it is unclear whether Btk overexpressing B cells have the capacity to breach tolerance of quiescent selfreactive T cells, which would ensure sufficient reciprocal stimulation to enable positive selection[1,38]. However, it is conceivable that originally non-autoreactive B cells that enter GCs may acquire selfreactive BCR's through somatic hypermutation, whereby Btk overexpression impairs negative selection of these cells. Influenza infection in CD19-hBtk mice showed that normal antiviral antibody titers were produced with normal antiviral crossreactivity, but selfreactive B cells emerged locally in the lung, leading to autoantibody secretion in BAL fluid. Based on the strictly anti-nuclear and not anti-cytoplasmatic specificity of these BALf autoantibodies in virus- infected CD19-hBtk mice and based on the comparable total IgG levels in BALf in CD19-hBtk and wt mice, it is less likely that these autoantibodies originate from a generally more robust antibody response in Btk transgenic mice. We propose a model for defective counterselection of autoreactive Btk-overexpressing B cells, based on recent findings showing that T cell help is the limiting factor in GC selection[39] (Supplementary Figure S6). In support of defective counterselection of selfreactive B cells, Btk overexpressing B cells proved to be insensitive to Fas-induced apoptosis once activated through their BCR, and this apoptosis resistance correlated to increased activation of NF-κB, a major downstream target of Btk that strongly promotes B cell survival[7-9].

To the best of our knowledge, Btk has not been directly implicated in the pathogenesis of autoimmune disease before. Although a previous study demonstrated that abrogated expression of miRNA's including miR185 in mature B cells increased Btk expression and induced systemic autoimmunity[23], many other defects in miRNA-deficient B cells may contribute to the development of autoimmunity. We previously reported that B cell-restricted expression of the constitutively active Btk^{E41K} mutant did not evoke autoimmune disease. We proposed that strong Btk^{E41K} mediated signaling may mimic self-antigen binding to the BCR, thereby instructing massive B cell deletion[26-28]. Nevertheless, residual B cells were hyperresponsive and spontaneously driven into GC-independent plasma cell differentiation. The critical difference between Btk^{E41K} transgenic and Btk overexpressing mice lies in the mode of increase in Btk kinase activity, which is constitutive versus conditional, respectively. Also other mouse models with constitutively increased BCR signaling often display aberrant B cell development without overt autoimmune pathology, e.g. mice with B cell-specific expression of the latent membrane protein 2A, an Epstein-Barr virus encoded protein that substitutes for BCR-derived activation signals[40-41], or mice overexpressing the stimulatory BCR co-receptor CD19[42]. In contrast, autoimmune pathology does develop in mouse models with conditionally enhanced BCR signaling, including mice deficient for the inhibitory BCR co-receptors FcγRIIB [43] or CD22 and Siglec-G[44], and mice deficient for Shp1 phosphatase[45-46] or the regulatory kinase Lyn[47]. But, direct linking of BCR signaling aberrations to autoimmunity is complicated in these models, since these molecules are not exclusively involved in BCR signaling or have additional functions in non-B cells. However, the finding that B cell-specific Btk overexpression affects BCR but not TLR signaling and results in autoimmunity, reinforces that enhanced

signaling upon BCR engagement in B cells is sufficient to induce autoimmune pathology.

Although mutations leading to enhanced Btk activity have not been identified in human subjects to date, our current study underlines that not hardwired mutations, but instead dysregulated expression of kinases downstream of the BCR may be the key to erroneous activation of selfreactive B cells. Moreover, the influence of TLR and CD40 signaling on Btk expression levels in mature B cells suggests that the commonly observed BCR hyperresponsiveness in SLE patients may not always directly result from intrinsic BCR signaling defects, but indirectly from aberrant signaling of other receptors that amplify the BCR signaling strength through upregulation of Btk. Targeting Btk in systemic autoimmune disease may therefore present an attractive new therapeutic approach, and the strong *in vivo* effects of Btk-inhibitor treatment on B cell activation in Btk overexpressing mice confirm the high potential of these new therapeutic agents in autoimmune disease, as well as B cell malignancies, including chronic lymphocytic leukemia and diffuse large B cell lymphoma[24-25]. It is reassuring that no adverse effects of Btk inhibition on B cell development were observed, possibly due to the limited inhibitor half-life and short time window of exposure. On the other hand, the finding of in vivo pathology associated with Btk overexpression may have important implications for the development of future strategies for gene therapy for XLA[14].

Acknowledgements

We thank the Erasmus MC experimental animal facility for their care for our mice and assistance at experiments, and we thank Allard Kaptein and Hans van Eenennaam for their advice with Btk inhibitor treatment experiments. This work was partly supported by the Dutch Arthritis Association (grants 08-1-103 and 09-1-302).

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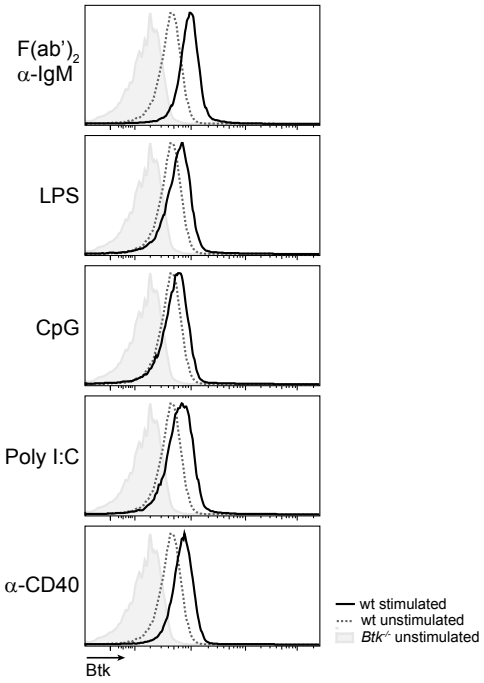
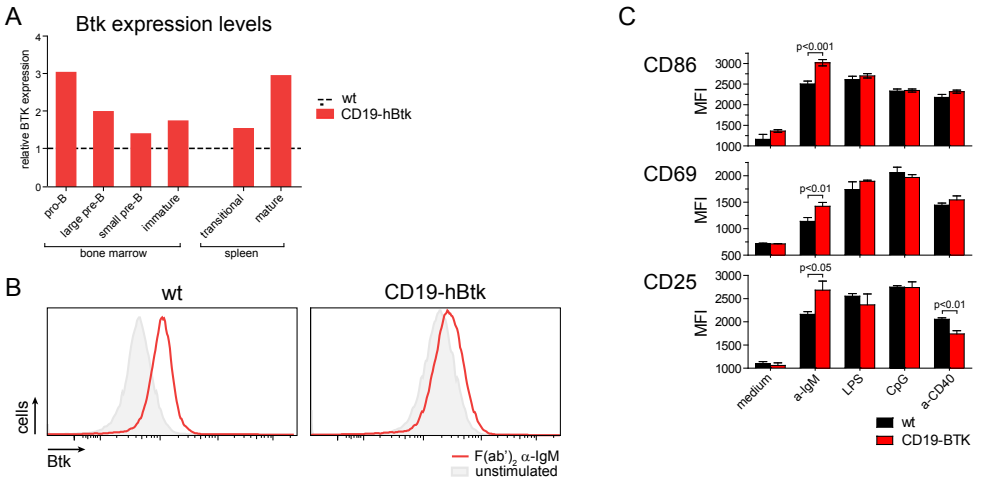
Supplemental data

Figure S1. Expression levels of Btk are upregulated upon B cell stimulation.



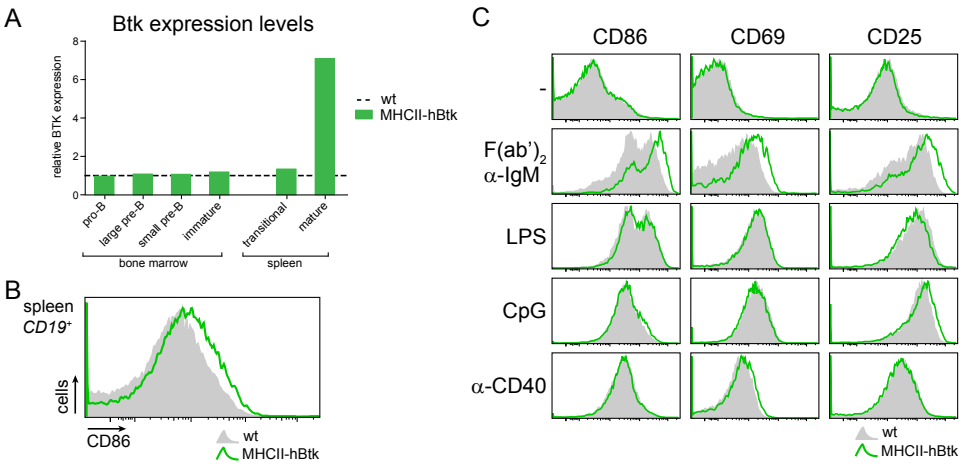
Intracellular flow cytometric detection of Btk protein in wt splenic B cells after 3 days of *in vitro* culture in the presence of the indicated stimuli. Only lymphocytes with low FSC values were included in this analysis for comparison of cells with comparable cytoplasmic content. Data are displayed as histogram overlays and are representative of 8-10 mice analysed. Mice were 10-14 weeks old.

Figure S2. Btk expression levels and its effects on B cell activation in CD19-hBtk mice.



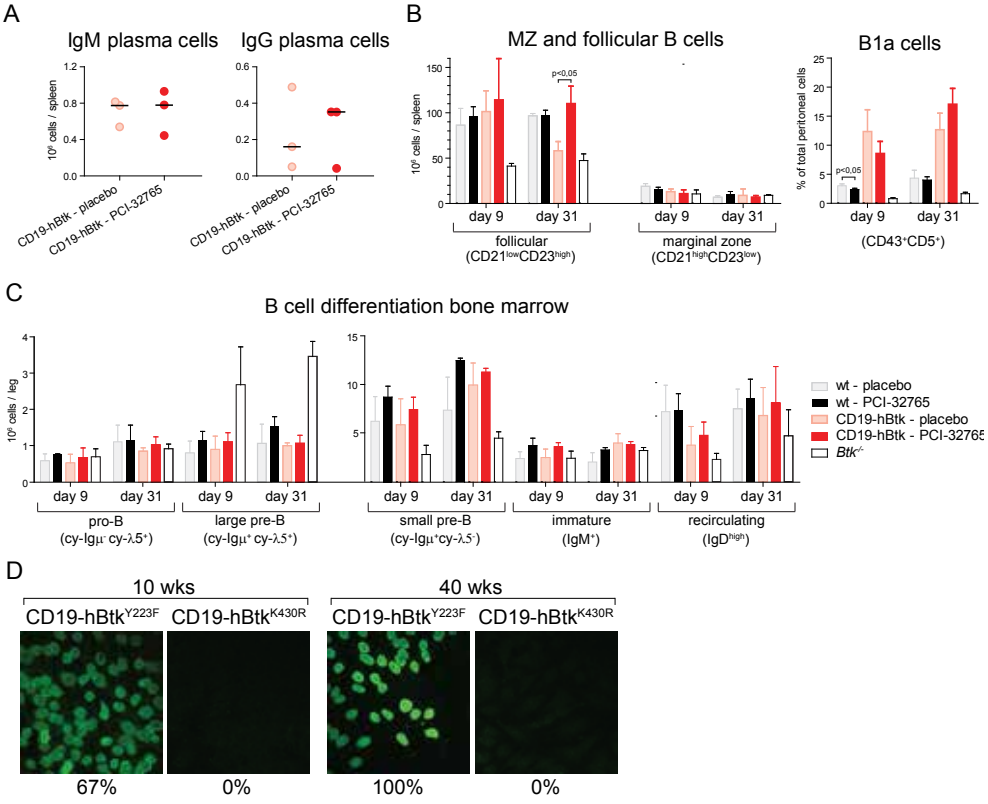
(A) Intracellular flow cytometric detection of Btk protein. From wildtype (wt) mice, *Btk*$^{-/-}$ mice, and CD19-hBtk mice deficient for mouse Btk (n=2 per group), bone marrow B cells were gated into pro-B (Igκ^{-}Igλ^{-}cy-Igμ^{-}cy-λ5^{+}), large pre-B (Igκ^{-}Igλ^{-}cy-Igμ^{+}cy-λ5^{+}), small pre-B (Igκ^{-}Igλ^{-}cy-Igμ^{+}cy-λ5^{-}), and immature (IgM^{+}IgD^{low}) B cell fractions, and splenic B cells were gated into transitional (IgM^{high}IgD^{low}) and mature (IgM^{low}IgD^{high}) B cells. Per B cell fraction, relative median levels of Btk expression by the CD19-hBtk transgene were calculated in comparison to average Btk levels in wildtype B cells (set to 1), and background levels in *Btk*$^{-/-}$ cells (set to 0). (B) Intracellular flow cytometric detection of Btk expression levels in splenic naive B cells from CD19-hBtk mice and non-transgenic littermates that were cultured for 72 hours with or without F(ab')$_2$ α-IgM. (C) Flow cytometric analysis of MACS-purified wildtype (black) and CD19-hBtk (red) naive B cells that were cultured in the presence of the indicated stimuli for 24 hours. Data are displayed as histogram overlays in Figure 3C. Graphs summarize the MFI levels of the indicated markers (n=4 per group). Mice were 10-14 weeks old.

Figure S3. Btk expression levels and its effects on B cell activation in MHCII-hBtk mice.



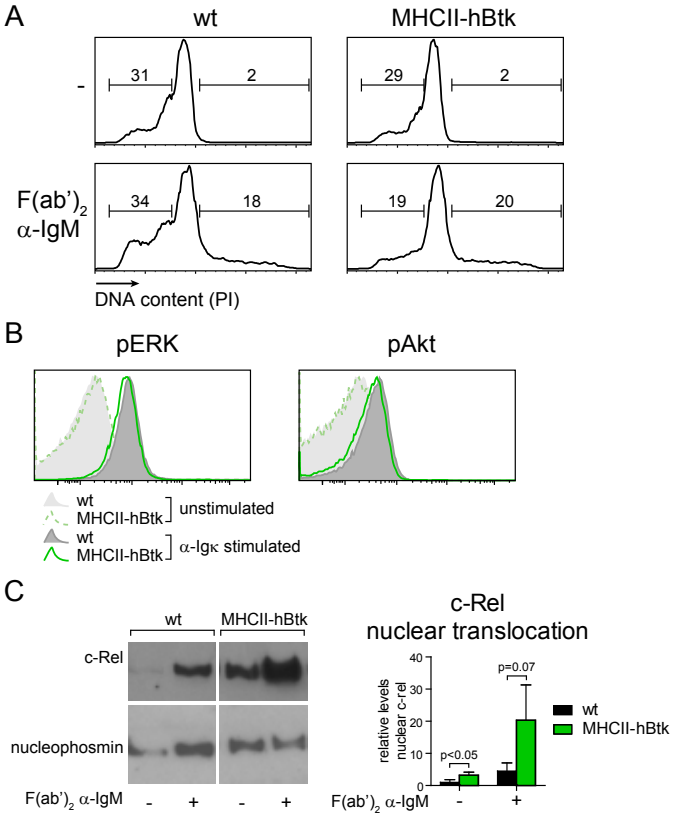
(A) Intracellular flow cytometric detection of Btk protein (see legend to Figure S1 for gating of B-lineage subpopulations and for calculation of Btk levels). (B) Flow cytometric analysis of CD86 expression on splenic B cells from young (10-14 weeks) MHCII-hBtk mice and non-transgenic littermates. (C) MHCII-hBtk B cells are hyperresponsive only to BCR stimulation. Flow cytometric analysis of various expression markers on MACS-purified naive splenic B cells that have been cultured *in vitro* for 24 hours in the presence of the indicated stimuli.

Figure S4. Effects of inhibiting Btk kinase activity on B cell development and autoimmunity.



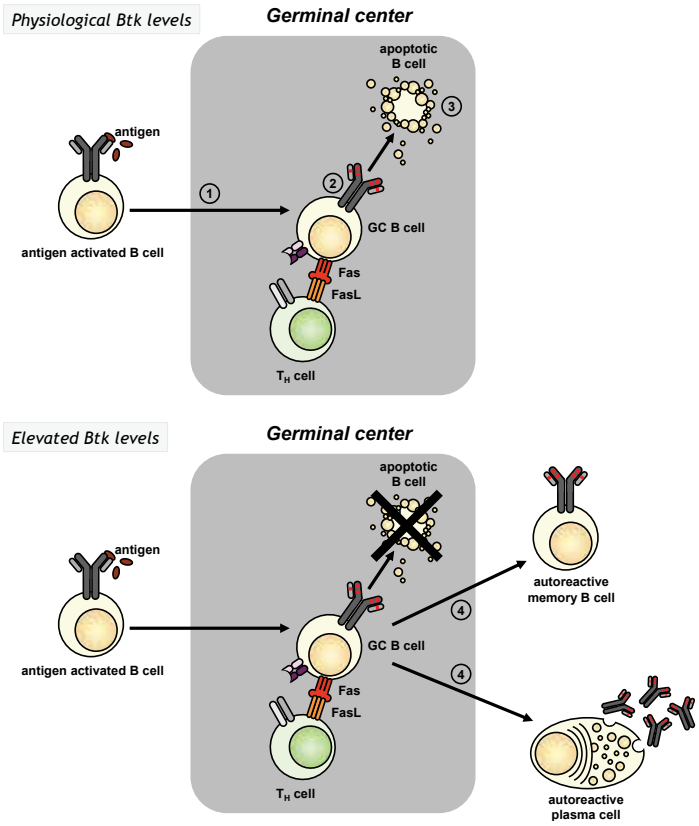
(A) Flow cytometric quantification of splenic IgM$^+$ and IgG$^+$ plasma cells numbers at 23 days after termination of treatment of CD19-hBtk mice with PCI-32765 versus placebo. (B) Quantification of splenic and peritoneal B cell populations and (C) bone marrow B-lineage subpopulations in wildtype and CD19-hBtk mice after 8 days of treatment with Btk-inhibitor PCI-32765 versus placebo. *Btk*$^{-/-}$ mice were included for comparison. For quantification of B cell subpopulations using flow cytometry, B cells (CD19$^+$) were further gated using the indicated markers. Data are from 3 mice in each group. (D) IgG reactivity with HEp2 cells in serum samples obtained from CD19-hBtk^{Y223F} and CD19-hBtk^{K430R} mice of 10 and 40 weeks old (n=3 per genotype per age; objective 40x).

Figure S5. Enhanced B cell survival and NF-κB activation in BCR-stimulated MHCII-hBtk B cells.
(A) Flow cytometric cell cycle analysis using DNA staining by propidium iodide (PI) of MACS-sorted splenic B cells



of the indicated mice after 36 hours of culture in the absence (-) or presence of F(ab')$_2$ α-IgM. Numbers indicate the proportions of apoptotic cells (sub-G1, *left gate*) or cycling cells (S/M/G2, *right gate*). (B) After α-Igκ (20 μg/ml) stimulation of MACS-purified naive splenic B cells from wildtype (wt) and MHCII-hBtk mice, phosphorylation of ERK and Akt (pERK and pAkt) was detected by intracellular flow cytometry. (C) Western blotting analysis of c-Rel in nuclear lysates of unstimulated (-) or F(ab')$_2$ α-IgM-stimulated (+) B cells from wildtype (wt) and MHCII-hBtk mice. Nucleophosmin levels were measured as loading control. Graph shows relative nuclear c-Rel levels (normalized for nucleophosmin expression, whereby c-Rel levels in unstimulated wt B cells were set to 1; n=3 per group).

Figure S6. Defective counterselection of autoreactive Btk overexpressing B cells.



Under physiological circumstances (*upper panel*), B cells may enter GCs after antigen recognition by the BCR (1), where somatic hypermutation of the variable region of the BCR can change the affinity of antigen-activated B cells for their antigen. Some mutations may abolish the antigen specificity of the BCR and even render the BCR autoreactive (2). Such autoreactive B cells will not be able to present antigen peptides to antigen specific T_H cells, and will thus be deprived of T_H cell-derived costimulation. Instead T_H cells will instruct apoptosis of the autoreactive B cells through FasL-Fas interactions (3).
Enhanced BCR signaling established by increased expression levels of Btk (*lower panel*) can rescue autoreactive B cells from apoptosis, and the abundance of apoptotic material in GCs may further support the selective survival of B cells with anti-nuclear BCR specificities. After escaping apoptosis, these autoreactive B cells are allowed to differentiate into memory B cells or plasma cells (4) that drive SLE-like autoimmune disease.

Supplemental tables

Table S1. B cell development and mature B cell subsets in CD19-hBtk mice.

	Wt (n=9)	CD19-hBtk (n=7)	P-value
Spleen			
Total cells [1)]	194,3 ± 31,9 [2)]	168,6 ± 20,4	n.s.
Total B cells ($CD19^+$)	106,5 ± 20,6	82,8 ± 10,2	0,015
B1a cells ($CD43^+CD5^+$)	8,2 ± 1,4	14,0 ± 1,2	<0,001
B1b cells ($CD43^+CD5^-$)	2,1 ± 0,5	1,7 ± 0,2	n.s.
B2 cells ($CD43^-$)	99,5 ± 20,2	66,4 ± 9,7	0,001
Follicular ($CD23^{high}CD21^{low}$)	90,5 ± 16,5	58,8 ± 8,5	<0,001
Marginal zone ($CD23^{low}CD21^{high}$)	12,2 ± 3,1	7,1 ± 1,5	0,001
$CD23^{low}CD21^{low}$	8,1 ± 2,4	16,1 ± 2,1	<0,001
Mature (IgD^{high})	86,9 ± 15,6	59,1 ± 8,5	<0,001
Transitional ($IgD^{low}IgM^{high}$)	9,9 ± 2,7	10,0 ± 2,2	n.s.
$IgD^{low}IgM^{low}$	9,7 ± 2,8	13,7 ± 2,2	0,009
Bone marrow			
Total cells	66,1 ± 13,5	77,8 ± 12,6	n.s.
Total B cells ($CD19^+$)	17,6 ± 3,1	18,1 ± 3,0	n.s.
Pro-B ($cy\text{-}Ig\mu cy\text{-}\lambda5^+$)	0,61 ± 0,19	0,55 ± 0,20	n.s.
Large pre-B ($cy\text{-}Ig\mu^+cy\text{-}\lambda5^+$)	0,68 ± 0,15	0,63 ± 0,14	n.s.
Small pre-B ($cy\text{-}Ig\mu^+cy\text{-}\lambda5^-$)	6,5 ± 1,6	7,8 ± 1,6	n.s.
Immature (IgM^+IgD^{low})	2,4 ± 0,5	3,6 ± 0,8	0,002
Recirculating (IgD^{high})	4,9 ± 1,7	2,9 ± 0,8	0,013

[1)] Using flow cytometry, B cell numbers in 10-14 wks old CD19-hBtk mice and wildtype littermates were quantified per subset or differentiation stage in spleen or bone marrow (bone marrow cells obtained from one leg). B cell subpopulations were gated using the indicated markers;
[2)] Numbers represent mean ± standard deviation (x 10^6 cells).

Table S2. Autoantibody reactivity in aging CD19-hBtk and MHCII-hBtk mice.

Genotype		Reactivity of autoantibodies [1])
wt	#1	-
	#2	-
	#3	-
CD19-hBtk	#1	Histones (++)
	#2	Histones (++)
	#3	Histones (++), RNP-C (++)
MHCII-hBtk	#1	Histones (++), RNP-C (++), SS-B (++)
	#2	Histones (++), RNP-C (++)
	#3	Histones (++), RNP-C (++)

[1]) Using a line immunoassay (LIA), the reactivity of autoantibodies to nuclear autoantigens was determined in serum obtained from >40 weeks old CD19-hBtk, MHCII-hBtk and wildtype mice (n=3 per group).

Table S3. Antibodies used in this study, grouped per experimental procedure.

Antigen	Clone	Conjugate	Manufacturer
Flow cytometry			
Akt(pS473)	M89-61	AlexaFluor®647	BD biosciences
B220	RA3-6B2	AlexaFluor®700	eBioscience
B220	RA3-6B2	FITC	eBioscience
BTK	53/BTK	PE	BD biosciences
CD5	53-7.3	APC	BD biosciences
CD19	ID3	PerCP-Cy™5.5	eBioscience
CD21/CD35	7G6	FITC	BD biosciences
CD23	B3B4	PE-Cy™7	eBioscience
CD25	PC61.5	PE-Cy™7	eBioscience
CD43	S7	PE	BD biosciences
CD69	H1.2F3	PE	BD biosciences
CD80	16-10A1	PE	BD biosciences
CD86	GL1	APC	eBioscience
CD95	Jo2	PE	BD biosciences
CD138	281-2	PE	BD biosciences
CD179b	LM34	biotin	BD biosciences
ERK1/2(pT202/pY204)	20A	AlexaFluor®488	BD biosciences
IgD	11-26c.2a	FITC	BD biosciences
IgG1	A85-1	FITC	BD biosciences
IgG2a/b[1]	R19-5	biotin	BD biosciences
IgG3	R40-82	biotin	BD biosciences
IgM	II/41	PE-Cy™7	eBioscience
MHCII	M5/114.15.2	FITC	eBioscience
PNA[2]	-	biotin	Sigma-Aldrich
Streptavidin[3]	-	APC-eFluor®780	eBioscience
MACS purification			
CD5	53-7.3	biotin	BD biosciences
CD11b	M1/70	biotin	BD biosciences
CD43	S7	biotin	BD biosciences
CD138	281-2	biotin	BD biosciences
Gr-1	RB6-8C5	biotin	BD biosciences
TER-119	PK136	biotin	BD biosciences

Table S3 (continued). Antibodies used in this study, grouped per experimental procedure.

Antigen	Clone	Conjugate	Manufacturer
Immunohistochemistry			
B220	RA3-6B2	-	Bioceros
CD3	KT3	-	Bioceros
CD138	281-2	PE	BD biosciences
GL7	GL7	FITC	BD biosciences
IgG1	A85-1	FITC	BD biosciences
IgG2a[4]	R19-15	FITC	BD biosciences
IgG3	R40-82	FITC	BD biosciences
PNA[2]	-	biotin	Sigma-Aldrich
Anti-hamster IgG	-	alkaline phosphatase	Jackson ImmunoResearch
Anti-rat IgG	-	alkaline phosphatase	Jackson ImmunoResearch
Anti-FITC	-	peroxidase	Rockland Immunochemicals
Anti-PE	-	peroxidase	Rockland Immunochemicals
Streptavidin[3]	-	peroxidase	Sigma-Aldrich

1 Reactive with IgG2b/c in C57BL/6 mice
2 PNA (peanut agglutinin) recognizes Gal-β(1-3)-GalNAc carbohydrates
3 Used for labeling of biotin
4 Reactive with IgG2c in C57BL/6

5

Enhanced Btk signaling in B cells drives autoimmunity by disrupting T cell homeostasis

Laurens P. Kil, Marjolein J.W. de Bruijn, Odilia B.J. Corneth, Patrick Asmawidjaja, Erik Lubberts, and Rudi W. Hendriks

Abstract

Inhibition of the B cell receptor (BCR) signaling molecule Bruton's tyrosine kinase ameliorates lupus and experimental arthritis in autoimmune mouse models. T cells do not express Btk but nevertheless exhibit a less activated phenotype upon Btk-inhibitor treatment in these models. To determine whether T cells are crucial for Btk-induced autoimmunity, we used mice that spontaneously develop lupus due to B cell-confined transgenic Btk overexpression (CD19-hBtk). Here we show that Btk signaling in B cells enhances ICOS upregulation on T cells, follicular T helper (T_{FH}) cell formation and IFN-γ secretion by $CD4^+$ T cells. In a collagen-induced arthritis model Btk overexpression in B cells enhanced arthritis and promoted T_{FH} formation in draining lymph nodes. The enhanced T cell activation by Btk-overexpressing B cells relied on altered BCR signaling, because enhanced in vitro activation of T cells only occurred in co-culture with anti-IgM stimulated CD19-hBtk B cells. Pathogenic T cell induction was indispensable for autoimmunity in CD19-hBtk mice since CD40L-deficiency fully corrected IgG plasma cell formation and normalized TFH cell numbers. Furthermore, GC formation in these mice could be greatly exaggerated by introduction of a T cell receptor (TCR) transgene that corrupts the $CD4^+$ T cell TCR repertoire and minimizes regulatory T cell numbers. Collectively, these data demonstrate that increased Btk signaling selectively in B cells is sufficient to induce T cell activation and to enhance T_{FH} differentiation, resulting in the establishment of a T cell propagated autoimmune disease. Our findings imply that Btk-targeting therapies in SLE may effectively improve T cell tolerance.

This study is financially supported by the Dutch Arthritis Association (grants 08-1-103 and 09-1-302)
In preparation

Introduction

Systemic lupus erythematosus (SLE) is a rheumatic autoimmune disease characterized by a breakdown in B cell tolerance to nuclear self-antigens, resulting in anti-nuclear autoantibody production and subsequent immune complex-mediated systemic inflammation (1). Although genome-wide association studies in patients and genetic mouse models indicate that B cell-intrinsic defects may be central to SLE development, the aberrant emergence, activation and persistence of autoreactive B cells in SLE is assisted by non-B cells (2). While in murine models B cell-specific single genetic defects in inhibitory co-receptors or signaling molecules of the B cell receptor (BCR) can induce SLE (3), it is largely unknown if and how B cells in these models propagate disease through the corruption of other immune cells or whether they mainly act solo.

Vice versa, the contribution of pathogenic follicular T_H cells (T_{FH} cells) to SLE development through autoreactive B cell activation in germinal centers (GCs) resulting in the formation of plasma cells producing high-affinity auto-antibodies is well-characterized (4). Antigen-presenting dendritic cells (DCs) prime T_{FH} responses by inducing upregulation of CXCR5 and inducible costimulator (ICOS) on T_{FH} cells required for their follicular localization and differentiation respectively (5). B cells however are thought not to prime but to consolidate T_{FH} differentiation and to support further expansion and functioning of these T_{FH} cells (6). B cells do not only guide T_{FH} responses through the production of IL-6 (7) or ICOS-L expression (8), but B cells mainly provide antigenic stimulation to T_{FH} cells when antigen availability is waning during GC reactions (9). Since T_{FH} cells dictate the positive and negative selection of GC B cells by providing differentiation and activation signals through IL-21 and CD40L and apoptotic signals through CD95L, disturbances in T_{FH} function therefore lead to severe lupus in mice (10, 11). In parallel, in SLE patients blood $CXCR5^+$ T_H cell numbers that probably represent the circulating memory counterpart of T_{FH} cells correlate with disease severity and auto-antibody diversity (12, 13), and high ICOS expression is observed on circulating and kidney-infiltrating T_H cells (14).

Given the contribution of B cells to the activation of pathogenic T_{FH} cells, in combination with the extensive evidence for a pathogenic role for dysregulated B cell activation in SLE, it is essential to identify B cell molecules that may drive pathogenic co-stimulatory loops between T_{FH} cells and autoreactive B cells. We recently found that enhanced expression of the BCR signaling molecule Bruton's tyrosine kinase (Btk) confined to B cells by B cell-targeted transgenic Btk overexpression is sufficient to induce a lupus-like disease in CD19-hBtk mice through enhanced BCR-mediated B cell activation, enhanced NF-kB induction and a resulting apoptosis resistance (15). A pathogenic role for Btk in rheumatic diseases is further emerging from murine studies demonstrating that the selective Btk inhibitors PCI-32765 (Ibrutinib), CGI1746 and RN486 prevent or ameliorate lupus nephritis and experimental arthritis (16-21). Btk functions in other immune cells as well downstream multiple receptors including chemokine receptors and toll-like receptors (22). Therefore, Btk-inhibition in these models dampens – next to BCR-induced B cell proliferation and autoantibody production – also FcγR signaling in monocytes and macrophages, FcεR signaling in mast cells and joint infiltration by myeloid cells. Strikingly, apart from direct effects on Btk-expressing cells diminished activation of T helper (T_H) cells, which do not express Btk, is also observed (16), raising the question how these T_H cells are indirectly activated in a Btk-dependent manner.

To elucidate how Btk signaling may indirectly engage pathogenic T cells in SLE, we investigated T cell activation, differentiation and B-T cell interactions in CD19-hBtk mice. Here, we demonstrate that in these mice T_{FH} populations are expanded, not only during SLE development but also following induction of experimental arthritis and upon T-dependent immunizations. Importantly, we show that increased Btk signaling selectively downstream of the BCR could enhance T_H cell activation in vitro. The induction of pathogenic T cells proved to be absolutely required for Btk-driven autoimmunity, since blocking T cell help to B cells by CD40L-deficiency completely abrogated IgG plasma cell formation. As collectively these data demonstrate that enhanced Btk signaling in B cells can establish a T cell driven pro-inflammatory loop resulting in autoimmunity, this implies that Btk-targeting therapies in SLE patients may effectively improve T cell tolerance.

Materials & methods

Mice and genotyping

The generation of CD19-hBtk (23), OT-II (24) and *Cd40l*$^{-/-}$ (25) mice has been described previously. Prior to intercrossing and analysis, these mouse strains were backcrossed to the c57bl/6 genetic background for >10 generations. Mice were genotyped by PCR using primers specific for the CD19-hBtk transgene (5'-CCTTCCAAGTCCTGGCAT-3' forward primer and 5'-CACCAGTCTATTTACAGAGA-3' reverse primer), the OT-II construct (5'-AAAGGGAGAAAAAGCTCTCC-3' forward primer and 5'-ACACAGCAGGTTCTGGGTTC-3' reverse primer), *Cd40l* wildtype alleles (5'-GTTCCTCCACCTAGTCATTCATC-3' forward primer and 5'-CCCAAGTGTATGAGCATGTGTGT-3' reverse primer) and *Cd40l* knockout alleles (5'-GCCCTGAATGAACTGCAGGACG-3' forward primer and 5'-GGGTAGCCAACGCTATGTC-3' reverse primer). All mice were kept at specified pathogen free conditions at the Erasmus MC experimental animal care facility, and all experimental procedures were evaluated and approved by the Erasmus MC committee for animal experiments.

Preparation of single cell suspensions

Lungs and salivary glands were cut into small pieces and dissociated by incubation with 20 µg/mL Liberase™ (Roche Applied Science) in RPMI1640 medium (Life Technologies) for 45 minutes at 37°C. Single cell suspensions were prepared of dissociated lung and salivary gland tissues or intact spleens and lymph nodes over 100 µm cell strainers (BD Falcon™) in MACS sorting buffer (PBS/0.5% BSA/2mM EDTA).

Flow cytometry procedures

Fluorescent labeling of cell membrane molecules was performed as published previously (15). Fluorochrome or biotin-labeled monoclonal antibodies were used specific for murine CD3e (145-2C11, eBioscience), CD4 (GK1.5, eBioscience), CD19 (1D3, eBioscience), CD25 (PC61.5, eBioscience), CD95 (Jo2, BD Biosciences), CD138 (281-2, BD Biosciences), B220 (RA3-6B2, eBioscience), CXCR5 (2G8, BD Biosciences), Foxp3 (236A/E7, eBioscience), and PD-1 (J43, BD Biosciences). 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 isotype-specific staining of intracellular immunoglobulins in plasma cells, extracellularly stained cells were fixed and permeabilized using BD Cytofix/Cytoperm™ Buffer and BD Perm/Wash™ Buffer respectively (BD Biosciences) according to manufacturer's instructions. Immunoglobulins were stained using antibodies specific for IgG1 (A85-1, BD Biosciences), IgG2a/b (R19-5, BD Biosciences), IgG3 (R40-82, BD Biosciences), and IgM (II/41, BD Biosciences). For the measurement of intracellular cytokines in T cells, after membrane marker staining cells were fixed in PBS/2% PFA and were permeabilized and stained in MACS sorting buffer containing 0.5% saponin (Sigma-Aldrich) and anti-IFN-γ (XMG1.2, BD Biosciences) and anti-IL-4 (11B11, BD Biosciences) antibodies. Flow cytometric measurements were performed on a LSRII™ flow cytometer (BD Biosciences) and data were analysed using FlowJo software (Tree Star Inc.).

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 in Lubberts et al. (26).

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

Serum and lymphoid organs were analyzed from mice 7 days after intraperitoneal injection with 0.1 mg TNP-KLH (Biosearch Technologies) and 0.8 mg Imject alum adjuvant (Thermo Scientific) in PBS. Serum IgG1-specific anti-TNP-KLH antibody levels were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (15). 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).

Splenic B and T cell purification and co-culture

Naive splenic B cells were MACS-sorted using a negative selection strategy as described previously (15) Briefly, non-B cells, activated B cells, B1 cells and plasma cells were depleted using indirect labeling with biotinylated anti-CD5 (53-7.3), anti-CD11b (M1-70), anti-CD43 (S7), anti-CD95 (Jo2), anti-CD138 (281-2), anti-Gr-1 (RB6-8C5), and anti-TER-119 (PK136) antibodies (BD Biosciences) and streptavidin-conjugated magnetic beads (Miltenyi Biotec). MACS-sorting of splenic T helper cells was performed using the murine $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions. Purity of sorted splenic B and T cells typically exceeded 95% as verified by flow cytometric analysis. MACS-sorted naive B and T cells were co-cultured at a 1:1 ratio ($1{,}25{\cdot}10^6$ cells/mL) for 4 days at standard conditions (37 ºC / 5% CO_2) in 96-well plates (BD Falcon) in non-polarizing T helper cell culture medium (RPMI 1640 / 10% FCS / 50 µg/ml gentamycin / 0.05 mM ß-mercapto-ethanol) containing 10 µg/mL anti-IFN-γ (XMG1.2 hybridoma), 10 µg/mL anti-IL-4 (11B11 hybridoma) and anti-TGF-β (1D11, R&D systems). For T cell stimulation, culture plates were coated with 30 ng/well anti-CD3e (145-2C11, BD Biosciences). B cells were stimulated with 10 µg/mL $F(ab')_2$ goat anti-mouse IgM (Jackson Immunoresearch) or 5 µg/mL LPS (own production).

Immunohistochemistry

Immunohistochemical stainings were essentially performed as published (15). Five µm spleen cryostat sections were incubated with PE-conjugated anti-CD19 (1D3, BD Biosciences) and biotin-conjugated anti-IgM (II/41, BD Biosciences) followed by incubation with alkaline phosphatase-anti-PE antibodies (Rockland) and peroxidase-streptavidin (Sigma-Aldrich).

Statistical analysis

Levels of significance of continuous data were calculated using the Student's T-test.

Results

Expansion and activation of pro-inflammatory T_H cell subsets in aging CD19-hBtk mice

Since it is unknown from Btk inhibition studies in which cell type Btk exerts in vivo its pathogenic function in autoimmune arthritis (17, 19, 20), we studied T cell activation and differentiation in CD19-hBtk mice that exhibit spontaneous GC formation and SLE-like autoimmunity due to B cell-restricted enhanced Btk signaling (15, 23). In young (8-12 weeks old) CD19-hBtk mice in which GCs spontaneously arise while autoimmune pathology is not yet developing, flow cytometric characterization of $CD4^+$ splenic T cells revealed that T_{FH} numbers were not increased ($p=0.975$) compared to wildtype littermates (Figure 1A). However, in aging (33-37 weeks old) CD19-hBtk mice with fully developed lupus, spleen T_{FH} numbers were significantly ($p<0.001$) increased (Figure 1A). Importantly, large populations of PD-1^{high}CXCR5^{high} $CD4^+$ T cells were found in lungs and salivary glands of aging CD19-hBtk mice (Figure 1B).

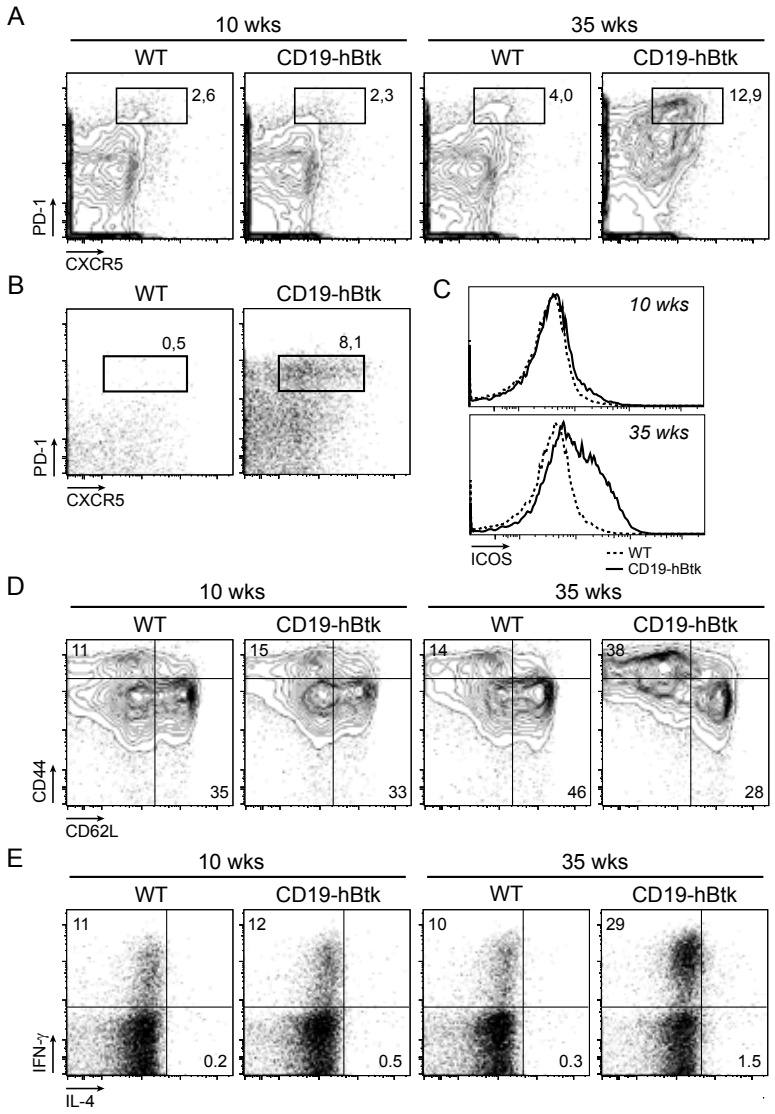
We further evaluated splenic $CD4^+$ T cell activation by measuring ICOS expression on these cells, and we noted a strong upregulation of ICOS on total $CD4^+$ T_H cells only in aging CD19-hBtk mice (Figure 1C). Furthermore, we measured CD44 and CD62L expression on splenic $CD4^+$ T cells. We observed that while in young CD19-hBtk mice CD44^{high}CD62L^{low} memory cells were only marginally increased compared to non-transgenic littermates, the numbers of memory $CD4^+$ T cells were strongly increased in aging CD19-hBtk mice (Figure 1D). We next used flow cytometry to determine which pro-inflammatory cytokines these $CD4^+$ cells produced. Although no significant increase in IL-17 production could be observed in aging CD19-hBtk mice (data not shown), a strong increase in IFN-γ-producing $CD4^+$ splenic T cells was observed in CD19-hBtk mice (Figure 1E).

In summary, the development of lupus-like disease in aging CD19-hBtk mice is characterized by expansion of T_{FH} cells, strong ICOS upregulation on $CD4^+$ T cells, memory $CD4^+$ T cell formation and strongly increased IFN-γ production. The complete absence of these T cell aberrations in young CD19-hBtk suggests that the induction of pro-inflammatory T cells is required for the development of autoimmunity in CD19-hBtk mice.

Amplified Btk signaling in B cells enhances arthritis and promotes T_{FH} formation

Since the induction of T_{FH} cells only occurred in aging CD19-hBtk mice, we further examined whether this T_{FH} induction may be associated with or required for the development systemic autoimmunity in aging CD19-hBtk mice. We therefore studied T_{FH} formation upon provoking autoimmune arthritis in young CD19-hBtk mice when autoimmunity is still absent and T_{FH} cell numbers are comparable to non-

Figure 1. T_H cell homeostasis and T_H activation is disturbed in aging CD19-hBtk mice.

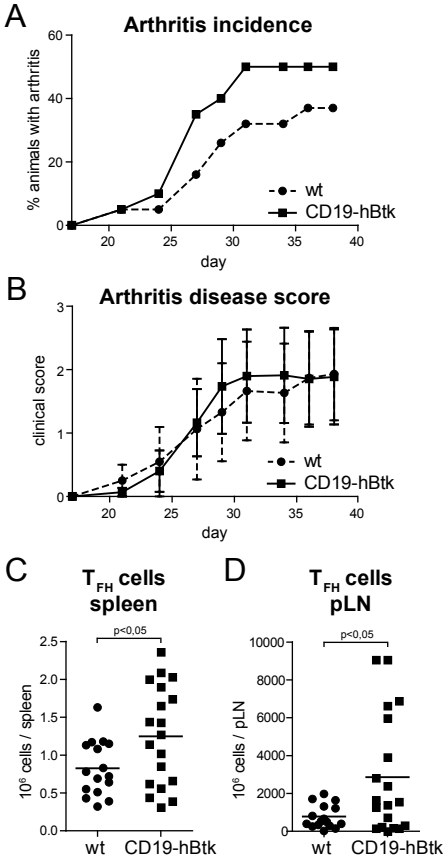


(A and B) In young (8-12 weeks) and aging (33-37 weeks) CD19-hBtk and non-transgenic (WT) littermates, T_{FHs} were identified as PD-1^{high}CXCR5^{high} cells. Numbers indicate the frequencies of gated PD-1^{high}CXCR5^{high} cells among gated $CD4^+$ T cells from spleen (A) or lung (B). (C) Flow cytometric evaluation of ICOS expression levels on gated CD4+ splenocytes from CD19-hBtk and WT littermates. (D) Relative frequencies of memory ($CD44^{high}CD62L^{low}$) and naive T_H cells ($CD44^{low}CD62L^{high}$) as determined by flow cytometry in gated $CD4^+$ splenocytes from young and aging CD19-hBtk and WT littermates. (E) Intracellular flow cytometric detection of IFN-γ and IL-4 cytokine production in gated $CD4^+$ splenocytes in young and aging CD19-hBtk and WT mice. Representative data are shown from >3 independent experiments in which in total > 8 animals per group were analyzed.

transgenic littermates (Figure 1A). Upon induction of arthritis by double immunization with collagen type II (CII) at day 0 and day 21 (collagen-induced arthritis; CIA) in CD19-hBtk mice and non-transgenic littermates, we observed a marked increase in the proportion of CD19-hBtk mice that developed arthritis symptoms (50%, compared to ~37% in non-transgenic littermates at day 38; Figure 2A). Strikingly, the arthritis scores of affected animals in both groups were similar (Figure 2B).

Thorough investigation of B and T cell activation and differentiation by flow cytometry in all CII-immunized CD19-hBtk and wildtype mice revealed increased numbers of GC B cells, IgM and IgG plasma

Figure 2. Enhanced arthritis induction and T_{FH} induction in CD19-hBtk mice.



(A) Proportion of CD19-hBtk mice (n=20) and wildtype (wt) littermates (n=19) developing arthritis symptoms upon a double immunization with collagen type II (CII) at day 0 and day 21. (B) Average arthritis severity scored as described in Lubberts et al. (26) in arthritis-positive CD19-hBtk and WT littermates from the same experiment as in (A). (C and D) Flow cytometric quantification of total follicular T helper (T_{FH}) cells as $CD4^+PD\text{-}1^{high}CXCR5^{high}$ cells in spleens (C) and popliteal lymph nodes (D) of CD19-hBtk (n=19) and wt (n=16) littermates from the experiment described in (A). Representative data from 1 out of 2 independent collagen-induced arthritis (CIA) experiments are shown.

cells, and enhanced upregulation of activation markers including CD86 on B cells in CD19-hBtk mice (data not shown). This phenotype however could not be distinguished from the spontaneous B cell aberrations in lupus-developing CD19-hBtk mice (15). In contrast, when quantifying the numbers of pro-inflammatory $CD4^+$ T cell subsets , we noted a ~1.5-fold increase in the numbers of splenic $CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells ($p<0.05$; Figure 2C). Importantly, this increase in T_{FH} induction in CD19-hBtk mice was even more outspoken in joint-draining lymph nodes, as we noted a ~3.7-fold increase in T_{FH} numbers in popliteal lymph nodes in CD19-hBtk mice compared to non-Tg littermates ($p<0.05$; Figure 2D).

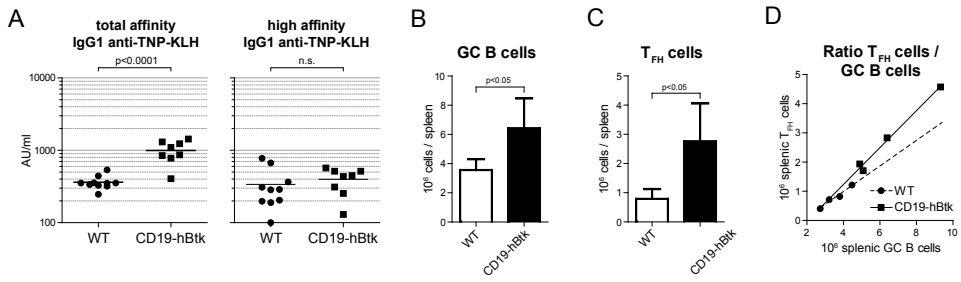
Taken together, these findings demonstrate that the Btk signaling strength in B lymphocytes is a key determinant for auto-immune arthritis susceptibility, possibly through enhanced local and systemic induction of pathogenic T_{FH} cells.

Increased BCR signaling through Btk promotes T_H cell activation and T_{FH} formation

To investigate whether the induction of T_{FH} cells by enhanced Btk signaling in B cells is not just secondary to the induction of systemic autoimmunity in these mice, outside the context of autoimmunity we studied the T-dependent immune response to TNP-KLH in young CD19-hBtk mice. Upon a single immunization with TNP-KLH in alum, 7 days after immunization we observed a ~2.9-fold increase in total serum levels of anti-TNP-KLH IgG1 antibodies in CD19-hBtk mice, compared to wildtype littermates ($p<0.001$; Figure 3A). Strikingly, the levels of high-affinity anti-TNP-KLH IgG1 antibodies were not increased accordingly (Figure 3A), indicating a relieved positive selection threshold in this T-dependent antibody response.

Next, we examined T cell differentiation and activation in TNP-KLH immunized CD19-hBtk mice. While splenic GC B cells were significantly increased by ~1.8-fold in CD19-hBtk mice compared to wildtype littermates ($p<0.05$; Figure 3B), a disproportional ~3.5-fold increase in splenic T_{FH} cells was

Figure 3. CD19-hBtk B cells exaggerate T_{FH} formation.

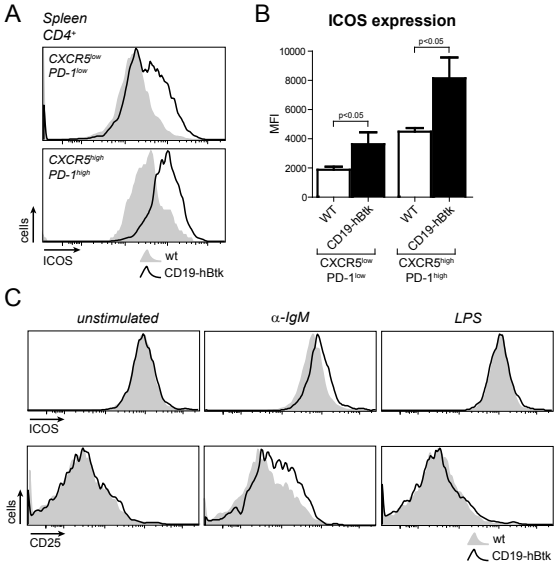


(A) IgG1 anti-TNP-KLH antibodies determined by ELISA in serum of (8-12-week-oldCD19-hBtk (filled squares) and wildtype (WT) littermates, 7 days after a single immunization with TNP(16)-KLH. Levels of IgG1 antibodies binding TNP(16)-KLH (total affinity; left graph) or TNP(5)-KLH (high affinity; right graph) are shown. (B and C) Numbers of splenic $CD19^+CD95^+PNA^+$ germinal center (GC) B cells (B) and $CD4^+PD\text{-}1^{high}CXCR5^{high}$ follicular T helper (T_{FH}) cells (C) determined by flow cytometry in young CD19-hBtk and wt mice (n=4 per group) 7 days after TNP(16)-KLH immunization. (D) Correlation graph of splenic GC B cell and T_{FH} cell numbers in individual CD19-hBtk and wt mice from figure (B) and (C).

observed, tightly correlating to GC B cell numbers in individual CD19-hBtk mice ($p<0.05$; Figure 3C and 3D). Parallel to our findings in aging CD19-hBtk mice (Figure 1C), TNP-KLH immunization in young CD19-hBtk mice could also provoke a stronger upregulation of ICOS expression on total $CD4^+$ T cells, with exceptionally high ICOS expression levels on T_{FH} cells when compared to wild-type littermates (Figure 4A, 4B).

Since Btk signals downstream a variety of receptors in B cells including the BCR, several TLRs and CXCR4/5 chemokine receptors (22), we set out to determine via which pathway enhanced Btk signaling in B cells could induce changes in B cell function that could boost $CD4^+$ T cell activation. To this end, we co-cultured anti-CD3e-stimulated T cells for 4 days in the presence of CD19-hBtk or non-transgenic B cells that were left unstimulated or stimulated through the BCR (α-IgM) or TLR4 (LPS). Differences in T cell activation marker upregulation induced by CD19-hBtk versus wildtype B cells were not as large as observed in vivo upon T-dependent immunization (Figure 4A). Nevertheless, higher expression levels of T cell activation markers ICOS and CD25 were induced in this in vitro co-culture system by CD19-hBtk B cells, selectively when these cells were stimulated through the BCR (Figure 4C).

Figure 4. and amplify TH activation upon BCR triggering.



(A) Flow cytometric measurement of ICOS expression on gated $CXCR5^{low}PD\text{-}1^{low}$ versus $CXCR5^{high}PD\text{-}1^{high}$ $CD4^+$ spleen cells in CD19-hBtk and wt littermates 7 days after TNP(16)-KLH immunization. (B) Quantification of ICOS levels shown in (A) on gated CD4+ splenic cells as mean fluorescence intensity (MFI) determined by flow cytometry. (C). Anti-CD3e stimulated (0.25 µg/mL) MACS-sorted splenic $CD4^+$ T cells from WT mice were co-cultured for 4 days with MACS sorted CD19-hBtk or wt splenic naive B cells that were left unstimulated (left graphs), or stimulated with 10 µg/mL F(ab')2 α-IgM (middle graphs) or 5 µg/mL LPS. At day 4, ICOS and CD25 levels on $CD4^+$ cells were evaluated using flow cytometry. Data are shown as histogram overlays.

In summary, these experiments not only show that enhanced Btk signaling in B cells promotes T_{FH} formation and global ICOS upregulation on $CD4^+$ T cells, but additionally that this enhanced T cell activation is dependent on Btk signaling in B cells downstream the BCR, indicating that the BCR signaling strength determines the extent of T cell engagement during T-dependent humoral immune responses.

Autoimmunity incited by increased Btk signaling in B cells is entirely T cell dependent

Next, we aimed to study whether the enhanced T cell activation and T_{FH} formation in lupus-developing aging CD19-hBtk mice and young CII-immunized CD19-hBtk mice is essential for B cell-driven autoimmunity and not just reflecting autoimmunity in these mice. To this end, we disabled the efficient delivery of T cell co-stimulation to B cells in CD19-hBtk mice by genetic ablation of *Cd40l*, which in particular should potently interfere with T_{FH} formation based on the complete absence of T_{FH} cells in *Cd40l*$^{-/-}$ mice (27). Indeed, whereas flow cytometry revealed spontaneous GC formation in spleens of *Cd40l* haplosufficient CD19-hBtk mice, $CD95^+PNA^+$ B cells could no longer be detected in young CD19-hBtk;*Cd40l*$^{-/-}$ mice (Figure 5A and 5B). In addition, the formation of splenic IgG plasma cells in CD19-hBtk mice fully relied on T cell co-stimulation since we observed a complete normalization of IgG plasma cell formation in CD19-hBtk;*Cd40l*$^{-/-}$ mice while the numbers of splenic IgM plasma cells were not reduced by *Cd40l* deficiency in CD19-hBtk mice (Figure 5B). Importantly, in aging CD19-hBtk;*Cd40l*$^{-/-}$ mice GC B cell numbers, IgG plasma cells, and anti-nuclear IgG auto-antibody production remained absent (data not shown), indicating that T cell costimulation represents a true prerequisite for lupus development in CD19-hBtk mice that cannot be overcome by establishing other pro-inflammatory loops between B cells and non-T cells. In accordance with a model of mutual B-T co-stimulation that is required for the development of germinal centers and autoimmunity, we observed no T_{FH} development in these mice, even at 35 weeks of age (Figure 5C).

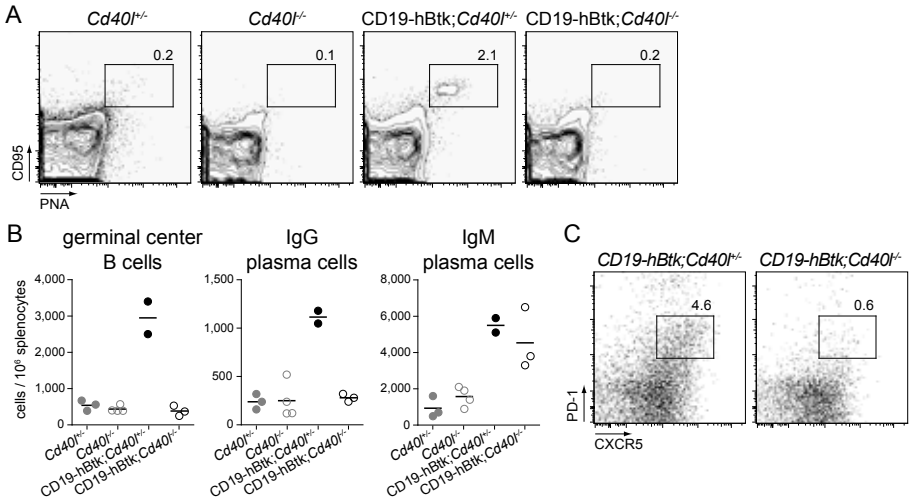
Although B cell-restricted overexpression of Btk can enhance B cell activation in CD19-hBtk mice (15), these data collectively show that the spontaneous GC and IgG plasma cell formation eliciting lupus in these mice requires a co-stimulatory positive feedback loop between B and T cells initiated by enhanced Btk signaling in B cells.

Enhanced induction of regulatory T cells by enhanced Btk signaling in B cells

While all aging CD19-hBtk mice develop a lupus-like syndrome with immune complex-mediated pathology of kidneys, lungs and salivary glands (15), this systemic auto-immune disease does not significantly affect survival up to 10 months of age. We therefore examined whether the expansion of pro-inflammatory T cell subsets could be counterbalanced by an effective regulatory T cell response. Indeed, we observed in aging CD19-hBtk mice a clear increase in $Foxp3^+CD25^+$ $CD4^+$ splenic regulatory T (T_{reg}) cells ($p<0.001$; Figure 6A), while T_{reg} numbers in young CD19-hBtk mice were only minimally and non-significantly increased (~1.1-fold; $p>0.05$).

To test whether the expansion of T_{regs} in CD19-hBtk mice parallels GC formation and autoimmunity , we investigated T_{reg} numbers following the induction of GCs in young CD19-hBtk and non-transgenic

Figure 5. Btk-induced GC and IgG plasma cell formation fully relies on T cell co-stimulation.

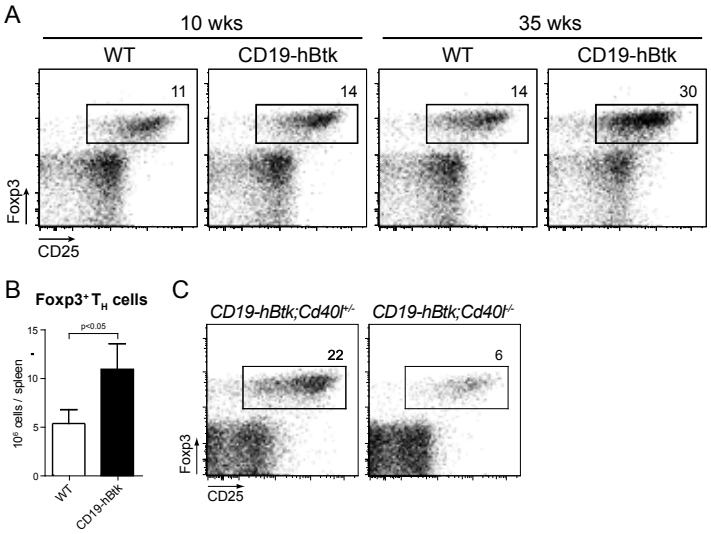


(A) Flow cytometric detection of CD95^{+}PNA^{+} germinal center (GC) B cells in gated CD19^{+} spleen cells from young (8-12 weeks) mice of the indicated genotypes (n=2-4 per genotype). Numbers represent the percentage of GC B cells within the CD19^{+} gate. (B) Quantification by flow cytometry of splenic GC B cells (left graph), IgG plasma cells (middle graph) and IgM plasma cells (right graph) in young CD19. IgG plasma cells were detected as CD138^{high} cells with high cytoplasmatic expression of either IgG1, IgG2 or IgG3. (C) Flow cytometric detection of follicular T helper cells as PD-1^{high}CXCR5^{high} cells among gated CD4^{+} splenocytes in aging (33-37 weeks) Cd40l-haplosufficient versus Cd40l-deficient CD19-hBtk transgenic mice. Representative data of 3 analyzed mice per group are shown.

littermates by a single T-dependent immunization with TNP-KLH. Indeed, while Treg numbers are very similar in naive CD19-hBtk and non-transgenic mice, 7 days after immunization we noted a significant increase in the numbers of splenic T_{regs} in CD19-hBtk compared with wildtype mice (Figure 6B). To further explore the hypothesis that increased Foxp3^{+}CD4^{+} T cell numbers in immunized or autoimmune CD19-hBtk mice are coinciding with the enhanced GC formation in these mice, we explored T_{reg} formation in Cd40l-deficient CD19-hBtk mice. When determining the number of Foxp3^{+}CD25^{+}CD4^{+} T cells in aging CD19-hBtk;*Cd40l*$^{-/-}$ mice, we observed a dramatic decrease but not complete absence of T_{reg} numbers compared to Cd40l-haplosufficient CD19-hBtk littermates (Figure 6C). Notably, T_{reg} numbers between CD19-hBtk transgenic and non-transgenic Cd40l-deficient mice were similar (data not shown).

In conclusion, while B cells with enhanced Btk signaling can disturb T cell homeostasis and reciprocally receive co-stimulatory signals essential for GC B cell and T_{FH} formation, the enhanced formation of T_{regs} that parallels GC formation and autoimmunity in CD19-hBtk mice likely restrains pro-inflammatory loops between B and T cells. However, from these data it is still unclear whether enhanced Btk signaling in B cells directly programs the differentiation or expansion of T_{regs}, or whether T_{regs} are formed in a Btk-independent way in response to the expansion of pro-inflammatory T cells.

Figure 6. Increased T_{reg} formation in CD19-hBtk mice.



(A) Measurement of relative frequencies of $Foxp3^+CD25^+$ regulatory T cells (T_{regs}) using flow cytometry among gated $CD4^+$ splenocytes in young and aging CD19-hBtk versus WT littermates (n=5 per genotype per age). Numbers indicate T_{reg} frequencies among $CD4^+$ spleen cells. (B) Quantification by flow cytometry of Foxp3-expressing $CD4^+$ splenic T cells in TNP(16)-KLH immunized young (8-12 weeks) CD19-hBtk and wildtype (WT) littermates (n=4 per genotype). (C) Relative numbers of $Foxp3^+CD25^+$ regulatory T cells (C) among $CD4^+$ gated splenocytes in aging (33-37 weeks) Cd40l-haplosufficient versus Cd40l-deficient CD19-hBtk transgenic mice. Representative data of 3 analyzed mice per group are shown.

Discussion

Studies on Btk inhibition in multiple murine models for SLE and rheumatoid arthritis have clearly demonstrated the enormous pathogenic potential of Btk in rheumatic diseases. Still, many questions regarding the mechanisms by which Btk corrupts immune tolerance are still withstanding, including through which cell types and which signaling pathways Btk acts in rheumatic disease pathogenesis, and whether Btk signaling may indirectly jeopardize tolerance in cells not expressing Btk themselves.

Here we report that the Btk signaling strength in B cells determines arthritis susceptibility in murine experimental arthritis (CIA), and that both autoimmune arthritis and spontaneous lupus in Btk-transgenic mice is characterized by enhanced T cell activation and expansion of pathogenic T_{FH} cells. Although enhanced Btk signaling in B cells suffices to directly induce B cell activation and IgM plasma cell formation, Btk-transgenic B cells could only break immune tolerance through acquiring costimulation from recruited pathogenic T cells. This is supported by our findings that GC formation and autoimmunity in CD19-hBtk mice were abrogated on a *Cd40l*$^{-/-}$. Our in vitro co-culture experiments demonstrated that the enhanced capacity of Btk-overexpressing B cells to induce T cell activation was dependent on amplified BCR signaling of these B cells, implying that the BCR signaling strength is crucial for the induction of T cell-driven autoimmunity.

While single genetic defects affecting B cells have been reported to be able to establish T-independent lupus-like disease (28, 29), and while many other monogenetic defects in murine SLE models drive disease development by targeting multiple cell types (3), to the best of our knowledge, we here provide the first evidence that a single genetic defect restricted to B cells is capable of establishing lupus-like disease that is dependent on T cells, based on the abrogation of IgG plasma cell development in CD19-hBtk;*Cd40l*$^{-/-}$ mice. Although the Btk transgene is selectively targeting B cells and the Cd40l deficiency is selectively affecting T cells, we cannot formally exclude that apart from mutual costimulation of B and T cells autoimmunity in CD19-hBtk mice relies on interacting other immune cells as well. For example, Btk-overexpressing B cells could influence DC activation, leading to altered T_{FH} priming, and Cd40l-deficiency would abolish such indirect Btk-driven T_{FH} priming by interfering with DC-mediated T_{FH} priming (30). However, we have not found signs of DC activation in young or aged CD19-hBtk mice (L.K. and R.H., unpublished data). Furthermore, if Btk-overexpression in B cells could enhance DC activation, this expectedly leads to the production of cytokines including BAFF and APRIL that support the extrafollicular Cd40l-independent formation of isotype-class switched plasma cells (31, 32). In CD19-hBtk;*Cd40l*$^{-/-}$ mice however, no increase in IgG plasma cells compared with non-transgenic *Cd40l*$^{-/-}$ mice could be observed, indicating that such T-independent DC-mediated formation of IgG plasma cells is not induced by enhanced Btk signaling in B cells.

Using an in vitro B-T cell co-culture system, we demonstrated that the increased T cell activating capacity of Btk-transgenic B cells relies on Btk signaling downstream of the BCR. In search for molecular targets of increased BCR signaling through Btk that could drive T cell activation and in particular T_{FH} formation, we checked several crucial T cell co-stimulatory factors possibly induced by amplified BCR signaling through Btk. Whereas the production of IL-6 following BCR stimulation was ~2-fold higher in CD19-hBtk B cells versus non-transgenic B cells, inhibition of IL-6 using α-IL-6 antibodies in our B-T co-culture system did not alter the selectively enhanced upregulation of ICOS and CD25 on T cells co-cultured with α-IgM stimulated CD19-hBtk B cells (L.K. and R.H., unpublished data). Furthermore, a possible role of enhanced IL-6 production by antigen-activated CD19-hBtk GC B cells in maintaining high T_{FH} numbers is uncertain since it has been reported that human GC B cells are incapable of producing IL-6 (33).

Alternatively, it could be speculated that altered expression of ICOS-L on CD19-hBtk B cells upon BCR triggering might increase T cell activation and especially T_{FH} differentiation that fully relies on ICOS-mediated induction of Bcl-6 (34). However, in vivo analysis of ICOS-L expression on CD19-hBtk B cells revealed that ICOS-L levels were actually lower, compared with wildtype B cells both within GCs and outside GCs (L.K. and R.H., unpublished data). This likely reflects increased ICOS-ICOS-L interactions in vivo since ICOS-L downregulation is observed both on healthy human B cells interacting with ICOS in vitro and on SLE B cells in the presence of ICOS-expressing T cells in vivo (14). Furthermore, observations that ICOS-L is constitutively expressed on B cells rather than induced upon activation (35) and that ICOS-L expression on non-cognate follicular B cells is critical for T_{FH} recruitment (8) further argues against a role for BCR-induced changes in ICOS-L expression that may underlie the enhanced T cell activation and T_{FH} differentiation in CD19-hBtk mice.

The inability of GC B cells to produce IL-6 in humans, the dispensable role for IL-6 in the enhanced

upregulation of ICOS on $CD4^+$ T cells activated by BCR-stimulated CD19-hBtk B cells, and the role of ICOS-L on bystander instead of cognate B cells in T_{FH} formation raise the question how antigen-activated CD19-hBtk B cells may increase T_{FH} formation and ICOS expression on T_{FH} cells in vivo. For T_{FH} expansion and maintenance, a critical role for antigen presentation itself by antigen-activated B cells has been demonstrated when antigen availability is limited (9). However, enhanced T_{FH} induction and T cell activation through more effective antigen presentation by Btk-overexpressing B cells is unlikely, since T cell ICOS upregulation was increased in B-T co-cultures by BCR-stimulated CD19-hBtk cells while in these co-cultures cognate antigen presentation could not occur. Therefore, it is likely that increased expression of other T cell co-stimulatory factors as B7 proteins may boost T cell activation by BCR-activated CD19-hBtk cells. In line with this, B7 proteins are crucial for T_{FH} induction since CD28-deficiency profoundly hampers T_{FH} formation in vivo (27). Moreover, we have previously shown that enhanced BCR signaling through Btk increases the expression of CD86. Still, the role of B7-driven enhanced T_{FH} formation and T cell activation is likely to be limited to early B-T interactions in CD19-hBtk mice since CD28 signaling is dispensable for T_{FH} number maintenance in GCs (36).

Our finding that the induction and activation of pathogenic $CD4^+$ T cells by enhanced Btk signaling in B lymphocytes affirms the view that abolishing pro-inflammatory cross-talk between multiple immune cell types may be more effective than simply depleting B cells. Moreover, it promises that effects of Btk inhibition therapy in human patients with rheumatic diseases will likely extend beyond the mere correction of activated Btk-expressing cells. When efficacy of Btk-inhibition would be evaluated in clinical trials in rheumatic patients, it should become clear whether in these patients correction of reported T cell aberrancies, including enhanced ICOS expression and increased numbers of T_{FH} cells (13, 14), indeed occurs.

Acknowledgements

This study is financially supported by the Dutch Arthritis Association (grants 08-1-103 and 09-1-302). We would like to thank Jeroen Essers (Erasmus MC) for his kind provision of Cd40l-deficient mice, and we would like to thank Ilke Ilgaz and the animal caretakers of the Erasmus MC experimental animal care facility for technical support during experiments and for carefully monitoring autoimmune disease development in experimental animals.

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6

Bruton's tyrosine kinase (Btk) and autoimmunity: Btk protein levels in B cells predominantly modulate B cell receptor signaling

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Abstract

In preclinical models of systemic lupus erythem atosus and rheumatoid arthritis, disease can be effectively treated by inhibition of Bruton's tyrosine kinase (Btk), a key signaling molecule of the B cell receptor (BCR). But since Btk signals downstream many more receptors in various immune cells, it is currently unknown how Btk exerts its pathogenic function in these rheumatic diseases. As B cell-restricted overexpression of Btk is sufficient to establish lupus-like disease in Btk-transgenic mice, we aimed to identify which signaling pathways in B cells are affected by enhanced Btk signaling leading to systemic autoimmunity. Using total mRNA deep-sequencing technology, we compared gene expression profiles of unstimulated and BCR-stimulated wild-type and Btk-transgenic naive B cells. We found that gene expression profiles of unstimulated Btk-transgenic B cells closely mirror a BCR-signaling signature, indicating that Btk protein overexpression *in vivo* predominantly enhances BCR signaling. Furthermore, we found that enhanced Btk signaling not only amplifies genes normally induced upon BCR activation, but in addition induces shifts in the gene targets of BCR signaling. Finally, enhanced Btk signaling downstream of the BCR proved to decrease Bcl6 expression and promote Blimp-1 induction, paving the road for exaggerated plasma cell formation and thereby possibly promoting autoantibody-driven autoimmune disease.

This study is financially supported by the Dutch Arthritis Association (grants 08-1-103 and 09-1-302)

Submitted

Introduction

The non-receptor protein tyrosine kinase Bruton's tyrosine kinase (Btk) - belonging to the Tec family of kinases - is expressed in all hematopoietic cells except T lymphocytes and signals downstream a variety of receptors in these cels (1). Despite its wide expression pattern in many immune cells, Btk signaling appears to be critical only for B cell differentiation: genetic defects in Btk in mice or humans result in the B cell-specific immunodeficiencies x-linked immunodeficiency (xid) or X-linked agammaglobulinemia (XLA), respectively (2). While XLA is characterized by an almost complete arrest of pre-B cell development leading to a virtually absence of peripheral B cells and immunoglobulin (Ig) in the serum , pre-B cell differentiation in Btk-deficient mice is mostly delayed (3-5). The ~50% reduction in peripheral B cell numbers that still occurs in these mice is not caused by a strong differentiation block but rather a survival defect of mature B cells (3, 6). Hampered maturation of peripheral B cells primarily arises from reduced Btk signaling downstream of the B cell antigen receptor (BCR) that provides critical survival signals. While many downstream signaling targets of Btk have not yet been identified, the BCR-mediated survival defect of Btk-deficient B cells largely results from the incapability to activate potent pro-survival transcription factor NF-kB (6-8).

Although originally only linked to immunodeficiency disease, new Btk inhibition therapies using multiple small molecule inhibitors demonstrate a pathogenic role for Btk in B cell malignancies and B cell driven autoimmune diseases. While the effectiveness of Btk inhibitors (Ibrutinib) is shown in multiple B cell malignancies in patient studies (9-11), the contribution of Btk signaling to autoimmune disease is currently evident from Btk inhibitor studies only in murine models of rheumatic autoimmune diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (12-17). Although the selective Btk inhibitors PCI-32765 (Ibrutinib), RN486 and CGI1746 all potently dampen BCR-induced activation and proliferation (13-15, 17), their anti-inflammatory effects *in vivo* do not exclusively rely on modulating B cell activation, since they also counteract signaling of stimulatory receptors on myeloid cells. In monocytes and macrophages, Btk inhibition strongly repressed FcγR-instructed TNF-α, IL-1ß and IL-6 secretion, whereas inhibition of FcεR signaling in human mast cells abolished the release of histamine and multiple pro-inflammatory cytokines (14, 15). Apart from FcR signaling, Btk inhibition likely modulates disease activity via more immune cell types through additional signaling pathways in which Btk is implicated, including CD38, CD303, IL-5R, IL-6R, IL-10R, CXCR4/5, and TLR4/8/9 signaling (18-26).

A direct B cell-confined role of Btk signaling in propagating systemic autoimmunity was found in CD19-hBtk mice, which spontaneously develop an SLE-like syndrome due to B cell-restricted overexpression of a human unmutated BTK transgene (27). While Btk overexpression physiologically occurs in activated murine B cells, the continuous Btk overexpression in CD19-hBtk mice induced spontaneous B cell activation, germinal center (GC) formation and IgG plasma cell formation, leading to IgG antinuclear autoantibody production and immune-complex mediated pathology of lungs, kidneys and salivary glands. This autoantibody production reflects a defect in negative selection of B cells, demonstrated *in vivo* by local autoantibody production in lungs of CD19-hBtk mice following influenza infection and by an *in vitro* resistance to pro-apoptotic Fas signals. Importantly, the *in vitro* Fas resistance, as well as enhanced upregulation of activation markers, could only be provoked in CD19-hBtk cells

stimulated by the BCR and not by TLRs. Moreover, the correction of BCR hyperresponsiveness of CD19-hBtk B cells by PCI-32765 treatment and the absence of autoimmunity in mice highly overexpressing a kinase-inactive Btk mutant proved that Btk overexpression itself does not constitutively changes B cell activation, but that Btk kinase activity downstream one or more B cell-expressed receptors is required for the induction of systemic autoimmunity. From these data, however, it is still unclear whether Btk has the capacity to induce SLE by corrupting BCR signaling, or whether signaling of multiple receptors, e.g. TLR or chemokine receptors, is affected in B cells.

To determine whether increased Btk levels selectively induce autoimmunity by modulating BCR signaling rather than other Btk-linked receptors expressed on B cells, we compared gene expression patterns induced by BCR stimulation and by Btk-overexpression using total RNA deep-sequencing technology. We found that the genes differentially expressed *in vivo* in Btk-overexpressing B cells distinctly mirror a BCR signature, based on their strong overlap with genes induced by BCR stimulation in wild-type (WT) B cells. Moreover, expression of a fraction of these BCR-induced genes in WT B cells is amplified or blocked in BCR-stimulated Btk-overexpressing cells, demonstrating that increased Btk levels alternatively program the outcome of B cell activation by the BCR. Finally, when examining transcription factors affecting terminal B cell differentiation, we observed a stronger downregulation of Bcl6 and enhanced upregulation of Blimp1 in BCR-stimulated CD19-hBtk cells, showing that Btk levels are decisive in BCR-orchestrated GC and plasma cell development.

Materials & methods

Mice and genotyping

CD19-hBtk mice have been generated previously (28) and were backcrossed onto the c57bl/6 genetic background for >10 generations. Genotyping of CD19-hBtk mice was performed by PCR using primers specific for the CD19-hBtk transgene (forward primer: 5'-CCTTCCAAGTCCTGGCAT-3'; reverse primer: 5'-CACCAGTCTATTTACAGAGA-3'). Mice were kept and bred at the Erasmus MC experimental animal facility under specified pathogen free conditions. All experimental protocols were reviewed and approved by the Erasmus MC committee for animal experiments.

Naive B cell purification and in vitro stimulation

Spleen cell suspensions were prepared in magnetic-activated cell sorting (MACS) buffer (PBS/2mM EDTA/0,5%BSA) and naive splenic B cells were purified by MACS as described before (27). Non-B cells, B-1 cells, germinal center B cells and plasma cells were first labeled with biotinylated anti-CD5 (53-7.3), anti-CD11b (M1-70), anti-CD43 (S7), anti-CD95 (Jo2), anti-CD138 (281-2), anti-Gr-1 (RB6-8C5), and anti-TER-119 (PK136) antibodies (BD Biosciences) and subsequently with streptavidin-conjugated magnetic beads (Miltenyi Biotec). Naive B cells were collected by magnetically depleting labeled cells from splenic cell suspensions, and purity of naive B cells (typically >99% $CD19^+$ cells) was confirmed by flow cytometry. Purified naive B cells were cultured at standard conditions (37°C / 5% CO_2) in culture medium (RPMI 1640 / 10% FCS / 50 µg/mL gentamycin / 0,05 mM ß-mercaptoethanol) in the presence of 10 µg/mL $F(ab')_2$ a-IgM (Jackson ImmunoResearch).

RNA sequencing and data analysis

Either directly after purification or following $F(ab')_2$ a-IgM stimulation for 12 hrs, RNA from (cultured) naïve B cells was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total mRNA sequencing was performed on a HiSeq 2000 (Illumina), and raw reads were aligned using Bowtie (29) to murine transcripts (RefSeq) corresponding to the University of California at Santa Cruz (UCSC) mouse genome annotation (NCBI37/mm9). Gene expression levels as fragments per kilobase of a transcript per million mapped reads (FPKMs) were calculated using Cufflinks (30). The differentially expressed genes were further evaluated using edgeR (31) on TIGR Multiexperiment Viewer (MeV) software (32).

Quantitative real-time PCR

For the generation of cDNA from RNA isolated as described above, 1 µg of RNA was treated with DNAse I, and cDNA was synthesized from RNA using Superscript IIReverse Transcriptase (Invitrogen). For realtime PCR reactions, we used Taqman Universal Mastermix (Applied Biosystems) and probes from the Universal ProbeLibrary Set (Roche Applied Science). Quantitative real-time PCR (qRT-PCR) was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). mRNA levels of transcripts of interest were normalized to glycereraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA levels. Primers (Invitrogen) specific for *Prdm1* (forward: 5'-TGCGGAGAGGCTCCACTA-3'; reverse: 5'-GCTTTCCGTTTGTGTGAGATT-3') and *Bcl6* transcripts (forward: 5'-CTGCAGATGGAGCATGTTGT-3'; reverse: 5'-TTCTGCTTCACTGGCCTTG-3') for qRT-PCR reactions were designed using transcript sequences obtained from www.ensembl.org.

Statistical analysis

Statistical differences in FPKMs between the analyzed groups in total RNA deep sequencing experiments were calculated by ANOVA with Bonferroni correction (the threshold for statistically significant differences was set at $p<0,01$). For calculating differences in mRNA expression determined by qRT-PCR, a Student's T-test was employed.

Results

Btk overexpression *in vivo* predominantly amplifies BCR signaling

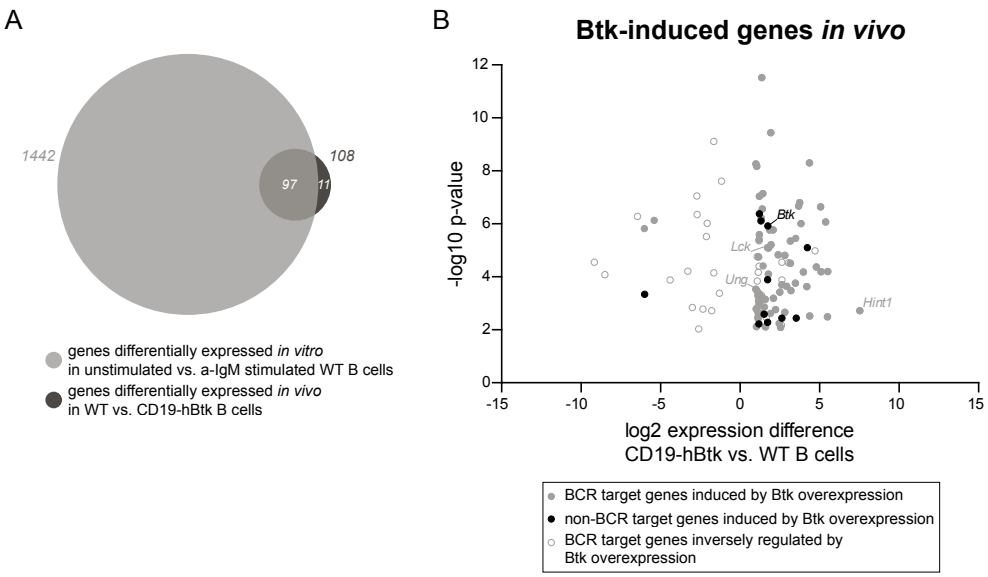
To determine whether increasing Btk expression levels in mature B cells selectively drive autoimmunity through enhanced BCR signaling, we compared genes targeted by BCR signals in WT B cells with genes differentially expressed between CD19-hBtk versus WT B cells in vivo. We therefore isolated splenic naive mature B cells from young (8-12 weeks; n=4) CD19-hBtk mice and non-transgenic (WT) littermates (n=4) and compared gene expression patterns using deep sequencing of total RNA obtained from these cells before and after 12 hrs of anti-IgM stimulation.

Using stringent cut-off levels for significance ($p<0.01$) and differences in expression levels (≥2-fold differences), a comparison of total mRNA expression profiles of unstimulated versus anti-IgM stimulated WT mature B cells revealed 1,442 statistically differentially expressed genes, of which 1011 genes were upregulated in anti-IgM stimulated B cells and 341 were downregulated. While classical BCR-signaling

targets, such as CD25 and CD69, did not meet the strict selection criteria of our list of 1,442 BCR-induced genes, we nevertheless observed an induction in CD25 and CD69 expression in anti-IgM stimulated WT B cells in our total RNA deep-sequencing data. Between unstimulated versus anti-IgM stimulated WT B cells, the fragments per kilobase of a transcript per million mapped reads (FPKMs) for CD25 increased from 0.38 ± 0.05 to 29.3 ± 0.9 in unstimulated versus anti-IgM stimulated WT B cells, while for CD69 FPKMs increased from 0.53 ± 0.26 to 28.8 ± 3.1.

When applying these stringent cut-off levels for significance and expression differences to a comparison of gene expression between unstimulated WT and CD19-hBtk B cells, only 108 genes were found to be differentially expressed. When examining the overlap of these 108 genes with the 1,442 identified BCR signaling targets in WT B cells, we noticed that 97 out of 108 genes (~90%) were overlapping (Figure 1A), suggesting that Btk overexpression primarily affects B cell functioning through BCR signals.

Figure 1. Selective amplification of BCR signaling *in vivo* by Btk overexpression.



A) Overlap of genes identified using total mRNA deep sequencing that are differentially expressed between unstimulated and 12-hrs anti-IgM stimulated WT B cells (1,442 genes, light grey circle) versus genes differentially expressed between unstimulated WT and CD19-hBtk B cells (108 genes, dark grey circle). Numbers correspond to the number of differentially expressed genes within each (part of a) circle. B) Expression level differences and corresponding p-values of genes differentially expressed between unstimulated CD19-hBtk B cells versus WT B cells. Grey filled circles indicate genes mirroring up- or downregulation upon 12 hrs of anti-IgM stimulation in WT B cells, black filled circles indicate genes not responding to 12 hrs of anti-IgM stimulation in WT B cells, and grey open circles indicate genes showing inverse up- or downregulation compared to up- or downregulation in 12 hrs anti-IgM stimulated WT B cells.

To test whether Btk overexpression simply amplifies the effects of BCR signaling, or whether enhanced Btk signaling differentially programs the outcome of BCR signaling in B cells *in vivo*, of these 97 overlapping genes we compared the up- or downregulation pattern between WT B cells upon anti-IgM stimulation versus unstimulated CD19-hBtk B cells. To cluster genes that exhibited a similar trend in up- or downregulation both upon BCR stimulation (in WT B cells) and upon Btk overexpression *in vivo*, we selected those genes of which the expression (1) increased/decreased by ≥2-fold in unstimulated CD19-hBtk versus WT B cells, with an absolute expression difference of ≥1 FPKM, and (2) increased/decreased by ≥2-fold in unstimulated WT versus anti-IgM stimulated WT B cells with an absolute expression difference of ≥1 FPKM. In line with an in vivo amplification of BCR signaling in CD19-hBtk cells in vivo, we found that among the 97 genes that are differentially expressed *in vivo* between WT and CD19-hBtk B cells and overlap with anti-IgM induced genes in WT B cells, 67 genes (~69%) exhibited a similar upregulation or downregulation pattern in CD19-hBtk B cells *in vivo* and in anti-IgM stimulated WT B cells (Table S1 and Figure 1B).

Among these BCR signature genes induced *in vivo* in CD19-hBtk B cells, we found Lck, reported to modulate the BCR signaling strength (33) and Ung, which contributes to isotype class switching and somatic hypermutation (34). We noted that the remaining 30 out of the group of 97 genes displayed a more or less "inverse" gene expression pattern, whereby genes upregulated upon BCR stimulation in WT B cells were downregulated by *in vivo* Btk overexpression or vice versa (Table S2 and Figure 1B).

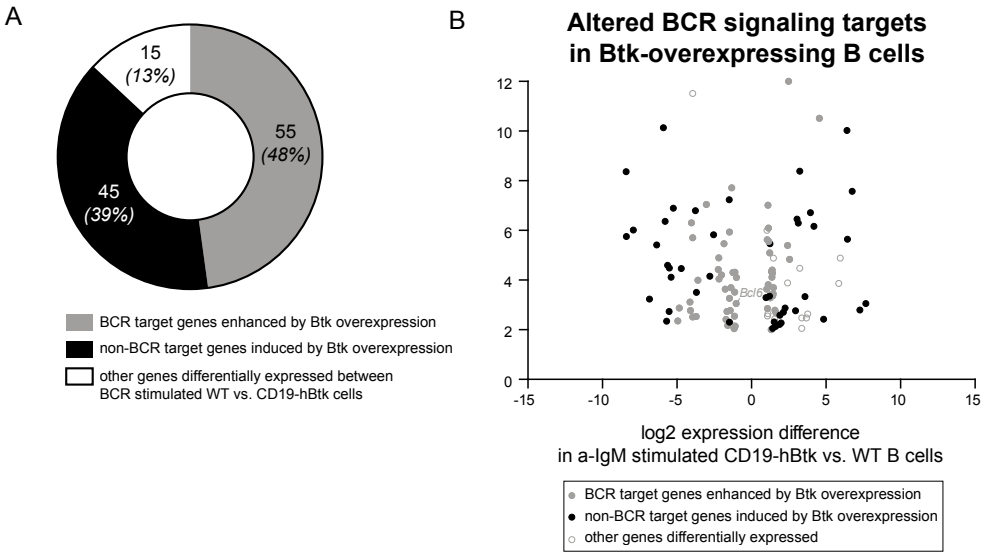
Among the 108 differentially expressed genes between WT and CD19-hBtk B cells *in vivo*, only 11 genes did not overlap with genes differentially expressed upon BCR stimulation in WT cells (Table S3 and Figure 1B). Notably, one of these genes proved to be Btk itself, which was ~3,3-fold overexpressed in unstimulated CD19-hBtk B cells *in vivo* but was not upregulated in anti-IgM stimulated WT B cells. The remaining 10 genes overexpressed *in vivo* in CD19-hBtk B cells but not belonging to BCR target genes mostly exhibited very low expression levels (typically <10 FPKM in all examined groups of (un)stimulated B cells), implying that these genes that seem independent of BCR signaling do not represent strong effectors of Btk signaling in CD19-hBtk mice.

Taken together, these analyses show that the genes differentially expressed in Btk-overexpressing B cells *in vivo* predominantly reflect a BCR signaling signature, suggesting that Btk signaling through the BCR is mainly responsible for the spontaneous autoimmune phenotype of Btk-transgenic mice.

Enhanced Btk signaling amplifies and alters the outcome of BCR signaling

To further examine how increased signaling capacity of the BCR through Btk would affect the expression of BCR target genes, we compared the gene expression profiles of anti-IgM stimulated WT versus anti-IgM stimulated CD19-hBtk B cells. With an increase/decrease of ≥2-fold and an absolute expression difference of ≥1 FPKM, we identified 115 genes that were differentially expressed. To examine whether enhanced Btk-mediated BCR signaling in CD19-hBtk B cells would mainly exaggerate up- or down-regulation of BCR target genes found in anti-IgM stimulated WT cells, we divided these 115 genes in subgroups.

Figure 2. Modulation of gene targets of BCR signaling by enhanced Btk expression.



A) Distribution of 115 genes differentially expressed between 12 hrs anti-IgM stimulated WT and CD19-hBtk B cells over three groups, containing genes that either exhibit enhanced up- or downregulation in CD19-hBtk B cells compared to the modulation observed upon 12 hrs of anti-IgM stimulation of WT B cells (55 genes, grey fraction), or exhibit an up- or downregulation in CD19-hBtk B cells that is inverse to the modulation observed upon 12 hrs of anti-IgM stimulation of WT B cells (45 genes, black fraction). The third group (15 genes, white fraction) contains genes that do not appear to be modulated by anti-IgM stimulation in WT B cells. B) Expression level differences and corresponding p-values of 115 genes differentially expressed between 12 hrs anti-IgM stimulated WT and CD19-hBtk B cells , divided over the three indicated groups.

The first subgroup was defined by a ≥2-fold increased/decreased expression and an absolute expression difference of ≥1 FPKM between unstimulated versus stimulated WT B cells. Using these selection criteria, we identified 55 genes (~48%) whose up- or downregulation upon BCR triggering could be exaggerated by Btk overexpression (Figure 2 and Table S4). This group of 55 genes included Bcl6, a critical factor for GC B cell differentiation (35-37). A second subgroup contained 45 genes (~39%) the expression of which was reversely decreased/increased by ≥2-fold in stimulated WT versus unstimulated WT B cells with an absolute expression difference of ≥1 FPKM (Figure 2 and Table S5). This observation (that the expression of 45 Btk-modulated genes was oppositely down- or upregulated in WT B cells upon BCR stimulation) showed that enhanced Btk signaling downstream of the BCR could establish shifts in BCR signaling targets. Finally, a third group contained 15 residual genes with expression patterns alternatively changed by Btk overexpression, almost exclusively comprising genes incompletely downregulated upon BCR stimulation in CD19-hBtk B cells compared to WT B cells (Figure 2 and Table S6).

Collectively, these data show that enhanced Btk signaling in B cells does not simply amplify BCR signaling, but instead induces important shifts in target genes induced by BCR signaling.

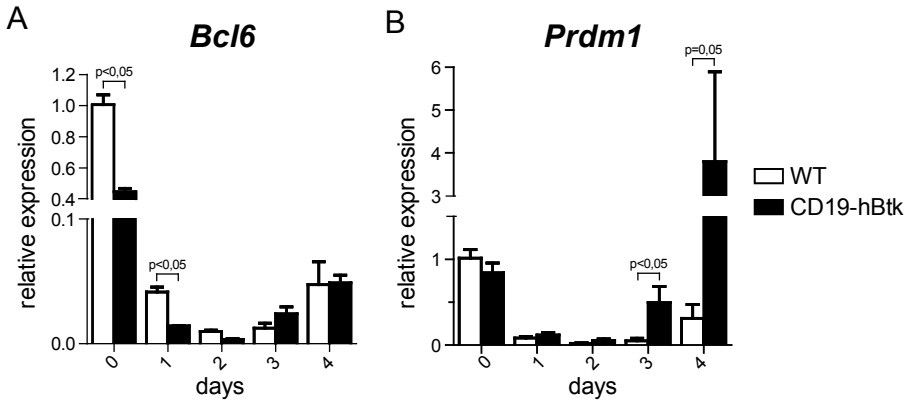
Enhanced Btk-mediated BCR signaling drives plasma cell differentiation via Bcl6 downregulation

As the molecular mechanism by which enhanced GC and IgG plasma cell formation drive autoimmunity in CD19-hBtk mice is still largely unknown, we searched the differentially expressed genes between (un)stimulated CD19-hBtk and WT B cells for transcription factors known to orchestrate terminal B cell differentiation into GC B cells and plasma cells. Indeed, from our RNA deep-sequencing data we observed that Bcl6, a critical factor for GC B cell differentiation and indirectly for plasma cell differentiation (38), was more potently downregulated in anti-IgM stimulated CD19-hBtk B cells versus WT B cells (4.3 ± 0.5 versus 8.9 ± 0.7 FPKM, respectively; Table S4). To verify the enhanced downregulation of Bcl6 by enhanced Btk-mediated BCR signaling, we used quantitative RT-PCR to measure *Bcl6* mRNA levels for 4 consecutive days upon *in vitro* anti-IgM stimulation of naïve WT and CD19-hBtk B cells. Indeed, we observed at day 1 a significantly stronger downregulation of *Bcl6* mRNA levels in anti-IgM stimulated CD19-hBtk B cells, compared with WT B cells (Figure 3A). Notably, in these experiments *Bcl6* mRNA levels were also found to be significantly down in CD19-hBtk versus WT B cells *in vivo* (day 0; Figure 3A).

Since Bcl6 represses Blimp-1 (encoded by the *Prdm1* gene) which commits B cell differentiation to the plasma cell lineage (39), an enhanced downregulation of Bcl6 by increased Btk signaling could lead to increased or faster induction of Blimp-1 and thereby promote plasma cell differentiation. To test this, we also determined *Prdm1* expression levels in WT versus CD19-hBtk cells that were stimulated for 4 days with anti-IgM. We detected significant upregulation of *Prdm1* transcripts in anti-IgM stimulated CD19-hBtk B cells at day 3 and 4, when compared to anti-IgM stimulated WT B cells (Figure 3B).

In summary, these data show that Btk signaling downstream of the BCR critically regulates Bcl6 expression, which was associated with enhanced induction of Blimp-1 that has the capacity or promote plasma cell differentiation.

Figure 3. Increased Btk expression strongly represses Bcl6 and induces Blimp-1 expression in B cells.



Quantitative RT-PCR analysis of *Bcl6* (A) and *Prdm1* (B) gene expression levels in WT (white bars) versus CD19-hBtk (black bars) naïve B cells prior to stimulation (day 0) and during 4 days of stimulation with 10 µg/mL $F(ab')_2$ a-IgM (day 1-4) *in vitro*. Expression levels were normalized to GAPDH levels and were calculated relatively to expression levels observed in WT B cells on day 0. Error bars indicate standard errors of the mean (SEMs).

Discussion

Although the therapeutic potential of the small molecule inhibitors PCI-32765 (Ibrutinib), CGI1746 and RN486 is well-demonstrated in pre-clinical rheumatic disease models (12-17), the cell type and signaling pathways through which Btk mainly exerts its pathogenic function are still unknown. Our recent finding that B cell-confined enhancement of Btk signaling through transgenic overexpression of wild-type human BTK in CD19-hBtk mice was sufficient to establish SLE-like disease highlights the strong contribution of Btk signaling in B cells to the development in autoimmunity (27). However, despite the observed B cell hyperreactivity and apoptosis resistance observed in BCR-stimulated CD19-hBtk B cells, in this study we were unable to dissect whether enhanced BCR signaling alone was sufficient to propagate autoimmunity in these mice.

Using a deep-sequencing approach of total mRNA expressed in WT and CD19-hBtk naive mature B cells, either unstimulated or stimulated through the BCR, we here report that increased Btk expression levels predominantly affect B cell function *in vivo* through the modulation of BCR signaling. While only a limited set of 108 genes was found to be differentially expressed in unstimulated CD19-hBtk versus WT B cells, 97 of these genes overlapped with 1,442 BCR target genes identified in WT B cells. Surprisingly however, the up- or downregulation of 30 of these 97 BCR-signature genes proved to be inverse compared to expression trends of these 30 genes in BCR-stimulated WT B cells. Although this could indicate that these 30 Btk-induced genes do not truly reflect BCR targets in CD19-hBtk B cells *in vivo*, these 30 BCR-signature genes constitute a disproportionally large fraction (~28%) of the 108 *in vivo* differentially expressed genes in Btk-transgenic B cells regarding the relatively small number of transcripts differentially expressed upon BCR stimulation (1,442 out of 24,452; ~6%). This would make it unlikely that many of these 30 genes are alternatively regulated by other Btk-employing receptors and coincidentally overlap with BCR-induced genes. While we cannot formally exclude that some of these genes may still be induced *in vivo* in CD19-hBtk B cells by pathways other than BCR signaling, it is conceivable that integrated signals from the *in vivo* environment to multiple receptors including the BCR induce differential modulation of expression of genuine BCR target genes.

An alternative explanation for seeming discrepancies in up- and downregulation of BCR target genes in CD19-hBtk B cell *in vivo* could be the different nature of kinetics of BCR activation *in vivo* versus *in vitro*. Compared to *in vivo* BCR-antigen interactions which are frequently of low affinity, in vitro BCR-antigen interactions mimicked by anti-IgM stimulation only resemble high-affinity interactions and are thus less intermittent, longer in duration and provoke stronger B cell activation. Indeed, remarkable differences in BCR targets genes following single-round BCR stimulation versus continuous BCR stimulation were demonstrated by Damdinsuren et al., showing that 9 hrs after the onset of single-round versus continuous BCR stimulation of WT B cells only 35-47% of BCR-induced genes were shared between these differentially stimulated cells (40). This suggests that in unstimulated CD19-hBtk cells the 30 BCR target genes exhibiting an inversed expression pattern compared to BCR-stimulated WT B cells may reflect true BCR target genes that are nevertheless differentially regulated due to the different nature or kinetics of BCR engagement *in vivo* versus *in vitro*.

From our total mRNA deep sequencing study, only 2 genes (Top2a and Spag5) not belonging to BCR signature genes could be observed whose expression was continuously increased or decreased

in CD19-hBtk B cells compared with WT B cells, irrespective of BCR stimulation (Table S3). This virtual absence of genes that are constitutively induced by Btk, independently of BCR signaling, indicates that Btk overexpression itself does not affect B cell functioning, but that instead changes in functioning of CD19-hBtk B cells are dependent on the kinase activity of increased Btk levels in the context of BCR signaling. In line with this, we previously observed that the phenotype of CD19-hBtk mice is fully dependent on the kinase activity of Btk, since PCI-32765 treatment could abolish spontaneous B cell activation and IgG plasma cell formation, and since autoimmunity was corrected in mice overexpressing the K430R kinase inactive mutant form of Btk (27).

Our finding that enhanced Btk-mediated BCR signaling represses rather than stimulates Bcl6 expression seems to contradict the observation of spontaneous GC formation in CD19-hBtk mice (27), since Bcl6 is critical for the induction of GCs (35-37). One of the functions of Bcl6 expression is to prevent premature plasma cell differentiation of GC B cells by repressing the plasma cell commitment transcription factor Blimp-1 encoded by the *Prdm1* gene (39). The enhanced IgG plasma cell formation in CD19-hBtk mice may thus result from less stringent Blimp-1 repression in Btk-transgenic B cells as observed after 3 to 4 days of *in vitro* anti-IgM stimulation. Importantly, a reduced threshold for Blimp-1 induction in Btk-overexpressing B cells may not simply drive plasma cell differentiation but also thwart the counterselection of autoreactive GC B cells as earlier or increased Blimp-1 induction may promote the premature exit of GC B cells as plasmablasts, thereby allowing newly arising self-reactive GC B cells to escape T cell-mediated negative selection. The autoantibody-producing cells appearing in lungs of influenza-infected CD19-hBtk mice may possibly originate from autoreactive plasmablasts that prematurely escape the GCs in inducible bronchus-associated lymphoid tissue (iBALT). On the other hand, we found that expression of the constitutive active E41K-Btk mutant in transgenic mice did drive B cells very efficienctly into IgM plasma cell differentiation (28, 41). Further experiments should show whether this phenomenon can be explained by the capacity of E41K-Btk to decrease Bcl6 and induce Blimp-1 expression in naive B cells outside germinal centers.

In summary, our mRNA deep-sequencing data indicate that the autoimmune-phenotype of CD19-hBtk mice is almost exclusively programmed by enhanced BCR signaling through Btk. This implies that inhibition of the BCR signaling pathway is already sufficient to block the development of SLE-like disease. This knowledge further warrants the therapeutic potential in the treatment of patients with rheumatic diseases, and pleads for further research for the development and clinical testing of Btk-inhibitors. In this light, the finding of off-target Itk-inhibition by Btk-inhibitor PCI-32675/Ibrutinib with effects on T cell activation and differentiation stresses the need for further development of Btk-inhibitors with higher Btk-selectivity (42).

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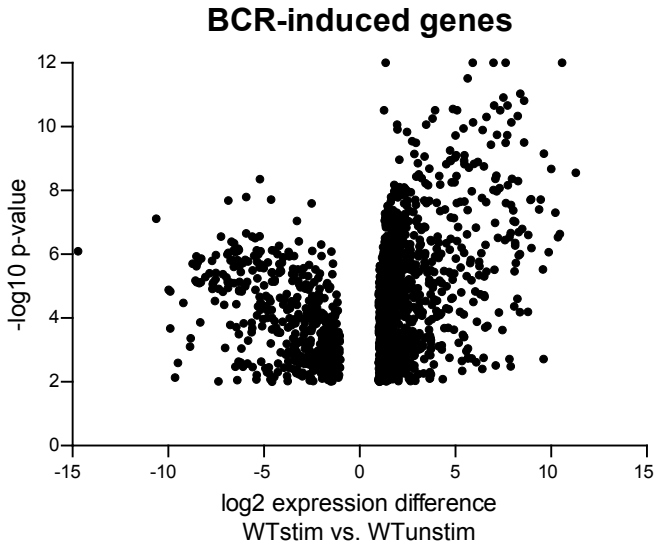
Supplemental data

Figure S1. Genes induced by BCR signaling in WT B cells.



Overview of all genes differentially expressed ($p<0,01$ and ≥2-fold expression difference) between naive splenic WT B cells before (WTunstim) and after 12 hours stimulation with 10 µg/mL $F(ab')_2$ a-IgM (WTstim).

Supplemental tables

Table S1. BCR target genes upregulated in CD19-hBtk B cells in vivo.

	Unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Hint1	0,325	76,39001	332,4875	401,78	0,001945	7,491665
Sdhd	0,125	9,9025	99,4675	120,0675	6,42E-05	5,474292
Vprbp	0	4,3	23,69	22,895	0,003285	5,459432
Commd4	0	3,97	94,12	86,24249	8,71E-07	5,346957
Rilpl2	0,925	34	338,5125	385,96	6,55E-05	5,056076
Cox5a	0,075	5,587501	247,0225	305,1775	2,29E-07	5,022368
Iars2	0	2,605	26,85	29,2125	4,31E-05	4,757557
Aarsd1	0,125	4,4575	30,8925	39,8	0,003058	4,340246
Prps1	0	1,8975	30,89	39,1975	5,06E-09	4,320124
0610010K14Rik	0,125	3,93	39,4925	38,44	2,38E-04	4,162783
Dlat	0	1,4425	15,35	16,715	6,76E-05	3,947199
Xpo5	0	1,2925	33,165	35,14	9,78E-07	3,799605
Pfn1	3,925	52,77	1449,5	1492,893	1,61E-07	3,715389
Mterfd1	0,025	1,4525	29,6075	29,65	2,15E-07	3,634593
Timm8a1	4,85	54,55	73,705	123,125	3,57E-06	3,464721
Hnrnpu	3,175	35,53	333,4975	365,6725	1,74E-04	3,443526
Ndufb9	3,675	33,7175	157,73	184,175	3,35E-04	3,163221
Rrp9	1,275	11,96	104,4025	126	4,54E-06	3,132726
Isg20l2	2,625	23,5575	167,895	197,9175	3,14E-05	3,117969
Pomp	8,499999	63,7275	261,02	317,8775	2,30E-04	2,89177
Cfl1	28,075	192,69	1136,85	1248,2	0,002213	2,774543
Cct3	7,9	54,52	317,395	365,22	1,56E-05	2,771357
Rps27a	0,225	1,85	7,94	22,3675	2,03E-04	2,584962
Ddx10	1,3	7,8825	28,435	29,0925	0,008202	2,511414
Alg9	0,225	1,685	14,025	15,9575	3,82E-04	2,457412
Mrps24	3,2	17,6425	43,7675	50,785	0,00585	2,426671
Tmc8	13,625	70,1625	269,58	251,785	1,49E-05	2,355949
Cox17	7,775	35,9225	204,2175	288,6875	0,001773	2,193546
Mrps18a	5,925	25,115	58,3575	70,2825	6,55E-04	2,065249
Larp4b	0,625	2,8725	54,7925	59,65	1,73E-06	2,035624
Lck	4,025	15,4125	51,2975	55,77	6,24E-06	1,910965
Prmt3	0,3	1,3875	46,19	45,2175	3,65E-10	1,894818
Rhof	12,125	44,755	50,9175	62,185	0,002452	1,875434
Il1r2	0,35	1,495	6,045	10,54	1,73E-06	1,82556
Ifitm1	4,1	14,175	51,625	66,59	8,30E-06	1,76503
Deptor	0,9	3,24	18,2625	19,0275	8,12E-05	1,739848
Mrpl12	11,6	37,7175	173,2225	203,6025	7,98E-06	1,692545
Gapdh	2,825	8,5175	36,715	50,2525	0,007925	1,558833
Eif5a	29,15	85,39751	570,5225	690,4525	7,23E-04	1,547446

Table S1. (continued)

	Unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Olfr1342	1,75	5,13	5,2475	11,19	0,001396	1,499286
Dtl	0,95	2,675	4,35	11,535	3,91E-05	1,402099
Nop56	0,95	2,6475	31,795	27,995	7,30E-08	1,38773
Eif3c	12,7	32,7775	214,52	234,37	2,80E-07	1,360957
Ccdc25	0,575	1,5875	33,535	2,0875	3,07E-12	1,321928
Cyb5b	4,725	11,96	29,055	42,1475	6,15E-04	1,321629
Angptl6	4,3	10,6825	177,13	192,7025	6,29E-07	1,293117
Atp5g3	42,425	97,705	559,285	645,8525	9,09E-04	1,201597
Parp11	1,025	2,445	8,514999	7,1675	9,22E-08	1,177741
E2f5	1,075	2,545	11,825	10,2425	2,58E-06	1,170607
Banf1	14,575	32,6725	396,5625	462,6525	4,07E-06	1,159125
AI467606	1,975	4,5075	12,1025	8,775	4,37E-04	1,150873
1110004E09Rik	3,475	7,835	24,0725	28,88	0,001094	1,150287
Ntng1	2,175	4,915	8,0875	7,2	0,00125	1,140383
Hmgn3	4,875	10,795	31,4225	35,0625	8,06E-04	1,130898
Nhp2	16,7	36,3475	219,83	285,0425	1,80E-05	1,117359
Adrm1	19,425	41,63	108,9375	127,17	0,002248	1,095762
Ndrg1	1,925	4,205	6,4275	6,7	0,003903	1,088091
Tmem8	0,875	1,97	4,58	3,2725	0,003315	1,086157
Mpnd	1,85	4,02	6,5675	15,835	5,17E-04	1,07917
Casc5	1,35	2,885	5,13	14,3375	3,61E-04	1,041678
Gse1	2,15	4,43	481,51	530,9125	6,77E-09	1,009586
Caprin1	2,1	4,275	28,0225	34,81	0,007616	0,991779
Serpinb9	2,15	4,3225	12,265	36,6775	0,001637	0,974937
Ung	1,325	2,6825	12,3175	22,2425	2,99E-04	0,96542
Idh3a	1,1	2,22	33,6575	41,2075	5,53E-09	0,95109
Hgs	287,4	6,53	6,8175	6,3375	7,52E-07	-5,43841
Cspg5	9,25	0,04	0,0125	0,0225	1,54E-06	-6,06147

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ unstimulated} + 0{,}1) / (FPKM^{WT;\ unstimulated} + 0{,}1))$

Table S2. BCR target genes inversely expressed in CD19-hBtk B cells in vivo.

	unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Fam117b	1,75	47,3375	6,4725	5,3675	1,07E-05	4,680431
Klk14	1,675	14,12	0,05	0,0575	2,97E-05	3,002031
Gm6793	1,1	7,1575	0,0925	0,13	2,82E-05	2,596438
Hspa9	8,225	49,905	24,4425	30,1625	1,35E-04	2,58655
Pag1	2,925	17,725	7,985	5,08	0,006705	2,558895
Agphd1	3,375	10,5475	0,03	0,0075	0,003488	1,61543
Sdc1	1,575	4,1175	3,565	2,6575	0,002408	1,332227
Derl3	4	10,0475	1,5975	3,2275	5,08E-04	1,307429
Ckb	5,6	12,675	0,3725	0,995	4,02E-05	1,164289
Tifab	1,5	3,475	0,1025	0,135	4,38E-06	1,159871
Fgl2	1,25	2,8	0,2425	0,46	6,81E-05	1,103093
Ccnd1	2,725	5,7275	0,5375	0,595	1,75E-05	1,044626
AF251705	5,925	12,2875	0,4875	0,4875	1,45E-04	1,039852
Havcr2	2,1	4,39	0,16	0,1225	0,001573	1,029212
Sept1	11,425	4,895	130,19	119,975	2,50E-08	-1,20621
Glrx5	9,5	3,705	58,695	70,3225	4,22E-04	-1,33514
Mybbp1a	7,3	2,1925	244,4525	265,655	7,88E-10	-1,6906
Olfr24	2,375	0,6625	47,3725	49,7875	7,15E-05	-1,69862
Ube2e1	4,475	1,1875	47,2275	52,1325	0,001915	-1,8292
Hspbp1	4,1	0,875	80,7375	77,4675	9,66E-07	-2,10692
Ndufa4	99,675	22,305	492,755	550,66	3,07E-06	-2,15486
Ddx24	18,525	3,5075	73,0425	81,835	0,001689	-2,36817
Hnrnpa0	33,675	5,33	137,0875	144,62	0,009352	-2,63693
Mrpl28	1,95	0,205	47,2875	55,875	4,50E-07	-2,74874
Spg11	8,175	1,1275	2188,04	2348,45	8,97E-08	-2,75304
Tsc22d3	1,85	0,095	10,1825	5,6075	6,28E-05	-3,32193
Sf3b5	26,25	1,1075	162,4675	178,9825	1,34E-04	-4,44771
Clp1	8,9	0	23,96	24,6875	5,25E-07	-6,49185
Cct2	37,225	0	357,0325	429,3675	8,50E-05	-8,544
Npm1	59,075	0	970,43	1265,99	2,84E-05	-9,20884

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ unstimulated} + 0{,}1) / (FPKM^{WT;\ unstimulated} + 0{,}1))$

Table S3. In vivo differentially expressed genes in CD19-hBtk B cells not induced by BCR signaling.

	Unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Gfm1	0,55	11,83	0,0525	0,065	8,12E-06	4,198011
Gpm6b	0,1	2,155	0,0375	0,2675	0,003638	3,495056
Stac	0,825	5,4475	0,005	0,03	0,003695	2,584312
Btk	73,85001	242,79	62,7	100,45	1,21E-06	1,715681
Spc24	1,125	3,875	2,095	8,665001	0,005258	1,698173
Cdk1	0,725	2,565	1,4925	8,4775	1,30E-04	1,69167
Top2a	4,4	12,475	5,38	20,4625	0,002584	1,482562
Clec4a4	0,975	2,49	0,0975	0,1725	7,77E-07	1,268615
Prss30	0,775	1,875	0,01	0,095	4,25E-07	1,174498
Spag5	1,125	2,6175	0,87	3,515	0,006164	1,149498
Wnt1	6,825	0,005	6,1225	74,97	4,60E-04	-6,04335

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ unstimulated} + 0{,}1) / (FPKM^{WT;\ unstimulated} + 0{,}1))$

Table S4. BCR target genes stronger up/downregulated by enhanced Btk signaling.

	unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Sp140	2,325	4,195	5,7725	138,2875	3,07E-11	4,558595
Ms4a4b	7,3	9,5575	16,005	94,315	1,46E-05	2,551507
Sp110	17,775	27,09	45,51	256,555	0	2,492408
Acss1	0	0	2,9575	16,28	4,06E-06	2,421511
Gm11428	14,475	19,3425	61,49	192,5225	0,002079	1,645008
Serpinb9	2,15	4,3225	12,265	36,6775	0,001637	1,572561
Rps27a	0,225	1,85	7,94	22,3675	2,03E-04	1,482572
Casc5	1,35	2,885	5,13	14,3375	3,61E-04	1,464938
Exo1	0,45	0,8525	1,5075	4,2	4,31E-04	1,419518
Pabpc4	0,1	0,13	4,955	13,3325	5,26E-05	1,409945
Dtl	0,95	2,675	4,35	11,535	3,91E-05	1,386594
Cst7	0,65	1,6275	6,335	16,675	7,69E-05	1,382301
Dhfr	0,7	1,2025	1,775	4,6625	4,47E-05	1,344828
Gmnn	6,675	10,8925	13,8775	34,89	0,009783	1,323836
Mpnd	1,85	4,02	6,5675	15,835	5,17E-04	1,256981
A630055G03Rik	0	0,18	1,85	4,4425	8,00E-06	1,220012
Noc3l	5,425	8,5825	12,0725	27,21	2,77E-06	1,165804
Prkca	2,9	4,685	11,5	25,51	7,96E-07	1,142582
Lig1	6,8	10,9175	14,3175	31,24	1,53E-04	1,120184
Mcm4	0	0	27,89	60,35	9,67E-08	1,110831
Olfr1342	1,75	5,13	5,2475	11,19	0,001396	1,078109
Ckap2	0,65	1,4175	1,96	4,2025	2,38E-06	1,062531
Triobp	0,15	0,38	1,315	2,77	2,32E-04	1,020249
Rbl1	43,175	47,7	17,845	8,8125	7,92E-05	-1,00968
Bcl6	28,45	30,345	8,934999	4,3425	8,88E-04	-1,02415
Bcl9l	21,425	16,485	7,295	3,3575	0,007173	-1,09682
Rgs18	114,725	165,555	19,4	8,9175	4,82E-05	-1,11267
Cib1	57,825	54,0975	5,537499	2,4725	3,08E-04	-1,13188
Exoc6	42,125	38,1425	14,8275	6,6475	0,002833	-1,14555
Cmah	18,55	16,135	2,9625	1,2675	0,008934	-1,16317
Cyp4f18	80,4	65,9575	6,34	2,6225	4,95E-05	-1,24213
Klf2	543,175	560,59	22,03	8,735001	1,95E-08	-1,3247
Cnr2	49,575	47,01	8,82	3,32	2,02E-04	-1,38305
Daf2	48,2	37,25	6,88	2,4875	0,001686	-1,43167
Ltb	349,2	352,2075	27,3425	9,785	1,17E-06	-1,4731
C4bp-ps1	177,35	135,0875	21,22	7,56	5,38E-04	-1,47679
Dpt	8,95	10,695	3,2825	1,0175	0,006637	-1,59782
A430078G23Rik	23,45	21,2025	3,1225	0,935	0,001856	-1,63855
Adrb2	25,5	23,57	4,665	1,3625	0,003886	-1,70404
Plekhm1	112,2	107,32	18,5575	5,4425	2,31E-04	-1,75115
Rasgrp2	132,725	122,005	7,6225	2,05	3,43E-06	-1,84473

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Pdlim7	192,775	201,2475	28,6025	7,0275	6,20E-05	-2,00971
Il9r	36,9	32,515	5,22	1,0725	8,97E-05	-2,18184
Copg2	15,1	16,4075	2,27	0,42	1,28E-05	-2,1883
Bcl7a	21,425	18,8775	1,2525	0,19	3,72E-05	-2,2215
R3hdm2	25,625	32,8525	2,57	0,2275	9,11E-08	-3,02727
Vsx1	15,2	14,8375	1,11	0	1,00E-04	-3,59694
D630039A03Rik	32,9	51,0925	5,48	0,3275	0,002928	-3,70627
Ms4a1	415,925	401,9175	158,28	10,1975	1,95E-06	-3,94302
Ccdc142	8,475	8,6175	1,87	0,0275	0,003055	-3,94963
Rfwd2	6,625	7,67	1,5625	0,0025	4,94E-07	-4,01966
Oit1	125,35	123,9425	11,8225	0,6025	0,001668	-4,08504
Gsg1	41,925	47,555	3,8125	0,125	7,62E-04	-4,12009
Mpzl1	105,225	108,51	10,595	0,2725	0,001341	-4,84355
Eif2ak3	28,975	29,3675	2,98	0	0,004428	-4,94486

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ a\text{-}IgM\ stimulated} + 0{,}1) / (FPKM^{WT;\ a\text{-}IgM\ stimulated} + 0{,}1))$

Table S5. Shifting BCR target genes induced by enhanced Btk signaling.

	unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
Dctn2	1,35	1,1875	0,0375	28,08	8,87E-04	7,679096
Sfi1	0,075	0,11	0,0775	27,4725	0,001597	7,279267
Strn4	0,1	0,1475	0,0825	19,5925	2,67E-08	6,753606
Tiprl	0	0,06	0	8,6	2,26E-06	6,442943
Slc25a36	0	0,005	0	8,3825	9,51E-11	6,406418
Ermap	24,1	25,315	0,7	22,8525	0,003764	4,842507
Naca	34,2	34,865	33,595	612,6725	6,80E-07	4,184745
Sec16b	0,2	0,2675	0,0275	1,875	1,91E-07	3,953284
Wnt1	6,825	0,005	6,1225	74,97	4,60E-04	3,59267
Mlycd	8,5	9,33	6,5425	62,38	4,12E-09	3,233596
Evi2a	4,85	7,3925	3,7925	34,1975	5,10E-07	3,139335
4930455F23Rik	1,225	1,2575	1,585	13,875	3,51E-07	3,052028
Serpinb1c	2,9	2,7025	3,285	26,115	0,001737	2,953165
BC030867	0,25	0,7	0,48	2,6875	0,001338	2,264847
Spry1	0,775	0,985	1,03	4,86	0,001961	2,134017
Spc24	1,125	3,875	2,095	8,665001	0,005258	1,997533
Top2a	4,4	12,475	5,38	20,4625	0,002584	1,907768
Spag5	1,125	2,6175	0,87	3,515	0,006164	1,897939
Dusp14	0,225	0,9275	1,1625	3,78	0,007267	1,619773
Tyms	0,35	1,04	1,1325	3,465	0,004887	1,532314
Rad54b	0,375	0,84	0,725	2,105	0,008837	1,418313
Brca1	0,225	0,215	1,2225	3,04	3,41E-06	1,247497
Dzip3	1,25	2,2625	1,99	4,715	4,43E-04	1,204033
Derl3	4	10,0475	1,5975	3,2275	5,08E-04	0,971027
Xpo6	37,3	38,9925	49,0225	17,6025	0,004935	-1,47243
Nasp	13,825	16,8425	51,1425	18,12	5,85E-08	-1,49182
Fgd3	27,125	27,37	63,3675	10,7675	1,49E-06	-2,546
2310001A20Rik	10,2	11,43	15,835	2,2125	7,05E-05	-2,78467
Fdxr	8,175	9,6225	16,6575	1,195	3,12E-04	-3,69378
Utp6	9,224999	9,905001	29,1675	2,06	1,61E-07	-3,7602
Ccdc25	0,575	1,5875	33,535	2,0875	3,07E-12	-3,94261
Smad5	24,1	26,6675	32,26	1,14	3,45E-05	-4,7058
2900010M23Rik	37,05	43,645	179,6425	4,6275	1,27E-07	-5,24871
Pcyox1l	2,95	3,435	4,095	0	7,68E-05	-5,3906
Gpr161	14,5	20,645	46,265	0,9225	3,26E-05	-5,50286
Tnr	0	0,0125	4,96	0,01	0,00183	-5,52356
Naca	85,52499	82,4225	57,3725	1,07	2,55E-05	-5,61829
Cdh16	0,025	0,635	5,04	0	0,00449	-5,6837
Loxl3	0,65	1,1825	13,015	0,1375	4,30E-07	-5,78715
Mtap6	0	0	24,285	0,3075	7,36E-11	-5,90305
Tbx19	5,575	6,4425	8,8875	0,01	3,83E-06	-6,35234

Clstn2	15,7	15,57	11,4275	0	5,82E-04	-6,84894
Gpbp1l1	94,50001	119,8125	160,37	0,56	9,75E-07	-7,92562
Ict1	19,4	20,81	48,8675	0,045	1,75E-06	-8,39963
Zfp691	0,15	0,14	62,4325	0,085	4,34E-09	-8,40094

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ a\text{-}IgM\ stimulated} + 0{,}1) / (FPKM^{WT;\ a\text{-}IgM\ stimulated} + 0{,}1))$

Table 6. Other genes differentially induced by enhanced BCR signaling through Btk.

	unstimulated		*a-IgM stimulated*			
Gene	*WT*	*CD19-hBtk*	*WT*	*CD19-hBtk*	*p-value*	*log2 difference*[1]
5330437I02Rik	100	124,57	0	6,14	1,32E-05	5,963474
C030030A07Rik	34,275	49,3225	0,0075	6,1025	1,36E-04	5,850441
Mir1966	7,9	7,8575	0	1,275	0,002296	3,78136
Atp8a1	14,825	14,6225	0,12	2,7375	0,003377	3,689045
Letm2	9,400001	9,49	0,0125	1,0725	0,008812	3,381591
Urm1	45,2	53,39	0,4025	5,1325	0,003376	3,380305
Duox1	1717,825	2099,553	2,795	27,2775	3,38E-05	3,241355
Cdk1	0,725	2,565	1,4925	8,4775	1,30E-04	2,429264
2010001M06Rik	29,075	32,26	2,21	6,715	0,003679	1,560821
Sumo1	39,425	50,0975	0,805	2,405	1,30E-05	1,468821
Efcab4b	28,45	32,125	1,1	2,73	2,29E-04	1,237768
Mrpl53	28,05	31,61	2,0275	4,48	0,002268	1,106188
Lta	22,1	22,035	3,3775	7,2675	0,002786	1,083125
Spna2	61,15	72,6925	3,02	6,345	9,89E-07	1,046634
Rps19	624,325	638,2575	900,56	370,1525	0,006792	-1,28247

1) expression difference calculated with a correction for FPKM values of 0 using the following formula:
$\log_2((FPKM^{CD19\text{-}hBtk;\ a\text{-}IgM\ stimulated} + 0{,}1) / (FPKM^{WT;\ a\text{-}IgM\ stimulated} + 0{,}1))$

7

Dysregulated BTK expression in peripheral blood B cells from SLE patients

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Abstract

Pharmacological inhibition of the signaling molecule Bruton's tyrosine kinase (Btk), which delivers crucial survival signals from the B cell antigen receptor (BCR) in B cells, effectively prevents or ameliorates lupus nephritis in SLE mouse models. Conversely, increasing Btk signaling through Btk overexpression in murine B cells induces an SLE-like disease phenotype. Given this crucial role for Btk expression levels in murine SLE pathogenesis, in this report we compared BTK expression and regulation in peripheral blood B cells from SLE patients and healthy controls. In agreement with previous findings in the mouse, we observed that BTK protein expression in normal human B cells is upregulated upon BCR stimulation. Moreover, Btk expression levels are significantly higher in $CD27^+$ antigen-experienced B cells compared with naive $CD27^-$ B cells. By contrast, in SLE B cells we found only limited BTK upregulation upon BCR stimulation *in vitro* and did not detect significant differences in BTK protein levels between $CD27^+$ and $CD27^-$ B cells. BTK levels appeared particularly increased in naive B cells of SLE patients with anti-cardiolipin autoantibodies in the serum and in patients with hematologic cytopenias. Finally, BTK levels in total B cells correlated significantly with C4 hypocomplementemia. Taken together, this study delivers the first evidence that BTK expression is altered in SLE patients and may correlate with disease activity. High BTK levels particularly marked an SLE phenotype overlapping with anti-phospholipid syndrome (APS). We therefore conclude that our observations provide further support for testing newly developed BTK-inhibitors in SLE treatment.

In preparation

Introduction

Systemic lupus erythematosus (SLE) is a rheumatic autoimmune disease occurring in up to 1 in 2000 individuals, affecting predominantly women and more frequently people of African or Asian ancestry (1, 2). Although many immune cell types contribute to SLE, a central role is attributed to B cells and the autoantibodies they produce since these autoantibodies often appear in patient serum before the onset of clinical symptoms (3). These autoantibodies are directed against a multitude of nuclear self-antigens and form circulating immune complexes that upon deposition in organs as joints, eyes, skin, lungs and kidneys elicit inflammation (1, 2). Many B cell-targeted therapies have therefore been tested in SLE treatment, but results of B cell-depleting regimens mostly prove to be disappointing (4). However, in SLE the efficacy of belumimab, a monoclonal antibody neutralizing B cell survival cytokine BAFF/BLyS, indicates that intelligent modulation of B cell functioning and survival in SLE may be key to successful treatment(5, 6).

Promising new drug candidates are newly developed inhibitors of the signaling molecule Bruton's tyrosine kinase (BTK), which conveys essential survival and activation signals to B cells. The importance of BTK signaling to B cells is evident from the severe B cell deficiency X-linked agammaglobulinemia (XLA) occurring in Btk-deficient patients (7). In murine models for SLE promising results have been obtained with Btk small molecule-inhibitors Ibrutinib (PCI-32765) and RN486, which could prevent or ameliorate lupus nephritis by correcting BCR-mediated B cell activation and autoantibody production (8-10). The effectiveness of these Btk inhibitors may not solely rely on their effects in B lymphocytes since these inhibitors also dampen myeloid cell activation in murine models for autoimmune arthritis (11-13). Nevertheless, Btk signaling in B cells appears to be crucial for SLE pathogenesis, since Btk-overexpressing mice in which Btk signaling is selectively enhanced in B cells spontaneously develop lupus-like disease (14). In these mice, modest B cell-restricted Btk overexpression selectively increased signaling of the B cell receptor (BCR) and rendered these B cells apoptosis-resistant, thereby corrupting the counterselection of selfreactive B cells and facilitating their differentiation into autoantibody producing plasma cells.

The finding that a modest increase in Btk expression in B cells is sufficient to induce SLE-like disease prompted us to examine BTK expression levels and regulation in B cells from SLE patients. Here we show that in congruence to murine B cells, human B cells of healthy subjects upregulate BTK protein levels following BCR stimulation in vitro, and that BTK upregulation equally occurs in memory B cells in vivo. We here provide the first data on BTK expression levels in B cells from SLE patients. We show defective BTK upregulation in BCR-activated B cells *in vitro* and in memory B cells *in vivo*, while naive B cells exhibit heterogeneous and high BTK protein levels. Furthermore, we observed correlations between BTK protein expression levels and disease activity and clinical parameters of SLE patients.

Materials & methods

Patients and healthy control subjects

After informed consent (according to the Declaration of Helsinki) peripheral blood samples were obtained from 23 SLE patients and 20 healthy controls (HCs). SLE patients (22 females and 1 male) had a mean age of 41.1 years (range of 19.8 – 74.0 years). Patients had an average disease duration of 8.3 years (range of

0.1 – 33.5 years) and patients with disease flares were excluded from study participation (average SLEDAI score 1.5; total range 0-10). Of all 23 included patients, 20 were receiving one or more immunosuppressive drugs at the time of blood collection (17 receiving hydroxychloroquine, 3 prednisolone, 3 prednisone, 3 azathioprine, 3 methotrexate, 2 mycophenolate mofetil, 1 cyclophosphamide, and 1 indomethacin). This study was approved by the Medical Ethical Committees of the Erasmus MC Rotterdam and the University Medical Center Groningen.

Flow cytometry procedures

Fluorescent labeling of cell membrane markers was performed as described previously (14). Fluorochrome or biotin-labeled monoclonal antibodies were used to detect human CD19 (SJ25C1, BD Biosciences), CD21 (B-ly4, BD Biosciences), CD27 (M-T271, BD Biosciences), CD38 (HIT2, BD Biosciences), IgD (IA6-2, BD Biosciences) and IgM (G20-127, BD Biosciences). Indirect fluorescent labeling of biotin-conjugated antibodies was performed with fluorochrome-coupled streptavidin (eBioscience). After membrane marker staining, total intracellular BTK levels were measured by fixing peripheral blood mononuclear cells (PBMCs) in PBS/2% PFA and subsequent permeabilization and staining in MACS sorting buffer containing 0.5% saponin (Sigma-Aldrich) and BTK/53 monoclonal antibody (BD Biosciences). Flow cytometric measurements were performed on a LSRII™ flow cytometer (BD Biosciences) and data were analysed using FlowJo software (Tree Star Inc.).

Isolation and culture of human peripheral blood B cells

PBMCs were isolated by centrifuging blood samples diluted 1:1 in PBS over a Ficoll-Paque (GE Healthcare) density gradient for 20 min at 1200g at room temperature. Purification of naive B cells from PBMCs was performed using the human Naive B Cell Isolation Kit II (Miltenyi Biotec) according to manufacturer's protocol, and B cell purity (at least >95%) was verified using flow cytometry. B cells were cultured in the presence of 10 µg/mL $F(ab')_2$ goat anti-mouse IgM (Jackson Immunoresearch), 2 µg/mL recombinant CD40L (R&D Systems), 1 µg/mL imiquimod (InvivoGen), 2 µg/mL LPS (own production) or 5000 U/mL IFN-α2 (PBL Interferon Source) for 3 days.

Statistical analysis

For statistical analysis of differences in BTK mean fluorescence intensities (MFIs) of human peripheral B cell subsets, the Mann-Whitney U test was employed. Statistical analysis of B cell BTK MFI values versus categorical clinical data was performed using a Fisher's exact test. The association between serum complement levels and BTK MFI values in B cells was assessed using a Pearson's rank correlation. A Wilcoxon signed-rank test was used to compare BTK levels in $CD27^-$ and $CD27^+$ B cells in HCs and SLE patients. All calculations have been performed using SPSS software (IBM).

Results

In vitro and in vivo BTK upregulation in activated human B cells

As Btk upregulation commonly occurs in murine B cells stimulated through the BCR, CD40 or TLRs (14, 15), we examined whether BTK upregulation would also occur in activated human B cells. Therefore,

we stimulated B cells from PBMCs of healthy control subjects (HCs) for 3 days with α-IgM, recombinant CD40L (rCD40L), and several TLR ligands. In agreement with our findings in murine B cells, α-IgM stimulation could indeed induce robust BTK protein upregulation in human B cells, as detected by an intracellular flow cytometric assay (Figure 1). In contrast to murine B cells, other stimuli including CD40L, LPS, and imiquimod only induced a limited increase of BTK protein levels (Figure 1 and data not shown).

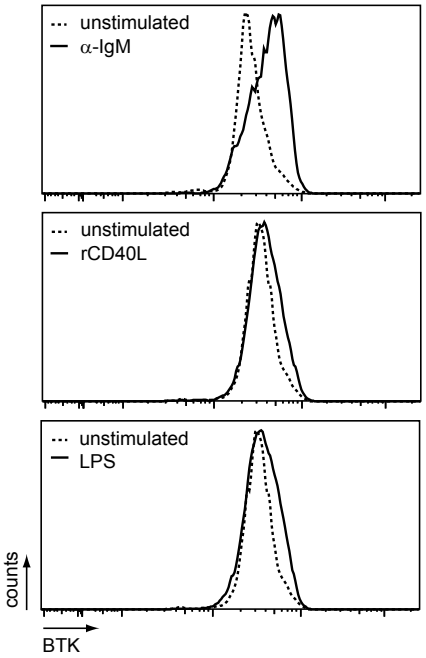
To test whether BTK upregulation also occurs in (previously) activated B cells *in vivo*, we compared BTK expression levels in HCs between B cells expressing memory B cell marker CD27 and B cells negative for this marker. In all HCs, we observed that BTK expression levels were significantly higher in antigen-experienced ($CD19^+CD27^+$) B cells compared with naive ($CD19^+CD27^-$) B cells (Figure 2).

These findings indicate that BTK is dynamically expressed in human B cells and predominantly induced by BCR signaling. The higher BTK expression levels in $CD27^+$ memory B cells *in vivo* suggest that BTK upregulation is long-lasting rather than transient.

Dysregulated BTK expression in SLE B cells

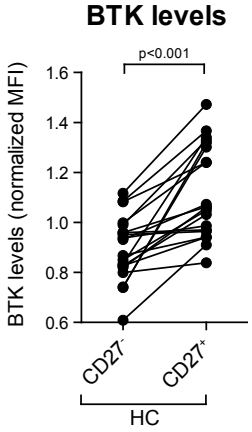
Since BTK is upregulated in activated human B cells, and since transgenic Btk overexpression in murine

Figure 1. BCR stimulation of human B cells induces BTK upregulation.



Intracellular measurement of BTK using flow cytometry in MACS-sorted peripheral blood B cells from healthy control subjects (HCs) after 3 days of stimulation with $F(ab')_2$ α-IgM (10 μg/mL), recombinant CD40L (rCD40L, 2 μg/mL) or LPS (2 μg/mL). Representative data from 1 out of 2 independent experiments are shown.

Figure 2. BTK is upregulated in vivo in antigen experienced CD27$^+$ B cells.



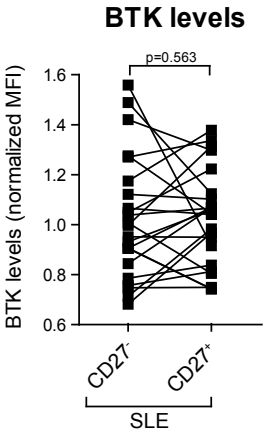
BTK expression levels in blood naive B cells (CD19$^+$CD27$^-$) and antigen-experienced CD27$^+$ B cells from HCs determined by MFI values using flow cytometry. Collective data of 3 independent experiments are shown. BTK MFI values were normalized between experiments by setting the average BTK MFI value for HCs in total CD19$^+$ cells at 1 per individual experiment and calculating BTK expression levels in SLE patients relatively to normalized BTK expression levels in HCs.

B cells induces a systemic autoimmune disease closely resembling SLE (14), we investigated whether BTK regulation could be disturbed in B cells from SLE patients. To test this, we purified peripheral blood naive B cells from SLE patients and age- and gender-matched HCs and examined BTK protein expression levels in these B cells after 3 days of culture with or without α-IgM stimulation. While after 3 days of culture no clear differences in BTK levels in unstimulated B cells from SLE patients versus healthy controls could be observed, we noted a significantly ($p<0.05$) weaker BTK upregulation in BCR-stimulated SLE B cells versus HC B cells (Figure 3).

This finding of defective induction of BTK upregulation following BCR stimulation prompted us to investigate BTK expression levels in CD27$^+$ antigen-experienced B cells *in vivo* in SLE patients. When we compared BTK protein levels between CD27$^+$ B cells from SLE patients and HCs, we found that relative BTK expression were slightly lower in SLE patients (1.04, compared with 1.12 in HCs; (1.04 and 1.12, respectively; $p=0.253$). In contrast, we noted a slight increase in BTK expression levels in CD27$^-$ B cells from SLE patients versus HCs (1.02 and 0.90 respectively; $p=0.113$). These findings not only suggest that newly developed B cells in SLE patients express high BTK levels, but furthermore show that BTK upregulation *in vivo* in antigen-experienced cells is defective. Indeed, when comparing BTK expression levels between CD27$^-$ and CD27$^+$ B cells in individual SLE patients, no trend in BTK upregulation could be observed ($p=0.563$; Figure 4), in contrast to our observations in healthy controls ($p<0.01$; Figure 2).

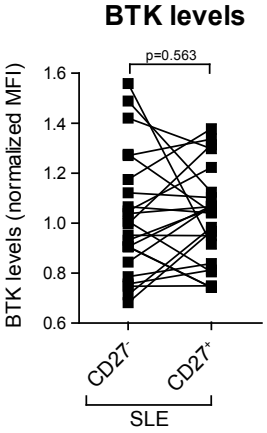
In conclusion, these data suggest that naive B cells in SLE patients may have elevated BTK expression levels and reveal that in SLE BTK upregulation is defective upon B cell activation both *in vitro* and *in vivo*.

Figure 3. Incomplete upregulation of BTK upon BCR stimulation of SLE B cells.



Flow cytometric characterization of BTK expression in MACS-sorted naive B cells from HCs and SLE patients that were cultured with/without 10 µg/mL $F(ab')_2$ α-IgM for 3 days. Representative histograms of 8 HCs and 12 SLE patients are shown in (A), and measured BTK levels (MFIs) are summarized in (B).

Figure 4. Increased BTK expression in naive $CD27^-$ B cells from SLE patients.



In naive B cells ($CD27^-$ $CD19^+$) and antigen-experienced B cells ($CD19^+$ $CD27^+$) from SLE patients BTK expression levels, determined as MFI values were quantified using flow cytometry. Collective data of 3 independent experiments are shown, and data were normalized between experiments to average BTK MFI levels in total $CD19^+$ cells from HCs as described in Figure 2.

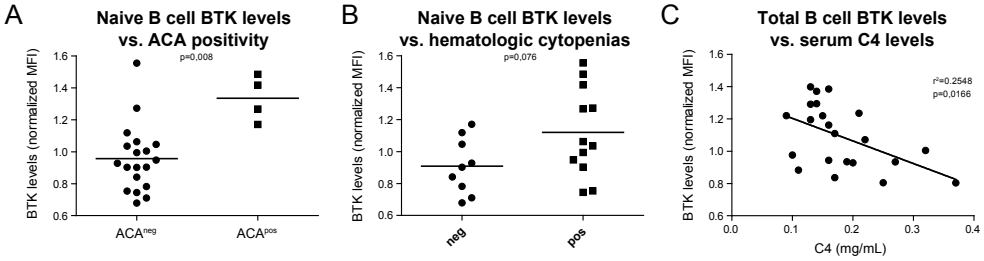
BTK levels in SLE correlate to C4 hypocomplementemia and anti-cardiolipin autoantbodies

While BTK levels in $CD27^-$ B cells were not significantly increased between SLE patients and HCs (p=0.113), in these B cells BTK levels were remarkably heterogeneous among SLE patients with a subgroup of patients exhibiting high BTK levels exceeding BTK levels observed in HCs (Figure 4 versus Figure 2). When we further examined BTK expression in other B cell subpopulations in peripheral blood of SLE patients, no increase or heterogeneity in BTK expression (as found in $CD27^-$ B cells) was observed in transitional B cells ($CD21^{low}CD38^{high}IgM^{high}$), plasmablasts ($CD38^{high}IgM^{low}$) or $CD21^{low}CD38^{low}$ B cells representing recently activated B cells (data not shown).

To examine whether BTK expression levels specifically in naive B cells are a suitable parameter to distinguish a subset of SLE patients with distinct disease characteristics, we correlated many clinical manifestations, disease activity, and laboratory findings to BTK levels in naive B cells in SLE patients. While naive B cell BTK levels failed to correlate significantly with almost all disease and treatment characteristics (including SLEDAI scores, disease duration, anti-dsDNA autoantibodies, anti-nuclear autoantibodies, presence of lupus anticoagulant, serum creatinine levels, proteinuria, anemia, leucopenia, lymphocytopenia, thrombocytopenia, LE cell presence, immunosuppressive medication, and clinical manifestations as butterfly rash, discoid skin lesions, UV hypersensitivity, Raynaud's phenomenon, oral ulcers, arthritis, pleuritis, pericarditis, epileptic seizures, and psychosis), we noted a highly significant association between the presence of anti-cardiolipin autoantibodies (ACAs) and high BTK levels in naive B cells (p=0.008; Figure 5A). Notably, a near-significant positive correlation was observed between the hematologic cytopenias (anemia, leukopenia, lymphocytopenia, and/or thrombocytopenia) and high BTK levels in $CD19^+CD27^-$ B cells (p=0.076), a finding that could be recapitulated when correlating BTK levels in total $CD19^+$ B cells (p=0.078; Figure 5B and data not shown).

Based on the correlation between hematologic cytopenias and BTK expression levels in total $CD19^+$ cells in SLE patients, we also assessed whether the disease and treatment characteristics mentioned above may correlate to BTK levels in total B cells. Again, most parameters did not correlate significantly

Figure 5. BTK expression is dysregulated in SLE and correlates with hypocomplementemia.



(A) Comparison of normalized naive B cell BTK levels as MFIs (calculated as described in Figure 2) in SLE patients that were negative versus positive for anti-cardiolipin autoantibody production (ACA^{neg} versus ACA^{pos}, respectively). (B) BTK expression levels in naive B cells in SLE patients that exhibited no hematologic cytopenias (neg) versus patients with anemia, leukopenia, lymphocytopenia and/or thrombocytopenia (pos). (C) Relative BTK expression levels in total $CD19^+$ B cells from SLE patients were calculated as described in Figure 2 and were plotted versus serum C4 complement levels determined in these patients at the time of blood collection for BTK level measurements.

with BTK expression levels in total CD19$^+$ cells. However, we found a significant inverse correlation between B cell BTK levels and serum C4 levels (p=0.0166; Figure 5C), suggesting that higher BTK levels do correlate with disease activity. Importantly, C4 serum levels also displayed a near-significant correlation with BTK levels in naive CD27$^-$ B cells (p=0.0834; data not shown).

Collectively these findings demonstrate that BTK levels in SLE B cells correlate to C4 hypocomplentemia and thus disease activity. The observed correlation of ACA production and high BTK levels in naive B cells in SLE patients may identify a small patient subgroup with distinct disease characteristics that overlap with the related rheumatic disorder antiphospholipid syndrome (APS).

Discussion

While clinical trials with B cell depleting therapies in SLE mostly yield disappointing results, the response of SLE patients to BAFF/BLyS-neutralizing antibody Belumimab implies that targeting B cell survival may be a highly effective new treatment strategy (4, 5). Another promising way to reduce B cell survival in SLE is by decreasing signaling of the BCR that provides crucial survival signals to mature B cells (16), possibly in conjunction with BAFF-R signals (17, 18). Moreover, targeting of BCR signaling in SLE may even causally correct B cell defects, since polymorphisms in several BCR molecules including BANK1 and BLK have been linked to SLE susceptibility in genome-wide association studies (19). Mutations in BCR signaling molecules as CSK that did not reach statistic significance in these linking studies now prove to associate with defective B cell functioning in SLE patients (20). It has been found that an intronic polymorphism in CSK increases its expression levels, leading to stronger inactivation of LYN and thereby enhanced BCR signaling. These results indicate that hardwired mutations that fail to be detected in genome-wide association studies can nevertheless contribute to B cell defects in SLE. In addition, they stress the importance of studying the effects of dysregulated expression of BCR signaling molecules in SLE pathogenesis.

The finding that increased Btk protein levels in B cells are sufficient to establish systemic lupus-like autoimmunity, characterized by enhanced germinal center and plasma cell formation and perivascular inflammation in various organs, prompted us to investigate whether Btk-driven pro-inflammatory loops may also propagate SLE-development in humans. A strong requirement for the establishment of a pro-inflammatory loop involving Btk upregulation in B cells would be the ability to induce BTK upregulation in human B cells, preferentially through multiple activating receptors on B cells. Indeed, in agreement with previous findings in the mouse (14, 15), we observed that BTK protein expression in normal human B cells is upregulated upon anti-IgM stimulation *in vitro*. Moreover, Btk expression levels were significantly higher in CD27$^+$ antigen-experienced B cells, compared with naive CD27$^-$ B cells from healthy controls. By contrast, in SLE B cells we found only limited BTK upregulation upon BCR stimulation *in vitro* and did not detect significant differences in BTK protein levels between CD27$^+$ and CD27$^-$ B cells.

Whereas in murine B cells multiple receptors including TLRs and CD40 were capable of instructing significant Btk upregulation (14), in human B cells BCR signaling much more effectively induced BTK upregulation compared to CD40 and TLR signaling. Since all these receptors in murine and human cells target NF-kB which transcriptionally controls the BTK locus (21), the differential induction of BTK upregulation by these receptors in human and mice thus reflects species differences in transcription

regulation of the *BTK* gene or alternatively in (post-)translational processes controlling BTK levels.

In this study, we are the first to report BTK expression levels in B cells from SLE patients. While we found a non-significant increase in average BTK expression levels in B cells from SLE patients versus HCs, BTK proved to correlate significantly with C4 hypocomplementemia and thus probably disease activity. In line with a link between BTK expression levels and disease activity, a positive trend could be discerned between the occurrence of hematologic cytopenias and high B cell BTK levels especially in naive B cells. Moreover, high BTK levels in naïve B cells correlated strongly with the presence of ACAs in SLE patients. Since cytopenias as leukopenia and thrombocytopenia have been reported to affect APS patients with SLE overlap more frequently (22), the trend of cytopenia occurrence and significant association of ACA-production in SLE patients with high BTK levels in naive B cells indicates that especially in APS patients BTK levels may be constitutively increased and contributing to disease pathogenesis.

Here we show that the higher BTK levels in $CD27^-$ antigen-inexperienced SLE B cells and absent induction of BTK expression in $CD27^+$ antigen-experienced SLE B cells reflects dysregulation of BTK expression that most likely results from a B cell-intrinsic defect in SLE, since isolation and BCR-stimulation of SLE B cells could not induce proper BTK upregulation. Whereas in a B cell driven disease as SLE enhanced B cell activation and thus enhanced BTK upregulation in activated B cells may have been anticipated, the trend of increased BTK expression levels in antigen-inexperienced B cells in SLE may pose a larger threat. Based on the detrimental autoimmune effects of constitutively high Btk expression levels in CD19-hBtk transgenic mice (14), high BTK expression levels in human B cells prior to antigenic activation may alter their activation threshold and thereby propagate humoral responses against self-antigens.

The findings of dysregulated BTK levels in activated SLE B cells *in vivo* and *in vitro*, the finding that B cell BTK levels in a subgroup of SLE patients exceed the range of BTK expression observed in HCs, the correlation in SLE between BTK levels and C4 hypocomplementemia, and the (near-)significant associations of increased BTK expression levels in SLE with ACA production and hematologic aberrancies strongly plead for an extension of the current study to identify a clinical SLE subtype that is highly eligible for testing treatment with selective BTK inhibition. Importantly, due to the heterogeneity of SLE many symptoms and disease characteristics are only present in a small proportion in this cohort of SLE patients, implicating that extension of the current study will likely unveil more significant correlations between BTK expression levels and a disease (sub)phenotype. Such correlations can improve the identification of a distinct group of SLE patients in which BTK signaling is propelling disease pathogenesis, and thereby define patients more eligible to treat with newly developed Btk-inhibitors, such as Ibrutinib (PCI-32765), CGI1746 or RN486, which are currently evaluated in clinical trials for B cell malignancies (23, 24).

Acknowledgements

We would like to thank Ilke Ilgaz for her technical assistance during experiments.

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8

Bruton's tyrosine kinase mediated signaling enhances leukemogenesis in a mouse model for chronic lymphocytic leukemia

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Abstract

In chronic lymphocytic leukemia (CLL) signals from the B cell receptor (BCR) play a major role in disease development and progression. In this light, new therapies that specifically target signaling molecules downstream of the BCR continue to be developed. While first studies on the selective small molecule inhibitor of Bruton's tyrosine kinase (Btk), Ibrutinib (PCI-32765), demonstrated that Btk inhibition sensitizes CLL cells to apoptosis and alters their migratory behavior, these studies however did not address whether Btk-mediated signaling is involved in the process of CLL leukemogenesis. To investigate the requirement of Btk signaling for CLL development, we modulated Btk expression in the IgH.ETμ CLL mouse model, which is based on sporadic expression of the simian oncovirus SV40 T-antigen in mature B cells. To this end, we crossed IgH.ETμ mice on a Btk-deficient background or introduced a human Btk transgene (CD19-hBtk). Here we show that Btk deficiency fully abrogates CLL formation in IgH.ETμ mice, and that leukemias formed in Btk haplo-insufficient mice selectively expressed the wild-type Btk allele on their active X chromosome. Conversely, Btk overexpression accelerated CLL onset, increased mortality, and was associated with selection of non-stereotypical BCRs into CLL clones. Taken together, these data show that Btk expression represents an absolute prerequisite for CLL development and that Btk mediated signaling enhances leukemogenesis in mice. We therefore conclude that in CLL Btk expression levels set the threshold for malignant transformation.

Published in Am J Blood Res. 2013;3(1):71-83.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world with incidence rates as high as ~4 per 100.000 individuals in the USA(1). Approximately half of the CLL cases carry unmutated immunoglobulin heavy (IgH) chain variable (V) regions, which is associated with an unfavorable prognosis (see for review: Kil *et al.*(2)). Recently reported transcriptome analyses of CLL and normal B cell subsets revealed that unmutated CLL derives from unmutated mature $CD5^+CD27^-$ B cells and mutated CLL derives from a distinct, previously unrecognized, $CD5^+CD27^+$ post-germinal center B cell subset(3).

In CLL, defects in apoptosis lead to the accumulation of $CD5^+$ mature B lymphocytes that express low levels of surface Ig, suggestive of continuous engagement of the B cell receptor (BCR) by antigens (4, 5). Such chronic BCR signaling may provide strong anti-apoptotic signals that support both malignant transformation of mature B cells and persistence of these transformed B cells(6). In support of a central role for chronic BCR signaling in CLL pathogenesis is the high recurrence of stereotypic BCRs among CLL samples, pointing to a probably limited set of allo- or autoantigens that stimulate these stereotypic BCRs(7).

Interestingly, it was very recently reported that most CLL BCRs can recognize an internal BCR epitope that additionally provides an abundant cell-intrinsic source of antigenic stimulation(8). In line with chronic BCR stimulation, CLL B cells exhibit a distinct BCR signaling profile that closely resembles signaling of anergic B cells, characterized by incomplete activation of downstream BCR signaling pathways and weaker responses to BCR triggering using soluble antibodies(9, 10). The importance of continuous BCR signaling for CLL cell survival is further demonstrated by the pronounced pro-apoptotic effects of new drugs that specifically target BCR signaling molecules(2, 11).

A novel CLL therapeutic target downstream of the BCR is Bruton's tyrosine kinase (Btk)(2). Although widely expressed in hematopoietic cells, except T cells, only B cells greatly rely on Btk for their development, activation and survival, as Btk deficiency in man or mice results in the B cell specific immunodeficiencies X-linked agammaglobulinemia (XLA) or x-linked immune deficiency (*xid*), respectively(12). The strong reduction of mature B cell numbers in *xid* mainly results from hampered survival of mature B cells, which may largely be explained by the ineffective activation of Btk's primary signaling target NF-κB(13, 14). Conversely, transgenic B cells overexpressing wild-type human Btk were selectively hyperresponsive to BCR stimulation and showed enhanced NF-κB activation, resistance to Fas-mediated apoptosis and defective elimination of selfreactive B cells *in vivo*(15). Consistent with the critical role for Btk in B cell survival, CLL cells lose their resistance to apoptosis *in vitro* when treated with the selective Btk-inhibitor PCI-32765(16, 17). Furthermore in a murine transfer model the *in vivo* expansion of transplanted CLL cells was markedly reduced by PCI-32765 treatment(17). These effects of Btk inhibition probably extend beyond dampening of BCR signaling alone, since PCI-32765 treatment also affected CLL cell adhesion, as well as migration directed by CXCR4 and CXCR5 that both employ Btk as downstream signaling molecule(17-20).

Since the levels of Btk represent a rate-limiting step in BCR signaling and thereby B cell activation and survival(15), the recent finding of Btk overexpression in CLL samples(16) prompted us to investigate the influence of Btk expression levels on CLL development. To this end, we used IgH.ETμ mice, a transgenic

mouse model that exhibits spontaneous CLL development driven by sporadic expression of the simian oncovirus SV40 T-antigen in mature B cells(21). In these mice, B-cell development is unperturbed in young mice, but in aging mice $IgD^{low}CD5^{+}$ monoclonal leukemic B cells accumulate with frequent usage of the Ig heavy (IgH) chain V_H11 family. Here, we demonstrate that Btk-deficiency completely abrogated CLL development in IgH.ETμ mice. Conversely, B cell specific overexpression of Btk accelerated CLL development, increased overall CLL incidence, and altered the Ig light (IgL) chain repertoire in CLL.

Materials & methods

Mouse tumor cohorts and genotyping

IgH.TEμ mice(21), Btk-deficient mice(22) and CD19-hBtk transgenic mice(23) were all on the C57BL/6 background for >10 generations and were crossbred to generate IgH.TEμ mice tumor cohorts that were haplo-insufficient or deficient for Btk, or transgenically overexpressing human Btk. The mice were genotyped with a polymerase chain reaction (PCR) using genomic DNA with the following primers (Life Technologies Europe BV) for the IgH.TEμ construct (forward 5'-GGAAAGTCCTTGGGGTCTTC-3', reverse 5'-CACTTGTGTGGGTTGATTGC-3'), *lacZ* (Btk-knockout) alleles (forward 5'-TTCACTGGCCGTCGTTTTACAACGTCGTGA-3', reverse 5'-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3'), wild-type Btk alleles (forward 5'-CACTGAAGCTGAGGACTCCATAG-3', reverse 5'-GAGTCATGTGCTTGGAATACCAC-3'), and the CD19-hBtk transgene (forward 5'-CCTTCCAAGTCCTGGCAT-3', reverse 5'-CACCAGTCTATTTACAGAGA-3'). CLL development in animals in tumor cohorts was monitored every 3-6 weeks by screening peripheral blood for monoclonal B cell expansion using flow cytometry (see below, "General flow cytometry procedures"), and were sacrificed after detection of CLL by severe monoclonal B cell lymphocytosis, or after a maximum period of 60 weeks of disease-free survival. Mice were bred and kept in the Erasmus MC experimental animal facility and the experiments were approved by the Erasmus MC committee of animal experiments.

General flow cytometry procedures

Preparation of single-cell suspensions of lymphoid organs and lysis of red blood cells were performed according to standard procedures(15). Cells were (in)directly stained in flow cytometry buffer (PBS, supplemented with 0.25% BSA, 0.5 mM EDTA and 0.05% sodium azide) using the following fluorochrome or biotin-conjugated monoclonal antibodies or reagents: anti-B220 (RA3-6B2, eBioscience), anti-CD19 (ID3, eBioscience), anti-CD5 (53-7.3, eBioscience), anti-CD43 (R2/60, eBioscience), anti-CD138 (281-2, BD biosciences), anti-CD95 (Jo2, BD biosciences), anti-IgD (11-26, BD biosciences), anti-IgMb (AF6-78, BD biosciences), anti-IgMa (DS-1, BD Biosciences), anti-Igλ (R26-46, BD biosciences), anti-Igκ (187.1, BD biosciences), anti-CD21 (7G6, BD Biosciences), anti-CD23 (B3B4, eBiosciences), PNA (Sigma-Aldrich), using conjugated streptavidin (eBioscience) as a second step for biotin-conjugated antibodies. For measurement of intracellular Btk levels, cells were fixed in PBS/2% paraformaldehyde (PFA) and permeabilized with permeabilization buffer (0.5% saponin (Sigma-Aldrich) in flow cytometry buffer). For staining of Btk, cells were incubated for 1 hour at room temperature with PE-conjugated anti-Btk (53/BTK, BD Biosciences) in permeabilization buffer. All flow cytometric measurements were performed on

an LSRII flow cytometer (BD Biosciences), and prior to measurement cells were washed and resuspended in flow cytometry buffer.

Flow cytometric detection of Erk and Akt phosphorylation

CLL cells and wild-type splenic B cells were purified by magnetic-activated cell sorting (MACS) with anti-CD19 coated magnetic beads (Miltenyi Biotec). Sorted cells were starved for 30 minutes at 37°C in FCS-free "RPMI-plus" medium (RPMI media 1640, supplemented with penicillin-streptomycin, 1.2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol; all components from Life Technologies™) and subsequently stimulated with 20 μg/mL goat anti-mouse Igκ (SouthernBiotech) for 5 min. After stimulation, cells were fixed for 10 minutes in 2% PFA, washed with PBS, permeabilized with ice cold 70% methanol in PBS for 30 minutes, and stained with fluorochrome-conjugated anti-Akt(pS473) and anti-Erk1/2(pT202/pY204) antibodies (BD Biosciences).

Evaluation of *lacZ* gene (β-galactosidase) expression

Expression of the *lacZ* cassette (encoding β-galactosidase) incorporated in Btk knockout alleles was evaluated essentially as published in Hendriks *et al*.(22). Briefly, CLL cells or splenocytes were loaded with fluorescein-di-β-D-galactopyranoside (FDG; Molecular Probes) using a hypotonic shock at 37°C. FDG loading was stopped after 1 minute by washing the cells with ice-cold culture medium, and leaving them for >2 hours on ice to facilitate FDG degradation while minimizing fluorescein leakage from the cells. Further staining for membrane markers was performed on ice during this incubation step, as described above in "General flow cytometric procedures", and fluorescein levels in conjunction with membrane marker expression were evaluated on an LSRII flow cytometer (BD Biosciences).

Measurement of c-Rel nuclear translocation

CLL cells and wild-type splenic B cells were purified as described (see "Flow cytometric detection of Erk and Akt phosphorylation") and were stimulated with goat anti-mouse [F(ab')$_2$]α-IgM fragments (JacksonImmunoResearch) at 37°C in culture medium for 4 hours. Total nuclear protein extracts were prepared and processed as described previously(24). After washing in PBS, cell nuclei were obtained by incubating cells for 10 minutes on ice in hypotonic Buffer A (10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mM KCl, 0.5.mM DTT, 0.2 mM protease inhibitor PMSF) followed by vortexing. After centrifugation of nuclei, nuclear lysates were prepared by 5 minutes incubation on ice with lysing Buffer C (10 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) followed by centrifugation, after which the supernatant was stored at -80°C. Samples were resolved using SDS-PAGE and transferred to nitrocellulose membranes according to standard procedures. Membranes were stained with rabbit anti-mouse c-Rel (Santa Cruz Biotechnology, Inc.) or mouse anti-actin (Chemicon International) primary antibodies and peroxidase-coupled swine anti-rabbit Ig or rabbit anti-mouse Ig secondary antibodies (Dako).

Immunoglobulin (Ig) heavy and light chain sequence analysis

From CLL tumor material total RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the

manufacturer's protocol, and cDNA was made using the RevertAid H minus Fist Strand cDNA sythesis kit (Thermo Scientific). For PCR amplification of the Ig heavy chain, two FR1 region high-degeneracy primers MH1 and MH2(25) or one high-degeneracy primer IPP000009 (IMGT®, http://www.imgt.org) were combined with a primer targeting the 5' cμ region(26, 27). Primers used for PCR amplification of the Ig kappa(25) and Ig lambda light chains(28) have previously been described. PCR products were directly sequenced with these primers using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI PRISM 377 automated sequencer (Applied Biosystems). Sequences were analyzed using CLC DNA workbench (CLCbio) and blasted in IMGT/V-Quest (http://www.imgt.org, using Ig gene nomenclature as provided by IMGT on Nov 1st 2012), and all sequences were confirmed in at least one duplicate analysis.

Statistical analysis

For calculating levels of significance, the student's t test was used for differences between groups of continuous data, whereas Fisher's exact test was used for differences between group proportions. The log rank test was used for calculating the level of significance for incidence and survival differences between tumor cohorts.

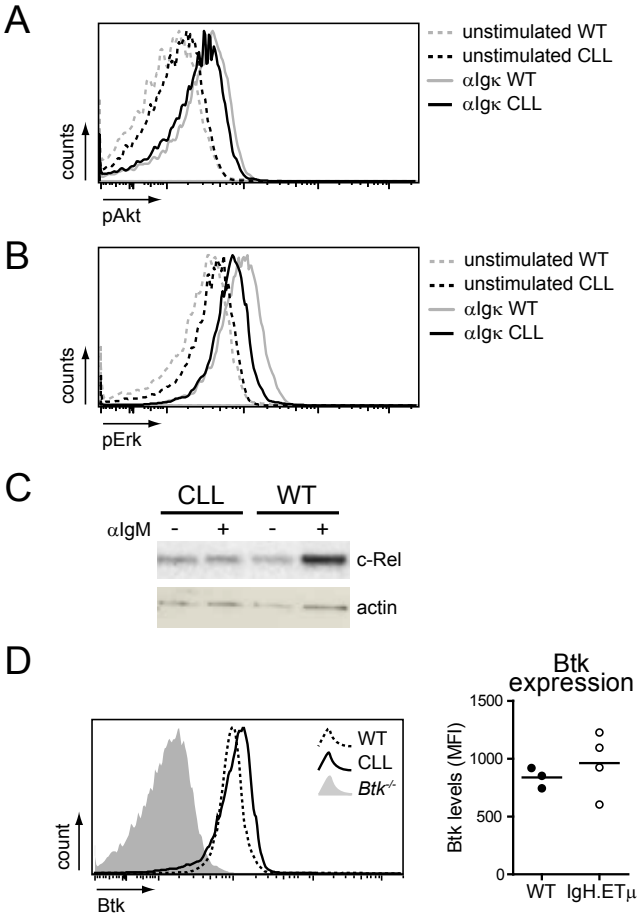
Results

Incomplete activation of BCR signaling pathways in IgH.ETμ CLL cells

There is substantial evidence for a significant contribution of Btk signaling to CLL formation or progression(16, 17, 20), but it remains unclear whether Btk signaling mainly exerts its pathogenic role downstream of the BCR, or downstream of other receptors, including chemokine receptors. We therefore aimed to characterize the engagement of different signaling pathways downstream of the BCR that are known to be activated rather independent of Btk (Akt), partially dependent on Btk (Erk), or greatly dependent on Btk signals (NF-κB)(2). To this end, we compared activation of these downstream signaling molecules in purified wild-type splenic B cells and primary mouse CLL cells isolated from diseased IgH.ETμ mice. In unstimulated IgH.ETμ CLL cells we observed a very basal phosphorylation level of both Akt and Erk, which was slightly higher than in normal splenic B cells (Figure 1A and 1B). Upon brief BCR stimulation *in vitro*, Akt phosphorylation in IgH.ETμ CLL cells was almost indistinguishable from wild-type splenic B cells (Figure 1A), whereas phosphorylation of Erk was weaker in stimulated IgH.ETμ CLL cells (Figure 1B). In contrast to the normal or suboptimal activation of Akt and Erk respectively, we could not detect induction of phosphorylated Btk or any nuclear translocation of NF-κB (c-Rel) upon prolonged BCR stimulation of IgH.ETμ CLL cells *in vitro* using western blotting (Figure 1C and data not shown).

The weak activation of downstream BCR signaling targets of Btk prompted us to investigate whether Btk expression levels were subphysiological in IgH.ETμ CLL cells, since Btk expression levels are tightly regulated upon stimulation by the BCR or other activating receptors, through NF-κB and miR185-mediated pathways(15, 29-31). Using intracellular flow cytometry, we measured Btk expression levels in primary IgH.ETμ CLL cells in comparison to wild-type splenic B cells. We found that Btk expression levels in IgH.ETμ CLL cells were more variable than Btk levels in non-malignant B cells. We did not find

Figure 1. Altered activation of downstream BCR signaling pathways in IgH.ETμ tumors.



Flow cytometric measurement of phosphorylation of Akt (A) and Erk (B) upon BCR stimulation in IgH.ETμ CLL cells versus wild-type (WT) B cells. MACS-purified CD19$^+$ cells were stimulated for 5 minutes with 20 μg/mL goat anti-mouse Igκ and subsequently stained for phosphorylated Akt or Erk. (C) Protein levels of c-Rel determined by western blotting in nuclear lysates of unstimulated (-) and αIgM stimulated (+) CD19$^+$ MACS-sorted CLL cells and wild-type (WT) splenic B cells. (D) Left: Btk expression levels in gated CD19$^+$ wild-type (WT) splenic cells versus IgH.ETμ CLL cells were evaluated using flow cytometry. Background staining levels were determined in *Btk*$^{-/-}$ cells. Right: flow cytometric determination of median Btk expression levels in wild-type (filled circles) and IgH.ETμ CLL cells (open circles). MFI: median fluorescence intensity. For all analyses at least 4 CLL samples and 3 wild-type splenic B cell fractions were included; representative results are shown.

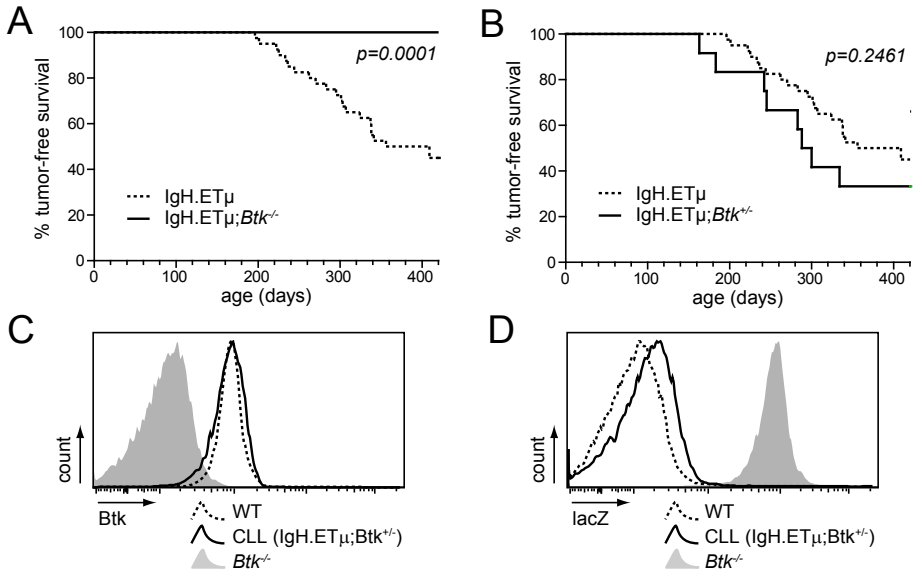
evidence for a decrease in Btk expression, since two out of four CLL samples analyzed displayed Btk overexpression (Figure 1D).

These results demonstrate aberrant BCR signaling in primary CLL cells compared with wild-type B cells and furthermore indicate that Btk activation downstream of the BCR is weak and associated with incomplete activation of known Btk signaling targets, particularly NF-κB.

CLL development is fully dependent on Btk

To test whether Btk signaling is critical for the formation of CLL, we monitored CLL development in cohorts of Btk-deficient IgH.ETμ mice versus Btk-sufficient IgH.ETμ mice, which were backcrossed onto the C57bl/6 genetic background for >10 generations. In contrast to the 100% CLL incidence and median survival of 161 days reported in IgH.ETμ mice on a mixed C57bl/6 x 129/Sv genetic background(21), we now observed a 55% CLL incidence with a median survival of 356 days in this cohort (Figure 2A). Strikingly, in none of the 19 IgH.ETμ mice on a Btk-deficient background we observed CLL formation (Figure 2B), nor did we detect any other expansion of monoclonal B cells with or without CLL-like features in peripheral blood samples that were obtained from these mice every 3-6 weeks. We also did

Figure 2. Btk is essential for CLL development.



(A) Kaplan-Meier survival curves of IgH.ETμ mice (dotted line; n=40) and Btk-deficient IgH.ETμ littermates (continuous line; n=19). (B) Kaplan-Meier survival curves of IgH.ETμ mice (dotted line; n=40) and Btk-haplo-insufficient IgH.ETμ littermates (continuous line; n=12). (C) Comparison of Btk expression levels in IgH.ETμ;*Btk*$^{+/-}$ CLL cells as in Figure 1D. (D) Flow cytometric measurement of β-galactosidase expression from *lacZ*-inserted Btk-knockout alleles in IgH.ETμ;*Btk*$^{+/-}$ CLL cells versus *Btk*$^{-/-}$ B cells, as determined by release of fluorescein upon FDG substrate degradation. Wild-type (WT) B cells were used as negative control. In (C) and (D) representative results are shown of 4 CLL samples analyzed.

not detect increased cellularity in lymphoid organs, including lymph nodes, spleen and bone marrow, after sacrificing mice at 60 weeks of age.

To further confirm the absolute requirement for Btk expression in CLL development, we followed CLL development in IgH.ETμ females that were haplo-insufficient for Btk. The *Btk* gene is located on the X-chromosome, and random X-inactivation occurring in all female somatic cells will therefore stochastically restrict Btk expression to one allele. In $Btk^{+/-}$ females this results in the generation of B cell precursors in the bone marrow that either express the normal Btk gene from the wild-type X chromosome, or express the *lacZ* reporter inserted in the targeted Btk allele from the mutated X chromosome(22). In peripheral B cells in $Btk^{+/-}$ female mice the distribution of Btk versus *lacZ*-expressing mature B cells is however largely skewed towards Btk-expressing cells, since lack of Btk expression poses a selective disadvantage for circulating immature B cells. Nevertheless, ~30% of $IgM^{high}IgD^{low}$ immature B cells in $Btk^{+/-}$ females do not carry the wild-type *Btk* locus on their active X chromosome and thus are Btk-deficient(22).

In IgH.ETμ;$Btk^{+/-}$ mice (n=12) we observed a CLL incidence comparable to IgH.ETμ mice (67% versus 55%, p=0.2461; see Figure 2B). In agreement with this finding, flow cytometric analysis for intracellular Btk in all IgH.ETμ;$Btk^{+/-}$ CLL samples revealed normal Btk expression in these tumors (Figure 2C) and accordingly no *lacZ* expression from the Btk mutant allele was detected (Figure 2D). Thus, in IgH.ETμ;$Btk^{+/-}$ mice only Btk-expressing cells were susceptible to CLL development by SV40 large T antigen mediated oncogenic transformation.

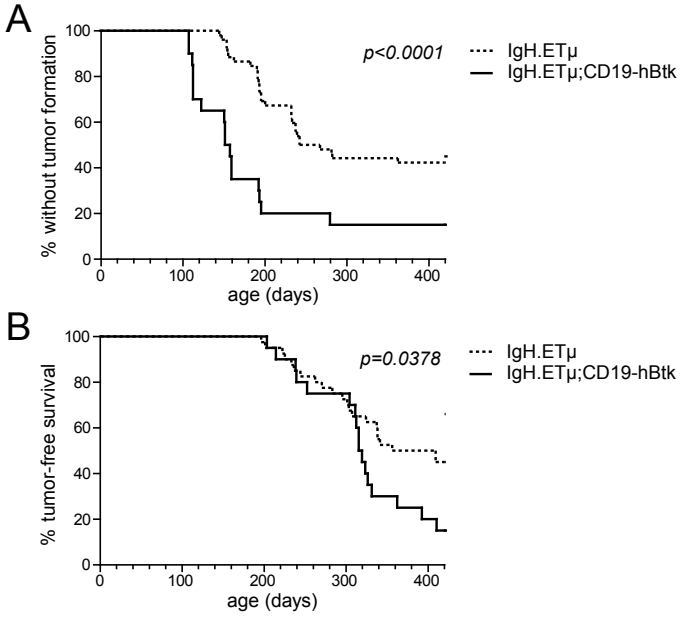
Taken together, these experiments show that Btk expression is an absolute prerequisite for CLL development in mice.

High-level Btk expression enhances CLL formation

Although variable overexpression of Btk is observed both in human CLL(16) and in our IgH.ETμ CLL samples (Figure 1D), it is unclear whether increased expression levels of Btk may increase the risk of CLL development or promote disease progression in CLL. To investigate this, we generated IgH.ETμ mice that overexpress a human Btk transgene selectively in the B-cell lineage under the control of the CD19 promoter region (CD19-hBtk)(23). We recently found that enhanced BCR signaling through Btk increases B cell activation and promotes B cell survival(15). When CD19-hBtk transgenic mice were followed over time up to 1 year of age, we did not find evidence for spontaneous B cell malignancies, but instead observed selective persistence of self-reactive B cells(15). In aging mice, we detected spontaneous differentiation into germinal center B cells and plasma cells *in vivo*, associated with auto-antibody formation and an SLE-like auto-immune disease. CD19-hBtk transgenic B cells were selectively hyperresponsive to BCR stimulation and showed enhanced αIgM-induced Ca^{++} influx and NF-κB activation(15). Btk overexpression did not affect *in vitro* vascular cell adhesion molecule-1 (VCAM1) mediated migration of B cells in response to CXCL12 or CXCL13 (data not shown), all of which is impaired in the absence of Btk(18-20).

Follow-up of a cohort of IgH.ETμ;CD19-hBtk mice demonstrated an earlier onset of CLL formation (median onset at 152 days, compared with 243 days in IgH.ETμ control mice) as well as an increase in CLL incidence (85% versus 55%, p<0.0001; Figure 3A). Moreover, the mortality was higher in IgH.ETμ;CD19-

Figure 3. Btk overexpression enhances CLL formation.



(A) Incidence curve of CLL formation in IgH.ETμ mice (dotted line; n=40) versus CD19-hBtk transgenic IgH.ETμ mice (continuous line; n=20). CLL formation was defined by the first appearance of monoclonal $IgMb^+$ B cells in peripheral blood of these mice ($IgMb^+$:$IgMa^+$ ratio > 80:20 (21)). (B) Kaplan-Meier survival curve of IgH.ETμ;CD19-hBtk mice (continuous line; n=20) versus IgH.ETμ non-CD19-hBtk transgenic mice (dotted line; n=40).

hBtk mice and the median survival was decreased compared to IgH.ETμ mice (315 versus 356 days). We noticed, however, a larger average interval between CLL diagnosis and CLL-associated death in IgH.ETμ;CD19-hBtk mice (151 versus 67 days; Figure 3B).

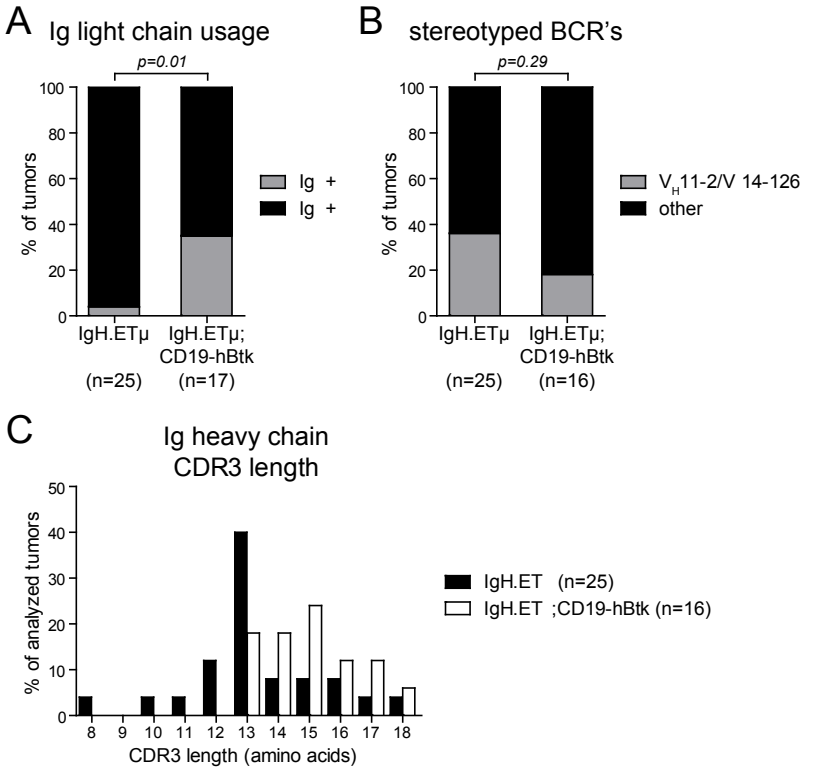
Taken together, these data show that Btk-mediated signaling enhances CLL lymphoma-genesis in mice.

High-level Btk expression is associated with CLL with non-stereotypic BCRs

To determine whether enhanced Btk-mediated signaling may lower the threshold for BCR-based selection of certain B cells into CLL clones, we characterized the BCRs expressed by CLLs from IgH.ETμ, IgH.ETμ;*Btk*$^{+/-}$ and Btk-overexpressing IgH.ETμ;CD19-hBtk mice. Flow cytometric and BCR sequencing analyses showed that 35% of IgH.ETμ;CD19-hBtk tumors expressed an Ig lambda (Igλ) light chain, compared with only 4% of tumors from IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (p=0.01; Figure 4A). This cannot be explained by an effect of Btk overexpression on Igλ usage in normal B cells, because in young non-diseased CD19-hBtk mice the proportion of Igλ expressing mature B cells was not increased: ~9.2% in CD19-hBtk mice versus ~8.5% in wild-type mice.

DNA sequence analysis of IgH, Igκ and Igλ chains revealed that 36% of tumors (9 out of 25) from

Figure 4. High Btk levels promote atypical BCR clone selection in CLL.



(A) Proportions of Igλ$^+$ (gray) and Igκ$^+$ (black) CLLs in IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; n=25) versus IgH.ETμ;CD19-hBtk mice as determined by flow cytometry (n=16). (B) Proportions of stereotypical (V_H11-2$^+$/$V_κ$14-126$^+$) BCR (gray) and non-stereotypical BCR (black) expressing tumors in IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; n=25) versus IgH.ETμ;CD19-hBtk mice (n=16). (C) Graph summarizing IgH CDR3 length determined by IgH sequencing of tumors from IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; black bars, n=25) and IgH.ETμ;CD19-hBtk mice (white bars, n=16).

IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice were highly stereotypical, using only one combination of V_H11-2 and $V_κ$14-126 genes and expressing a largely similar IgH complementarity-determining region 3 (CDR3) motif (Table 1, Figure 4B). In IgH.ETμ;CD19-hBtk mice we identified V_H11-2/ $V_κ$14-126 usage in 19% (3 out of 16) of CLL samples, indicating an increase in non-stereotypical BCR usage (Table 1, Figure 4B). The cohorts of IgH.ETμ mice on the C57bl/6 background showed infrequent development of mutated CLLs (M-CLLs) that express BCRs harboring >2% mutated nucleotides (2 out of 25 tumors) and no significant difference in M-CLL occurrence was noted in IgH.ETμ;CD19-hBtk mice (none out of 17 tumors). Further CDR3 characterization of IgH from tumors in IgH.ETμ;CD19-hBtk mice revealed a significant increase in CDR3 length, compared with IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (p=0.0394, Figure 4C). In addition, 3 out of 17 CDR3's from IgH.ETμ;CD19-hBtk tumors contained stretches of 3 or more tyrosine residues whereas

this was only observed in 1 out of 25 tumors from IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (data not shown).

These atypical BCR features of IgH.ETμ;CD19-hBtk tumors demonstrate that the increased CLL susceptibility of Btk overexpressing B cells is associated with enhanced transformation of B cells clones expressing non-stereotypic BCRs in IgH.ETμ mice. These BCR characteristics further comprise increased Igλ usage and the presence of long IgH CDR3 regions frequently containing tyrosine stretches, reminiscent of BCRs with self-reactive or poly-reactive specificities.

Discussion

Several recent reports have demonstrated an important role for Btk signaling in the survival and migratory behavior of human CLL cells, but from these studies it was not clear whether Btk signaling is indispensable for CLL development(16, 17, 20). Here we show in the IgH.ETμ CLL mouse model that CLL development requires Btk expression: CLL formation was absent in Btk-deficient IgH.ETμ mice, and all tumors derived from Btk haplo-insufficient IgH.ETμ females exclusively expressed the wild-type Btk allele. Although these findings do show that Btk is required for establishing CLL, they do not identify the main mechanism by which Btk promotes CLL development. Given the cardinal role of Btk signaling downstream of the BCR, previous studies initially focused on Btk in BCR signaling in CLL, but now it has become clear that Btk signaling downstream other receptors including CXCR4 and CXCR5 may greatly affect the risk of CLL and of disease progression(16, 17, 20). This view was affirmed by a transient lymphocytosis, observed in CLL patients and diseased mice alike, upon initiation of treatment with the selective Btk-inhibitor PCI-32765(17, 32). It is therefore conceivable that Btk controls CLL formation through multiple pathways including BCR and chemokine receptor signaling, and future research should elucidate the exact contribution of these Btk signaling pathways to CLL pathogenesis.

By examining the activation of the downstream effector molecules of BCR signaling that are activated to different extents by upstream Btk signals, we tried to identify a Btk signaling signature in BCR-activated CLL cells. Whereas the activation of Akt and Erk upon BCR stimulation was largely unaffected, the activation of Btk's primary signaling target NF-κB was clearly defective in IgH.ETμ tumor cells. These findings are largely in line with earlier characterizations of BCR signaling pathways in CLL that revealed a BCR signaling profile resembling that of anergic or chronically stimulated B cells (reviewed in Kil et al.(2)). Nevertheless, it is unlikely that in CLL Btk signaling is completely silenced downstream of the BCR, since it has been shown that PCI-32765 treatment can counteract the pro-survival effects of αIgM stimulation on CLL cells, accompanied by a reduced phosphorylation of Erk and Akt(17). Our failure to detect induction of NF-κB nuclear translocation in BCR-stimulated CLL cells may therefore be explained by a general retuning or rewiring of BCR signaling pathways in CLL, leading to a shift of Btk signaling targets away from classical targets including NF-κB, which were identified in non-malignant primary B cells. Alternatively, Btk signaling may effectively be weaker in CLL cells compared to non-malignant mature B cells. The possibility that Btk mainly functions as an adaptor protein(24) in signaling complexes in CLL cells is not very likely, because of the reported capacity of PCI-32765 to reduce αIgM-induced phosphorylation of Erk and Akt(16, 17, 20).

The finding that transgenic Btk overexpression (CD19-hBtk) could accelerate and increase CLL formation in IgH.ETμ mice does not directly point to a role for Btk exclusively downstream of the BCR in

CLL formation. However, a substantial contribution of Btk-mediated BCR signaling to CLL development is evident from the more frequent occurrence of CLL clones that expressed non-stereotypical BCRs that more frequently employed Igλ and harbored longer CDR3s, demonstrating altered selection based on BCR signaling. This is in line with the finding that, although Btk functions downstream multiple receptors expressed in B cells, increasing Btk expression levels selectively enhances the signaling strength of the BCR, and not of Toll-like receptors or chemokine receptors (Kil et al.(15), and L.P.K. and R.W.H., unpublished). The atypical features of the BCRs found in IgH.ETμ;CD19-hBtk mice also point to a different origin of the B cells that were selected into malignantly transformed CLL clones. It is known that beyond 10 weeks of age, CD19-hBtk mice produce anti-nuclear auto-antibodies, mirroring the presence of auto-reactive or at least poly-reactive cells. Since increased CDR3 length and tyrosine stretches are associated with a higher risk of BCR selfreactivity, it is likely that some of these CLLs expressing atypical BCRs are derived from auto-reactive or poly-reactive B cells.

The debate on which B cell subsets may contain or represent the precursor cells of CLL is still ongoing, and have led to formulation of hypotheses that these cells may be derived from poly-reactive B1-like cells(33), conventional B2 cells(34), marginal zone B cells(35) or memory B cells(36). A recent study however, that extensively compared human mature $CD5^+$ B cells to CLL B cells, revealed striking similarities in gene expression profiles and stereotype IgH V gene rearrangements between these two cell populations(3). Although human $CD5^+$ mature B cells do not represent the exact counterpart of $CD5^+$ mouse B cells which mostly are B1 B cells, this finding could imply that also in murine CLL models (including our IgH.ETμ mice) CLL clones are derived from $CD5^+$ expressing B cells. In this context, the absence of CLL formation in Btk-deficient IgH.ETμ mice could thus result from their lack of B1 B cells(22, 37), rather than from a signaling defect in mature B2 cells. Conversely, enhanced CLL development in CD19-hBtk mice could be related to their ~2-fold increase in B1 B cell numbers(15). However, it is not likely that the differences in CLL formation can be fully attributed to changes in B1 B cell numbers, since only a minor fraction of the analyzed CLL samples in our panels expressed V_H11 family members, which are vastly overrepresented in B1 B cell populations(38, 39). We did not observe a proportional increase in V_H11 expressing tumors in CD19-hBtk mice, which would reflect the increase in B1 cell numbers in these mice.

In summary, we have shown that Btk-deficiency fully abrogated CLL formation in mice and that Btk overexpression accelerated CLL onset, increased mortality, and was associated with selection of non-stereotypical BCRs into CLL clones. We therefore conclude that Btk expression levels set the threshold for malignant transformation, further supporting that Btk inhibition by small molecule inhibitors such as PCI-32765 represents an effective new treatment for patients with CLL.

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Tables

Table 1: Characteristics of stereotypic BCRs in CLLs from IgH.ETμ mice

	tumor	*IgH*			*CDR3*	*Igκ*	
	code	V_H	D_H	J_H		$V_κ$	$J_κ$
IgH.ETμ CLL							
	E01	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	E06	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	E07	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4
	E20	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	2
	E32	11-2	1-1	1	CMRY**GG**YWYFDVW	14-126	2
IgH.ETμ;Btk$^{+/-}$ CLL							
	EH02	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	EH03	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	EH08	11-2	1-1	1	CMRY**GSS**YWYFDVW	14-126	1
	EH10	11-2	2-5	1	CMRYSNYWYFDVW	14-126	1
IgH.ETμ;CD19-hBtk CLL							
	ET08	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	ET10	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4
	ET16	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4

CDR3: complementarity determining region 3. Bold amino acid symbols indicate differences from the CMRYSNYWYFDVW CDR3 motif.

9

The role of B cell receptor stimulation in CLL pathogenesis

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Abstract

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in adults in the Western world and is characterized by the accumulation of monoclonal CD5$^+$ mature B cells in the blood. The disease has a highly variable clinical course. CLL is subdivided into two disease subtypes, whereby leukemias with hypermutated immunoglobulin heavy chain variable (IGHV) genes have a more favorable prognosis than those with unmutated IGHV genes, who tend to show advanced, progressive disease, adverse cytogenetic features and resistance to therapy. The current view is that both CLL types derive from antigen-experienced cells. Based on the finding that the IGHV repertoire is highly restricted and biased, as compared to the normal adult B-cell repertoire, it has been hypothesized that CLL cells are selected by some sort of antigenic pressure. Hereby, either autoantigens or antigens derived from apoptotic cells or pathogens are essential to trigger CLL pathogenesis. Although different cytogenetic aberrations were shown to contribute to CLL leukemogenesis, it remains unclear which abnormalities are primary events. Very recently, whole-genome sequencing identified genes that are recurrently mutated and provided novel insights into the mechanisms of oncogenic transformation. Because of the impact on prognosis, it is important to unravel the role of antigenic selection in CLL. Interestingly, B cell receptor (BCR) signaling is aberrantly increased in CLL and expression of tyrosine kinase ZAP70, which is able to signal downstream of the BCR, is a prognostic indicator. In this context we discuss the functional significance of antigenic selection in CLL and describe emerging agents to target BCR signaling that are currently being tested as a novel therapeutic strategy for CLL.

These studies were partly supported by the Association for International Cancer Research (AICR, to R.W.H. and A.W.L.) and an unrestricted research grant from Roche/Genentech (to A.W.L.). We acknowledge members of the Langerak and Hendriks laboratories for their technical support in CLL studies and for critical discussions. Published in Curr Pharm Des. 2012;18(23):3335-55.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world, showing high incidences in Europe and North America (almost 4 per 100,000 individuals). Every year approximately 20,000 new CLL patients are diagnosed in Europe (reviewed in Ref. (1) and (2)). Notably, the incidence differs significantly between men and women, with CLL occurring twice as often in males compared to females. With a median age at diagnosis of >70 years, CLL is typically considered a disease of the elderly. CLL is characterized by the accumulation of small, rounded B lymphocytes with scanty cytoplasm in the blood. These lymphocytes have a typical $CD5^{+}CD23^{+}CD19^{+}CD20^{dim}$ immunophenotype and generally exhibit low expression levels of surface immunoglobulin (Ig), probably reflecting B cell receptor (BCR) internalization due to engagement of surface IgM (3). Consistent with this hypothesis, CLL B cells readily upregulate surface IgM expression *in vitro*, once they are isolated and thus deprived of their *in vivo* ligands. Circulating CLL cells are arrested in G0/G1 phase of the cell cycle, showing a low proliferation rate (4). Typically, proliferation of CLL cells occurs in so-called proliferation centers in bone marrow or lymph nodes, whereas circulating cells in the blood survive and accumulate due to disturbed apoptosis (5). Though the biological features and mechanisms are beginning to be explored and disclosed, the exact underlying pathogenic mechanisms in CLL are largely unknown. It is believed that next to cell-intrinsic events, such as genetic and epigenetic modifications, also external factors, e.g. micro-environmental influences and antigenic stimulation contribute to CLL transformation and progression (reviewed in (1) and (2)). Particularly, the lymph node was identified as the key site in CLL pathogenesis: compared with peripheral blood, lymph node resident CLL cells showed upregulation of gene signatures indicating BCR activation (6).

Despite the seemingly homogeneous nature of CLL cells, including the above-mentioned immunophenotype that is distinct from other mature B-cell malignancies, it has become clear that CLL is not a single disease entity. Rather, CLL is characterized by a remarkable heterogeneity, with some patients being asymptomatic at diagnosis, and others presenting with lymph node enlargement, bone marrow failure, fatigue or autoimmune hemolytic anemia (reviewed in (1) and (2)). A considerable fraction of CLL patients shows a relatively indolent clinical course over a period of 10-20 years, often without requiring any type of therapy. Other CLL patients have a clinically more aggressive form of the disease for which therapy is needed. The two major complications in CLL concern cytopenia (anemia, thrombocytopenia) and a secondary immunodeficiency, related to low serum Ig levels.

Based on the extent of their disease, CLL patients can be classified according to the Raj (0-IV) and Binet (A-C) clinical staging systems, which correlates with tumor aggressiveness and survival (7-8). Despite this clinical classification, a search has been conducted for biological parameters that would show predictive value for disease course and outcome at the time of diagnosis. Over the years several laboratory parameters (nodular vs. diffuse infiltration of bone marrow, lymphocyte doubling time, chromosomal aberrations, expression levels of particular membrane markers, serum levels of cytokines and other molecules) have been identified that all somehow correlate with differences in prognosis and survival. A major breakthrough in CLL prognostication came with the finding that the somatic hypermutation (SHM) status of the immunoglobulin heavy variable (IGHV) genes is a very informative prognostic parameter (9-10). In addition, expression of the tyrosine kinase ZAP70, which

has the capacity to signal downstream of IgM (11-12), and the surface molecule CD38, the expression of which correlates with surface IgM signaling (9) were also identified as prognostic indicators, pointing at a role for antigenic stimulation in CLL pathogenesis. This would be supported by the recent finding that the expression of BCR target genes was stronger in clinically more aggressive CLL, indicating more effective BCR signaling in this subtype *in vivo* (6). Together with the accumulating evidence that pharmacological inhibition of various BCR-associated signaling molecules promote pro-apoptotic signals in CLL (13-19), these findings substantiate a crucial role of BCR signaling in the pathogenesis and clinical outcome of CLL.

Chromosomal aberrations in CLL

In addition to IGHV mutational status, chromosomal aberrations also constitute an important prognostic marker in CLL. Chromosomal aberrations are traditionally detected by cytogenetic karyotyping. However, conventional karyotyping can only be performed in 40-50% of CLL cases, as it is difficult to culture CLL cells to obtain sufficient metaphases for analysis. Nevertheless, based on the limited karyotypic data and mostly on fluorescence in situ hybridization (FISH) data, it has become clear that – in contrast to other types of leukemia - no recurrent translocations are present in CLL. Moreover, there is no single CLL-defining chromosomal aberration. Rather there are multiple chromosomal aberrations that together occur in approximately 80% of CLL cases. These especially concern deletions of chromosomal regions 17p, 11q, or 13q, and trisomy of chromosome 12 (Table 1). This corroborates the idea that CLL is not a single disease, but that biological and clinical heterogeneity exist.

Indeed, the presence of these aberrations correlates with the clinical heterogeneity of CLL as documented by the Döhner group (1, 20). Deletions of chromosome band 13q14 are the most frequent genetic event in CLL (~50% of the cases) and correlate with a more favorable prognosis (median overall survival of 133 months) (Table 1). The 13q14 region encompasses the *miR15a* and *miR16-1* microRNA genes (see below), which have been implicated in apoptosis induction via BCL2 targeting (21). Trisomy 12 occurs in ~15% of CLL but the relevant genes for CLL pathogenesis are unknown. Initially trisomy 12 was suggested to be a poor prognostic factor, which was associated with a shorter time to progression. However, the less favorable effect of this aberration has not been convincingly confirmed in later studies; this is also evidenced from the median overall survival of 114 months that is very similar to CLL with 13q14 deletions (Table 1).

Another frequent deletion concerns (part of) the long arm of chromosome 11, which is observed in 10-20% of early stage CLL disease to >20% of refractory CLL. This 11q deletion identifies a CLL subgroup with a clearly inferior outcome (median overall survival of 79 months) as compared to 13q14 deletion CLL cases (Table 1). Within this deleted region (11q22.3-11q23.1) ATM has been identified as the most important candidate gene, although it cannot be fully excluded that other genes in the 11q region may play a role as well (22). Furthermore, mutations in the ATM gene are known to occur in ~9-19% of CLL (23-24) (Table 2) and in about one-third of CLL with a 11q23 deletion ATM mutations are present on the second allele (22). The ATM protein is involved in cell cycle regulation and p53 activation, which is essential for apoptosis induction in response to DNA damage-inducing agents. Hence, ATM dysfunction leads to loss of cell cycle control and p53 dysfunction.

Interestingly, the TP53 gene, encoding the p53 tumor suppressor, is the primary gene implicated in deletions involving band 17p13. In ~8-15% of CLL samples the TP53 gene itself harbors mutations (24-25) (Table 2), while in the subgroup with monoallelic 17p13 deletion >80% of the samples carries TP53 mutations in the second allele, thus leading to complete functional loss of p53 activity (26). CLL with 17p aberrations form a minor subgroup in early CLL (around 5%), but its proportion increases in refractory CLL (>30%) (Table 1). CLL with a 17p deletion often show therapy resistance, which is in keeping with the poor survival of CLL with such deletions. Not surprisingly, 11q and 17p deletions were almost exclusively found in U-CLL, whilst 13q14 deletions were clearly overrepresented in M-CLL (27). Trisomy 12 cases were divided over both groups.

Defects in either ATM or p53, which collectively may occur in up to 30% of CLL, lead to impaired DNA damage responses and are correlated to therapy resistance and poor survival. In this CLL subgroup impairment in the activation of p53 is the common event underlying resistance to drugs such as fludarabine, a purine analogue that inhibits DNA synthesis, through disrupted apoptosis induction. In a recent study deregulation of *miR-34*, being a direct downstream target of p53, was also implicated in this chemotherapy resistance network, including in CLL that show normal p53 activity (28). Interestingly, overactivation of the non-homologous end-joining (NHEJ) DNA repair pathway has been shown to impair DNA damage-induced apoptosis in CLL (29) (30). Increased levels of the NHEJ factor DNA-PK_{CS} in CLL with 17p or 11q deletions indicates that DNA-PK_{CS} may be involved in disease progression and resistance to e.g. the alkylating agent chlorambucil in CLL. Targeting the DNA-PK_{CS} by a specific DNA-PK inhibitor (NU7441) has indeed been shown to sensitize CLL cells to drug-induced DNA damage and subsequent apoptosis (30).

Genetic susceptibility to CLL

CLL is a disease with a strong familial component, showing an elevated risk of ~7% in first-degree relatives of CLL patients. To find susceptibility loci large-scale genome wide association studies (GWAS) have been performed on materials from CLL patients and controls. Based on SNP analysis a total of 10 CLL risk loci have been identified at several different chromosomal regions (31-32). As no high-penetrance (causative) genes were included, these findings support a polygenic model of several low-penetrance susceptibility loci. Among the candidate genes that showed the strongest association are MYC, IRF4 and possibly IRF8. In another susceptibility study, the main focus was on mapping SNPs in genes involved in DNA damage response and cell cycle pathways, implicating variants in the ATM-BRCA2-CHEK2 axis with risk of CLL (33). Activation of ATM leads to ATM-dependent phosphorylation of CHEK2, which in turn can bind p53; the relevance of this pathway is clear given the chromosomal deletions targeting the ATM and TP53 genes (see above). Based on the idea of a possible etiological role of (in)directly mutagenic chemical agents in CLL (34), polymorphisms in genes implicated in the three main DNA repair pathways, i.e base-excision repair (BER), nucleotide-excision repair (NER) and double-strand break repair (DSB), were recently studied. Clear differences were found for SNPs in ERCC2 (NER) and XRCC1 (BER) loci between CLL patients with unfavorable cytogenetic aberrations and controls, which suggests that DNA repair polymorphisms could be related to outcome in CLL (35).

Defective microRNA expression in CLL

CLL has been associated with defective expression of microRNAs (miRNAs). These are evolutionarily conserved short endogenous non-coding RNA molecules that regulate gene expression via translational repression or transcript degradation (36). miRNAs are transcribed by RNA polymerase II into long primary miRNA transcripts of different size (pri-miRNA). These are recognized and cleaved inside the nucleus by the RNase III enzyme Drosha, which results in a hairpin precursor form called pre-miRNA. Exportin 5 is responsible for exporting the pre-miRNA from the nucleus to the cytoplasm where it is further processed by another RNase enzyme called Dicer, which produces a transient 19–24 nucleotide duplex. Only one strand of the miRNA duplex (mature miRNA) is incorporated into a large protein complex, called RNA-induced silencing complex (RISC). The mature miRNA guides RISC to cleave the mRNA or stimulates translational repression, depending on the level of complementarity between the miRNA and its target. Although the most frequent site of interaction is the 3′ untranslated region (UTR) of the target mRNA, miRNAs have been described that bind to the open reading frame (ORF) sequences, as well as to the 5′ UTR. In addition, miRNAs can also bind to RNA-binding proteins or regulate gene transcription by binding directly to the target gene promoter or by modulating DNA methylation.

Garzon et al. (36) proposed that a miRNA can be described as a tumor suppressor when it is frequently lost in tumor cells and it downregulates the expression of an oncogene. Loss-of-function of such a miRNA may be caused by e.g. mutation, deletion or promoter methylation. Any abnormality in miRNA biogenesis might result in an abnormal expression of the target oncogene and thus contribute to tumor formation by inducing cell proliferation, invasion, angiogenesis or decreased cell death. Many of the proposed mechanisms for the inactivation of miRNAs in cancer have experimentally been proven, including the downregulation of miR-15a and miR-16-1 expression in patients with CLL that harbor homozygous and heterozygous genomic deletions. Calin et al. (37) first reported the involvement of miR15a-miR16-1 in CLL. This cluster is located at 13q14.3, which is a deleted in ~50% of CLL patients. Expression of miR15a-miR16-1 was found to be downregulated and the loss of this miRs cluster results in overexpression of survival genes, including BCL2 and MCL1, thereby inducing resistance to apoptosis (38). In addition, in some CLL patients germline mutations were identified in the miR15a-miR16-1 precursor, which resulted in lower miR-15a and miR-16-1 expression levels (37). Point mutation in the 3′ flanking region of miR16-1 in the New Zealand Black Mouse strain (see below) has been correlated with reduced miR16-1 expression and development of a B-cell lymphoproliferative disorder that serves as a model for human CLL (39). Also mice that harbor a deletion of the miR15a-mirR16-1 cluster develop a CLL-like disease and lymphoma (40). Conversely, Cimmino et al. (41) reported that overexpression of the miR15a-miR16-1 cluster resulted in the induction of apoptosis of leukemic cells. Targeting miR15a-mirR16-1 accelerates the proliferation of both human and mouse B cells by modulating the expression of genes controlling cell-cycle progression, in particular by upregulating proteins involved in G0/G1 phase transition. Downregulation of miR15a and miR16-1 has also been reported in other malignancies, including pituitary adenomas and prostate carcinoma. The discovery, functions, and clinical relevance of these miR in cancer, particularly in CLL have recently been reviewed (42).

On the other hand, amplifications of oncogenic miRNAs, including the miR-17-92 cluster, miR-21 and miR-155, have been associated with tumor initiation and progression, by inhibiting expression of

various tumor suppressor genes, such as PTEN, BIM and programmed cell death 4 (PDCD4). In particular, miR-21, which is located at 17q23 was found to be upregulated in CLL (37). miR-21 expression levels were significantly higher in patients with poor prognosis and poor predicted overall survival (43). Another study showed the involvement of miR-21 in down-regulation of PTEN in CLL (44). miR-155 up-regulation has been repeatedly reported in CLL (37, 45) and lymphomas (46-48). In addition, transgenic miR-155 overexpression in the mouse stimulates B-cell proliferation and frequent development of lymphomas (49). Interestingly, miR-155 is an oncogenic microRNA that has been shown to be critical for B-cell maturation and its primary transcript, B-cell integration cluster (BIC) is induced by BCR cross-linking. It has been shown in the human B-cell line Ramos that activation of BIC and miR-155 expression by BCR signaling occurs through the ERK and JNK pathway, but not by the p38 MAP kinase pathway (see below).

In addition, up- or downregulation of various miRs has been associated with tumor progression. For example, miR-29 has been described as a tumor-suppressor downregulated in CLL, lung cancer and breast cancer that targets MCL1, CKD6 and TCL1. Pekarsky et al (50) proposed that downregulation of miR-29 miRNA in CLL could contribute to the development of a more aggressive phenotype. Recent studies demonstrated the decrease of miR-29c, miR-181b and miR-223 levels in CLL cells during the progression of the disease (51-52). Elevated levels of miR-155 represents a candidate pathogenic factor in CLL (37, 45). Finally, miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities (53). Asslaber et al. (54) showed that miR-34a is significantly increased in CLL patients and in the leukemic phase in Eμ-TCL1 mice, but in CLL cells of patients with p53 aberrations, mIR-34a was consistently low. Together with the finding that overexpression of miR-34a induces apoptosis in primary CLL cells, this suggests miR-34a as a a sensitive indicator of the activity of the p53 pathway and as a possible therapeutic avenue. Recently, mIR-185 was implicated in the reduction of BCR signaling by down-regulating BTK via binding to the 3' UTR of BTK mRNA (55), while in other cancers miR-185 suppresses tumor growth and progression by targeting the cell-cycle proteins and Six1 transcriptional targets c-myc and cyclin A1 (56).

Taken together, these findings have led to rapidly expanding knowledge linking miRNAs expression levels to CLL. In this context, it is important that miRNAs are very stable in blood plasma and serum (57-58), enabling the identification of cancer-specific miRNAs in the blood to be used as potential diagnostic and prognostic biomarkers.

Recurrent gene aberrations identified in CLL

Next to chromosomal aberrations, various recurrent point mutations have been identified in CLL (Table 2). A fraction of cases, confined to M-CLL, displays mutations in the 5' UTR of the BCL-6 gene (59). B-CLL cells express low levels of BCL-6 protein regardless of the status of mutations in the BCL-6 and IGHV loci. Recently, mutations in NOTCH1, exportin-1 (XPO1), MYD88 and Kelch-like 6 (KLHL6) were identified by whole genome sequencing of two U-CLL and two M-CLL cases and further analyses of recurrent mutation in a cohort of 363 patients (60). In a recent study, using a powerful approach of high throughput sequencing of exosomes and genomes from 91 CLL samples (24), many established CLL-associated mutations were reproduced (including mutations in NOTCH1, TP53, ATM, and MYD88). In addition new frequently occurring mutations were identified in various genes, including SF3B1, ZMYM3, ERK/MAPK1,

FBXW7, and DDX3X (24). Although these genes have not been implicated in CLL pathogenesis before, they are functionally directly linked to genes and pathways that do play a critical role in CLL formation.

Mutations in the NOTCH1 gene were reported in some CLL patients (61) and have recently been confirmed in additional cohorts and studies (24, 62). Such NOTCH1 gene mutations generate a premature stop codon, resulting in a constitutive active protein lacking the regulatory C-terminal PEST domain (Table 2). Furthermore mutations in the ubiquitin ligase FBXW7, a known tumor suppressor that targets the degradation of oncogenes including NOTCH1(63), can also contribute to enhanced NOTCH1 signaling in CLL B cells(24).

The L265P mutation in MYD88, a protein that contributes in the IL-1 and Toll-like receptor (TLR) signaling pathways during immune responses (reviewed in (64)), has also been found in DLBCL. This gain-of-function mutation increases the production of IL-1, thereby recruiting macrophages and T-cells and creating a favorable niche for survival of the CLL cells. In addition, activation of TLRs in CLL cells promotes their proliferation and protects them from apoptosis. A downstream molecule of both TLR and BCR signaling is ERK/MAPK1, and mutations in ERK have recently been identified in CLL samples (24). Such mutations probably directly alter ERK signaling properties, since 2 out of the 3 identified mutations are located in the kinase domain of ERK.

Mutations have been found in the XPO1 gene, which is involved in the nuclear export of proteins and mRNA in yeast (65). Importantly, mutations have also been identified in the RNA helicase DDX3X that directly interacts with XPO1(24). The implications however of these mutations for CLL cells remain to be investigated. Puente *et al* (60) also reported mutations in the KLHL6 Fgene, encoding a protein involved in GC formation (66). Recent work from Havelange *et al* (67) showed the presence of recurrent heterozygous somatic mutation in the DNA-binding domain of interferon regulatory factor 4 (IRF4) (Table 2). This transcription factor has critical functions at several stages of B and T cell differentiation and is expressed in a graded manner in differentiating B cells, whereby higher concentrations have the capacity to induce the transition from a GC gene expression program to that of a plasma cell (68).

In addition, in CLL several genes show abnormal expression. CLL cells display aberrant nonfunctional transcripts of the E-cadherin gene, resulting in up-regulation of the Wnt/b-catenin pathway (69). Palacios et al. (70) reported that a subgroup of U-CLL patients display high levels of AID expression and ongoing clonally related IGH class switch recombination (CSR), associated with an even worse prognosis. Expression of lipoprotein lipase A (LPL) correlated to IGHV mutational status: increased LPL expression is associated with U-CLL (71-72). LPL expression is relatively stable in the majority of patients and provides prognostic information in early stage and in patients with advanced disease. Also expression of the BCR-associated molecule ZAP70 is aberrant in CLL (11-12) (see below).

The TOSO/*FAIM3* protein is overexpressed in CLL B cells (73) and has recently been identified as the long-sought-after FcR for IgM (FcμR) (74-75). FcμR is expressed on human $CD19^+$ B cells, $CD4^+/CD8^+$ T cells and $CD56^+/CD3^-$ NK cells. It localizes to the cell membrane, but large pools of FcμR accumulate in the *trans*-Golgi network (76). Aggregation of FcμR on CLL cells by IgM prompted rapid internalization of both IgM and FcμR. Upon internalization, FcμR transported IgM through the endocytic pathway to the lysosome, where it was degraded. It has been reported that BCR activation increases FcμR expression in CLL (74) (73), while Vire et al. (76) found that activation of TLRs strongly downregulated FcμR at both

the mRNA and protein levels. Through internalization of IgM bound immune complexes, FcμR may play a role in immune surveillance and contribute to B cell activation. TOSO/*FAIM3 acts as an anti-apoptotic factor, it is associated with U-CLL and* progressive disease (77) and is enhanced in the proliferative $CD38^+$ CLL subset. (73). The specific induction of TOSO via the BCR suggests autoreactive BCR signaling as a key mediator of apoptosis resistance in CLL. In addition, FcμR deserves study as a potential pathway for the delivery of therapeutic Ab–drug conjugates into CLL cells.

Antigenic selection of the CLL immunoglobulin repertoire

T cell-dependent and T cell-independent B cell activation

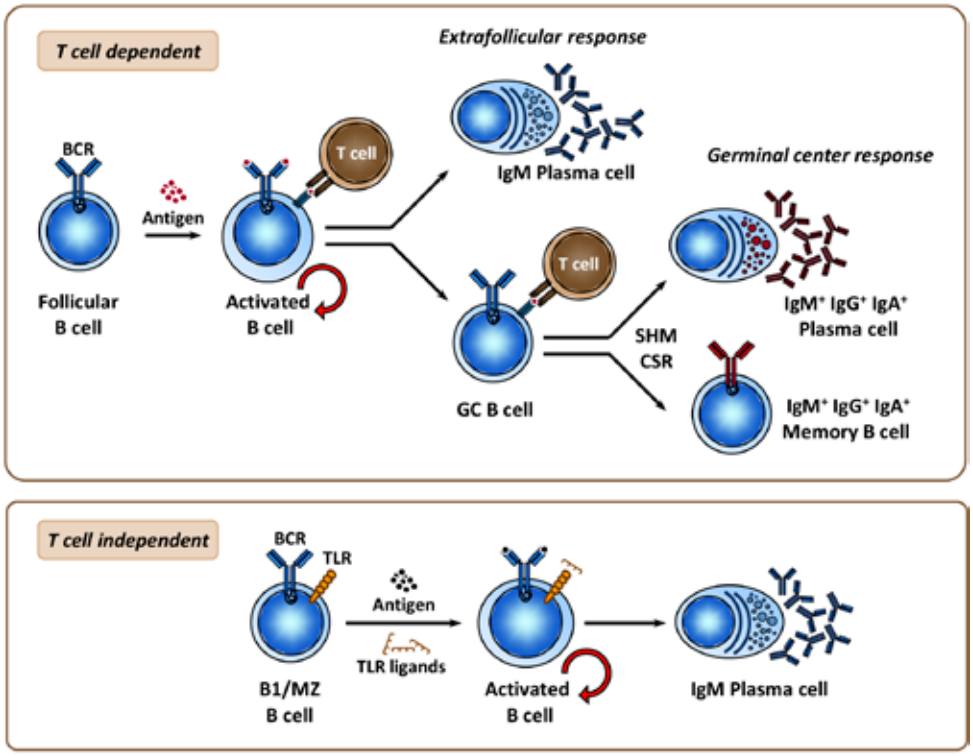
Activation of B lymphocytes during an immune response relies on antigen recognition by the BCR. The unique antigen specificity of the BCR of each B cell is ensured by a process called V(D)J recombination, whereby in developing B cells in the bone marrow V, D and J genes of the Ig heavy and light chain loci are randomly rearranged to generate a highly diverse Ig repertoire (Reviewed in Ref. (78-79)). While virtually endless combinations of V, D and J gene rearrangements guarantee the presence of B cells that can recognize newly encountered pathogens through their BCR, this random V(D)J recombination inevitably yields BCRs that are reactive to self-antigens (80). To prevent systemic autoimmune disease, BCR signals upon self-antigen binding either instruct developing autoreactive B cells to edit their BCR, to go into apoptosis during their development or render them anergic (hyporesponsive) once they have fully matured (81). This clearly illustrates that BCR signaling can dictate very opposing fates to a B cell, ranging from apoptosis or anergy versus activation, proliferation and survival.

Following the V(D)J recombination process in the bone marrow, these rearranged Ig genes may then be further modified by two distinct processes, SHM and class-switch recombination, that can be initiated upon antigen recognition in peripheral lymphoid organs. Both processes critically depend on the activation-induced cytidine deaminase (AID) enzyme (82-83), which is induced in activated B lymphocytes in germinal centers (GC) of lymph node follicles during a T cell-dependent response (Figure 1). SHM formation is a physiological process that is characterized by random introduction of mutations within rearranged Ig genes at rates of $\sim 10^{-5}$-10^{-3} mutations per bp per generation (which is $\sim 10^6$ fold higher than the spontaneous mutation rate). As the introduction of mutations is entirely random, this is followed by selection processes in the GC, resulting in further affinity maturation of the Ig molecules (84).

In addition, B cells can be activated in a T-cell independent way in the absence of a GC reaction, e.g. as is the case for marginal zone B cells (Figure 1). This subset of B cells is present at the interface between the non-lymphoid red pulp and the lymphoid white pulp in the spleen. MZ B cells have a pre-activated state, as is clear from high expression levels of CD40, CD80 and CD86 and low CD62L expression. The major role of these resident, non-recirculating, MZ B cells is to respond rapidly to blood-borne pathogens. Upon antigen recognition they move to the T-B cell border, proliferate and form plasmablasts that migrate through bridging channels into the red pulp within 2 days, as shown in mouse experiments (85). They generally respond in a T-cell independent way, and in such a T-independent response they do not perform class switch recombination and have limited somatic hypermutation (Figure 1).

Another B cell subset involved in T-cell independent responses is the B-1 lineage, which is present

Figure 1. T cell-dependent and T cell-independent B cell activation.



Upon antigen recognition in the presence of T cell help follicular B cells may undergo plasma cell differentiation in an extracellular response or differentiate into GC B cells that may form plasma cells or memory B cells (*top*). In contrast, B-1 B cells or marginal zone B cells can be activated by antigen and/or TLR ligands in the absence of T cell help, and differentiate into plasma cells in a T cell-independent fashion (*bottom*). CSR= class switch recombination; SHM= somatic hypermutation; MZ= marginal zone.

in peritoneal and pleural cavities and has extensively been studied in the mouse. Interestingly, many of these B-1 B cells express CD5, which is a negative regulator of BCR signaling and CD43, both of which are also present on human CLL cells. The $CD5^+$ B-1a cell population is responsible for circulating natural antibodies (see below), while $CD5^-$ B-1b cells have the capacity to provide a rapid T-cell independent IgM response to high molecular weight repetitive epitopes, such as bacterial capsular LPS, e.g. *Streptococcus pneumonia* (86) (Figure 1).

IGHV mutational status as prognostic factor

At the end of the nineties it was shown in two independent seminal studies by the Chiorazzi and Hamblin & Stevenson groups that CLL can be divided in two prognostically different biological subgroups,

based on the presence or absence of mutations in their rearranged IGHV genes (9-10). Initially, it was hypothesized that unmutated CLL (U-CLL) are derived from antigen-inexperienced naive B cells, and mutated CLL (M-CLL) would stem from post-germinal center memory B cells. However, gene expression profiling experiments have shown that both CLL subgroups have a memory-like transcriptome (4, 87). Both CLL subgroups are therefore now believed to be derived from antigen-experienced B-cells, which however differ in their SHM level and especially in the way they are selected for antigen-specificity (88-90), and extensively reviewed in (91).

M-CLL cells could be considered as the counterparts of memory B-cells (Figure 1), which mostly have undergone a GC-dependent developmental pathway. U-CLL would then also originate from an antigen-experienced memory-like B cell, possibly a human equivalent of the mouse B-1 cell or the MZ B cell, but that had not been able to alter its BCR by mutation during differentiation. Indeed, it is now well-established that CLL cases can be categorized into two clinically meaningful subsets, using the IGHV gene mutational status (reviewed in (1) and (2)). The U-CLL subgroup (with unmutated IGHV genes with >98% identity to the closest germline IGHV gene) often shows adverse cytogenetic features and clonal evolution, and generally a less favorable prognosis with short time to treatment, poorer response to therapy and an inferior overall survival. The M-CLL subgroup (with mutated IGHV genes with <98% identity to the closest germline IGHV gene) has a more favorable predicted outcome. It appeared that IGHV gene mutational status even has a prognostic value independent of the clinical stage (9-10). This has lead to its utilization for inclusion or stratification of CLL patients in many clinical protocols. Although in most cases the prognostic implications of IGHV gene analysis can be ascertained reliably using the 98% identity cut-off, caution is still warranted when dealing with CLL cases of 'borderline' IGHV gene mutational status, as for any mathematical cut-off value (92-93). Technical aspects and interpretation of IGHV gene mutational analysis have been standardized in guidelines (92-94), developed by the European Research Initiative on CLL (ERIC) (www.ericll.org). Such standardization is essential to generate reliable IGHV gene sequences and to correctly report the IGHV mutational status for individual CLL patients to the referring clinician.

Because not in every routine hematological or immunological laboratory involved in diagnosing CLL gene-sequencing technology is available to determine the IGHV gene mutational status, several surrogate markers for IGHV gene mutational status have been evaluated. These markers are partly based on gene expression profiling studies between U-CLL and M-CLL (4) and include CD38, ZAP70 and more recently also lipoprotein lipase and TCL1 (95-98). Although some of these markers look promising, they did not replace IGHV gene mutational status as a reliable and robust marker to be applied in multi-centre studies. Therefore, IGHV gene mutational status, though labor-intensive, is still the most informative marker available.

Another argument to still rely on IGHV gene mutational status as a prognostic marker is the fact that IGHV sequencing generates more detailed information than only the mutational status per se. Also the exact IGHV sequence and even Ig heavy chain D and J gene usage can be determined. This is important, since it was shown that CLL cases with IGHV3-21 usage that are mostly mutated (with ~2-5% mutation load) showed poorer overall survival than the other patients with M-CLL (99-101). This finding stresses that important prognostic information resides in the Ig molecules and has revitalized in-depth studies on the role of BCR in CLL.

Stereotyped BCRs in CLL

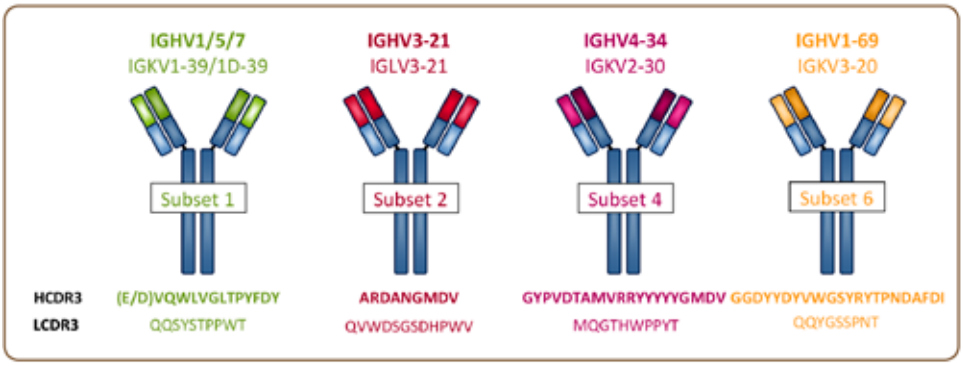
Already more than a decade ago, it has been hypothesized that CLL cells are selected by some sort of antigenic pressure (102). In this concept antigens derived from pathogenic bacteria or apoptotic cells or autoantigens are essential in triggering CLL development. This hypothesis emerged from early studies showing that the IGHV gene repertoire of CLL is highly restricted and biased, as compared to the normal adult B-cell repertoire (103) (104). In normal physiology the chances that two independent B cell clones would carry identical Ig receptors are virtually negligible, given the huge diversity of the normal Ig repertoire. Both the skewed gene usage and the SHM patterns in the IGHV genes thus implicate antigen selection processes via the BCR in CLL pathogenesis (see review by Stamatopoulos) (105).

Non-random IGHV usage was even more apparent when considering U-CLL versus M-CLL cases. In U-CLL increased usage of IGHV1-69 was observed, whereas IGHV4-34, IGHV3-23, and IGHV3-7 were more frequently seen in M-CLL (106). Since CLL typically occurs in elderly patients, similar shifts in IGHV usage of normal B cells upon aging were evaluated; no increase of IGHV1-69 was found in normal elderly, whereas IGHV4-34 could be increased, probably related to reactivation of herpes viruses.

Next to this asymmetric IGHV usage, differences in the entire IGH chain have also been found in CLL, e.g. an association between IGHV3-7 and IGHJ4 genes (~90%), between IGHV4-34 and IGHJ6 genes (~50%) (106). The IGHV1-69 gene also utilizes significantly more frequently the IGHJ6 gene in CLL and carries significantly longer IGH antigen-recognition complementarity determining region 3 (CDR3) than those noted for IGHV1-69 expressing normal B cells (107-108). The CDR3 region is the most variable part of the IgH and includes the junction of V, D, and J genes and can also vary because of the addition of random (N) nucleotides. In addition, an almost exclusive association with the same light chain, IGKV3-20, was noted in this group. Another example of a striking association concerns IGHV3-21 gene usage in combination with IGLV3-21 gene usage in a subset of CLL patients (109). In this context, another important observation was the finding of biased IGHV usage in combination with highly similar IGH CDR3 motifs (110-111) (112). This phenomenon of very similar Ig molecules (including HCDR3) was from then on referred to as stereotyped BCR (88). Analysis of large CLL cohorts has now revealed that up to 30% of CLL show very homogeneous gene usage and HCDR3 conformations and can be clustered in subsets with a stereotyped BCR (89-90, 113) (Figure 2). Interestingly, the frequency of stereotypy varies between different CLL groups, ranging from ~11% in M-CLL to >40% in U-CLL. It also varies significantly amongst certain IGHV genes (e.g. IGHV3-21, IGHV1-69 >> IGHV3-7 and IGHV3-74). Moreover, even CLL-biased deletions and particular somatic hypermutation patterns were found to be associated with stereotyped CLL subsets (90, 114). Remarkably, some stereotyped subsets show geographical differences. The best example is the stereotyped IGHV3-21 subset that seems more frequent in Northern-Europe than in the Mediterranean area (115).

In the most recent evaluation of almost 3,000 CLL IGHV sequences a few hundred stereotyped subsets have been defined, which included 2-21 (virtually) identical sequences each (113). Some subsets as defined in this cohort are relatively small, but might become more prominent subsets when larger cohorts are studied. Thus, CLL can now largely be divided into two different categories: one (~30% of primarily U-CLL cases) with a very restricted repertoire and BCR stereotypy (clustered cases) and a second with a heterogeneous BCR (non-clustered cases). Based on detailed bioinformatics analyses it

Figure 2. Clinico-biologically relevant stereotyped CLL subsets.



Indicated are the characteristic IGHV and IGKV / IGLV genes per stereotyped subset, as well as the amino acid residues in the respective CDR3 motifs, HCDR3 and LCDR3. Stereotyped subsets are based on data obtained by Stamatopoulos et al. (89) and Murray et al. (90). Figure obtained from dr. R. Rosenquist, Uppsala, Sweden and reproduced with permission.

has been hypothesized that the two CLL categories might have a distinct ontogeny. CLL cases with a heterogeneous BCR would likely derive from precursor cells of the conventional B-cell pool, whereas cases with a stereotyped BCR could originate from progenitor cells adapted to particular antigenic challenges (113). Since this latter precursor cell is somewhat intermediate between the true innate immune system and the conventional adaptive B-cell immune system, this would open the possibility that the cell of origin of stereotyped CLL would be a human functional equivalent of the mouse B1 cell.

Studies indicate that at least some stereotyped CLL not only have biological impact, but also seem to confer prognostic information in addition to - but perhaps also independently of - IGHV gene mutational status (89, 115-116). This might be particularly true for IGHV3-21 expressing CLL mentioned above. Cases belonging to the IGHV3-21 subset with stereotyped HCDR3 and highly similar IGLV3-21 light chains showed more progressive disease than cases of another IGHV3-21 subset (heterogeneous HCDR3 and variable light chain gene usage), which showed a more variable clinical course (115). Interestingly, stereotyped IGHV4-34 CLL cases also differ in outcome, but seem to have a better prognosis than heterogeneous IGHV4-34 cases.

Collectively, the current data strongly point towards a model that CLL development is not so much a random event in the elderly B cell population but rather represents a stepwise process in which antigen stimulation via the BCR is clearly implicated. BCR stimulation could act as a factor precipitating initial transformation and further promoting acquisition of additional genetic lesions (see review by Chiorazzi and Ferrarini) (91). The importance of BCR signaling is even further underscored by the recent observation of intraclonal diversity in an IGHV4-34 expressing stereotypic subset, which is suggestive of ongoing antigenic stimulation via the BCR (117).

Natural antibodies and semi-variant BCRs

Given the clear repertoire skewing of both CLL groups, antigen-specificity of the BCR thus seems a critical factor for biology and clinical course. Because of the importance of the BCR in CLL immunopathogenesis, the quest for the antigen(s) implicated in BCR stimulation has started. This is logically one of the most pressing issues in CLL, as it could tell more about the etiology and immunopathogenesis of CLL, and perhaps also provide clues to prevention and/or treatment of the disease. Based on differences in the SHM levels of their BCR, as well as differences in the stepwise process of leukemogenesis or even the cells of origin, it is not too surprising that U-CLL and M-CLL would recognize different types of antigens. M-CLL generally express oligo- or monoreactive antibodies associated with T-cell dependent GC-derived B cell responses. In contrast, U-CLL tend to express BCR with long IGH CDR3 motifs and amino acid residues that favor polyreactivity and therefore resemble natural antibodies (91).

Natural antibodies are produced at tightly regulated levels in the complete absence of external antigenic stimulation and provide immediate, early and broad protection against pathogens (118). They have evolutionarily important specificities (non-protein antigens, such as glycolipids or carbohydrates) and are produced mainly, if not exclusively, by a subset of long-lived, self-replenishing B cells termed B-1a B cells, which have been extensively characterized in mice (119-121). These B-1a cells develop mainly during fetal life in the liver and migrate to peritoneal and pleural cavities - strategic anatomical locations at interfaces with the environment - where they have self-renewal capacity. In particular, they rapidly respond in a T cell-independent fashion to host-derived innate signals, such as cytokines, or pathogen-encoded signals, such as LPS and phosphorylcholine, but they respond very poorly to BCR-mediated activation. In fact, they undergo programmed cell death following self-antigen recognition; this is thought to be related to their expression of CD5, which is a negative regulator of BCR signaling. B-1 cells are absent in lymph nodes and comprise a minor population in the spleen, but they rarely enter GCs and rarely undergo affinity maturation (120-121). Using chimeric mouse models, it was shown that serum IgM is mainly B-1 cell derived (122). Pulmonary influenza virus infection induces antiviral antibodies of IgM and other subclasses, but does not affect the serum levels of natural antiviral IgM (122). B-1 cells do respond to influenza infection locally in the lung (but not systemically) with increased IgM production, but this local B-1 B cell response is not antigen-driven (123).

B-1 B cells largely recognize non-protein antigens, such as glycolipids or carbohydrates. B-1 B cells have a limited repertoire with restricted Ig H and L chain V-gene usage; they carry a semi-variant BCR that is generally poly-reactive and lacks somatic hypermutation. In the mouse it has been shown that the BCR of B-1 B cells undergoes positive selection at multiple checkpoints during differentiation, both in the bone marrow and in the periphery, leading to restricted Ig H chain J gene usage and restricted Ig H and L chain combinations, e.g. Vh12-Vk4 (124) and Vh11-Vk9 (125). Remarkably, their development rests on positive selection by self-antigens, as clearly shown in transgenic mouse models (126). The poor response of B-1 B cells to BCR engagement is thought to minimize the risk of autoimmunity. In contrast to the considerable knowledge on B-1 B cells in mice, the nature of human B-1 cells has not been successfully defined to date and therefore the existence of human B-1 cells is controversial. Nevertheless, very recently a small population of $CD20^{+}CD27^{+}CD43^{+}$ cells, present in both umbilical

cord and adult peripheral blood, was shown to manifest B-1 cell properties (127). The elucidation of the relation between these cells and CLL cells awaits detailed molecular comparisons.

Nature of putative antigens recognized by BCRs in CLL

Natural antibodies are associated with autoreactivity and are often stereotyped, a feature which – though not exclusively- is seen more often in U-CLL than in the M-CLL group. However, polyreactivity is certainly not restricted to stereotyped antigen-binding sites. It has actually been suggested that both U-CLL and M-CLL originate from autoreactive B cell precursors, but that SHM plays an important role in M-CLL to alter this original BCR autoreactivity (128). This conclusion was based on DNA sequencing and ELISA analysis of cloned and *in vitro* expressed recombinant antibodies from U-CLL and M-CLL. In these analyses U-CLL expressed highly polyreactive antibodies, whereas most M-CLL did not. However, when nonautoreactive M-CLL antibody sequences were reverted *in vitro* to their germline counterparts, they encoded polyreactive and autoreactive antibodies.

Certain stereotyped BCR are known to recognize antigenic structures on apoptotic cells, for example myosin heavy chain IIA (129), which might reflect an intrinsic property of such B cells to clear cellular debris and/or dead micro-organisms. In other cases, the BCR expressed by the CLL cells shares features with autoantibodies. One example is the similarity between rheumatoid factor and the BCR in U-CLL of the IGHV1-69 / IGKV3-20 subset (89). Cross-reactivity between the IgG-Fc epitope recognized by rheumatoid factor and a viral epitope of the hepatitis C virus, could be the link between viral infection and autoimmunity (130). And, although the IGHV1-69 BCR in CLL may not directly recognize hepatitis C virus, this paves the way for a model in which particular infections could be a trigger in the stepwise process of CLL leukemogenesis.

Another intriguing example is the mutated IGHV4-34 CLL subset (reviewed in (105)), which shows a stereotyped BCR that is characterized by a long, positively charged IGH CDR3 motif (90). IGHV4-34 antibodies have been implicated in apoptotic cell removal through FR1-based recognition of the NAL antigenic determinant of the I/i blood group antigen and the CD45 molecule (131-132). Despite an abundance of IGHV4-34 B cells, IGHV4-34 antibodies in serum are low, which could be the result of counterselection because of intrinsic autoreactivity. However, in the autoimmune condition systemic lupus erythematodes (SLE) IGHV4-34 serum antibody levels are increased and constitute a considerable fraction of the characteristic anti-DNA antibodies in this disease. The IGHV4-34 CLL subset may therefore also have originated from a progenitor cell with anti-DNA specificity, whilst this reactivity would have been abrogated in the CLL cells through SHM-based editing. Interestingly, a rise in IGHV-34 antibodies is also seen in response to certain infections, including cytomegalovirus (CMV), Epstein-Barr virus (EBV) and *Mycoplasma pneumoniae* (133). Molecular evidence for EBV and CMV persistence in stereotyped IGHV-34 expressing CLL patients supports the model that CLL progenitor cells might be stimulated by viral antigens, thereby creating cells prone to undergo oncogenic transformation.

Finally, it has been shown that upon cloning ~60% of CLL BCR molecules were polyreactive, were mainly derived from the U-CLL group, and bound apoptotic cell surfaces (134). Structures that were recognized typically comprised two types of antigens: (i) native molecules, relocated to the external cell surface during apoptosis, and (ii) neoantigens, generated by oxidation during apoptotis. Some of

the oxidized epitopes appeared to be similar to microbial epitopes. These data imply that CLL may derive from B cells that normally function to remove cellular debris or to provide a first line of defense against pathogens. Similar conclusions were reached in another study in which several antigens for CLL were identified, including cytoskeletal proteins (vimentin, filamin B, and cofilin-1), cardiolipin, but also phosphorylcholine-containing antigens such as *Streptococcus pneumoniae* polysaccharides and oxidized low-density lipoprotein (135). It is thus conceivable that CLL is partly derived from the B cell compartment that produces natural antibodies, instrumental in elimination and scavenging of apoptotic cells and pathogenic bacteria.

In summary, even though the exact antigens are not yet known for all CLL cases, a picture is now emerging that many CLL progenitor cells might be implicated in recognition of microbial pathogens or cellular debris, and that sustained antigenic stimulation of their BCR and an activated cell state is a key step in CLL leukemogenesis, in which additional genetic lesions are acquired.

B cell receptor signaling in CLL B cells

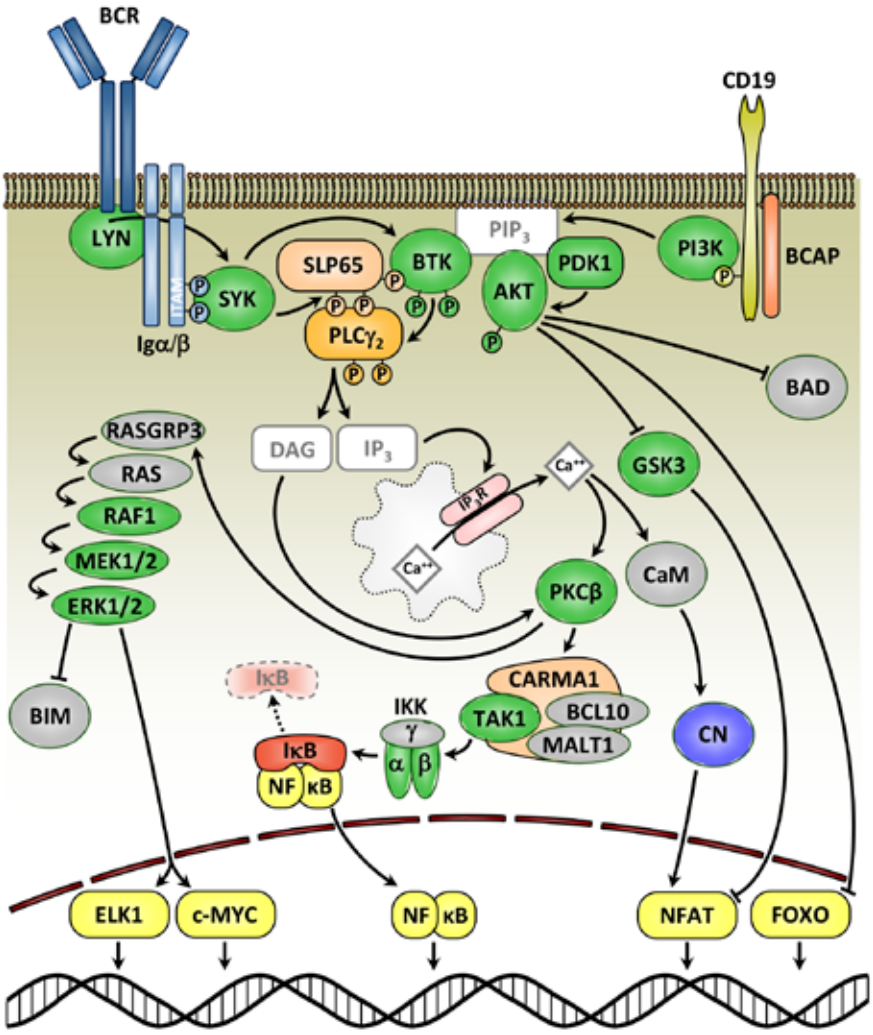
BCR signaling pathways in mature B cells

The identification of stereotyped or hypermutated BCRs in CLL cells overtly demonstrates that antigenic stimulation of the BCR is required for CLL leukemogenesis. Although it may be argued that the unique BCR specificities in CLL may merely indicate a strong selection of B cells early in pathogenesis, it is more likely that BCR signals continue to play a role in CLL progression, particularly because BCR signaling in CLL cells is markedly different from healthy mature B cells. Therefore, we will discuss BCR signaling in healthy versus CLL B cells below. Furthermore we will discuss the possible consequences of changes in BCR signaling in CLL B cells on cell fate in the context of the exploration of new CLL therapies targeting BCR signaling components.

The BCR complex is associated with a Igα/β (CD79a/b) heterodimer whose cytoplasmic tail contains immunoreceptor tyrosine-based activation motifs (ITAMs) that are required to initiate BCR signaling (see Figure 3). Recent evidence indicates that BCRs form dense clusters on the B cell membrane, which can be dispersed by antigen binding, thereby initiating BCR signaling (136). Upon antigen binding, Src-family protein tyrosine kinase Lyn phosphorylates the Igα/β ITAMs, thereby creating docking sites for SYK (spleen tyrosine kinase) (137-138). In parallel Lyn phosphorylates tyrosine residues in the cytoplasmic tail of the BCR co-receptor CD19, enabling the binding and activation of phosphatidylinositol-3 kinase (PI3K) and VAV (139) (140-141). VAV binds and activates Rac1, which subsequently can increase enzymatic activity of PI3K by inducing a conformational change of PI3K (142). Besides CD19, cytoplasmic adapter BCAP (B cell adapter for PI3K) can recruit PI3K as well (143), indicating a redundancy in sites for docking and activating PI3K following BCR triggering. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3) that attracts the TEC-family kinase Bruton's tyrosine kinase (BTK) (144) (145) to the cell membrane via interaction through its PH domain (139, 146) (147) (Figure 3).

After localization of BTK to the cell membrane, SYK and LYN transphosphorylate BTK in its kinase domain at the Y551 position (148-149), followed by BTK autophosphorylation at Y223 (150). Although disruption of the Y223F autophosphorylation site does not hamper B cell development significantly (151), BTK autophosphorylation might exert a regulatory role on BTK activity (150) or alter BTK's SH3

Figure 3. Overview of BCR signaling pathways.



Upon antigen binding by the B cell receptor, signaling is initiated by LYN-mediated phosphorylation of ITAMs in the cytoplasmic tail of Igα/β and CD19, resulting in recruitment of SYK and PI3K, respectively. PI3K generates PIP_3 to enable membrane recruitment of BTK. SYK then phosphorylates SLP65 to create a docking platform for BTK and $PLC\gamma_2$, leading to phosphorylation of $PLC\gamma_2$ by BTK. The formation of this BCR micro-signalosome forms the branching point from which 4 major signaling pathways originate, finally resulting in activation of ELK/c-Myc, NF-κB, NFAT and FOXO transcription factors. See text for details.

domain binding properties and thereby change interactions of Btk with other proteins (149). For the transphosphorylation of BTK and further signal propagation to downstream effectors, recruitment of SLP65 (SH2 domain-containing leukocyte protein of 65 kDa, alternatively named BLNK or BASH) is essential, as SLP65 serves as a scaffold for several tyrosine kinases including SYK and BTK (152-157). Activated SYK generates SLP65 docking sites by phosphorylation (156) that enable binding of BTK and phospholipase $C\gamma_2$ ($PLC\gamma_2$) to SLP65 (158-159). This final recruitment of $PLC\gamma_2$ completes the formation of so-called microsignalosomes, composed of VAV, PI3K, BTK, SLP65 and $PLC\gamma_2$, that initiate BCR signaling upon antigen binding (160) (Figure 3).

The detailed characterization of downstream BCR signaling pathways during the last decades has revealed that a few major signaling pathways diverge at different levels from the signaling cascade initiated by the BCR microsignalosome. The first major branching point is marked by activation of the serine/threonine kinase AKT (alternatively called PKB) pathway by PI3K, which occurs downstream of SYK but does not require $PLC\gamma_2$ activation (161)(Figure 3). All other signaling routes are dependent on $PLC\gamma_2$, but further branching of signaling routes is directed by the two PIP_2 cleavage products DAG and IP_3 generated by $PLC\gamma_2$. Whereas IP_3 induces Ca^{2+} influx that is particularly important for activation of NFAT transcription factors and JNK, DAG-mediated activation of PKCβ is required for p38, ERK and NF-κB activation (Figure 3).

After phosphorylation of AKT at the cell membrane by 3-phosphoinositide dependent kinase (PDK1; (162)), fully activated AKT returns to the cytoplasm to orchestrate an anti-apoptotic program (reviewed in (163)). Important primary targets of AKT are forkhead transcription factors (FOXOs) (164-165), NFAT via inhibition of glycogen synthase kinase-3 (GSK-3) (166) or NF-kB (167). AKT is a central component in pro-survival signaling downstream of the BCR, e.g. by blocking pro-apoptotic proteins, including the BH3-only protein Bad, releasing Bad from $Bcl\text{-}_{XL}$ (168-169) or by stabilizing MCL1 (170).

Activation of $PLC\gamma_2$ by BTK results in the generation of two second messengers, IP_3 and diacylglycerol (DAG), which activate partially overlapping downstream signaling pathways (Figure 3). IP_3 binds to IP_3 receptors located on the endoplasmatic reticulum (ER) membrane, leading to release of Ca^{2+} from the ER (171). Intracellular Ca^{2+} can activate calmodulin and subsequently calmodulin activates calcineurin phosphatase, which in turn activates NFAT transcription factors (reviewed in (172)) (Figure 3). Both intracellular Ca^{2+} and DAG can activate another downstream kinase, protein kinase C beta (PKCβ), resulting in NF-kB activation through a scaffold complex that includes CARMA1 (also called CARD11 or BIMP1), Bcl10 and MALT1 (173) (Figure 3). NF-κB regulates transcription of many genes that are directly involved in cell cycle progression and anti-apoptotic pathways (reviewed in (174)). Activation of PKCβ also induces the phosphorylation of ERK1/2 (extracellular signal-regulated kinase1/2), which promotes cell survival and especially cell cycle entry. Whereas Ras signaling is required to induce activation of ERK1/2, other MAP kinases p38 and JNK can be induced by $PLC\gamma_2$ without intermediate signaling via Ras (175).

It is thought that the balance of the parallel BCR signaling routes (Figure 3) determines the effects on B cell fate, ranging from proliferation and survival to apoptosis. The nature of the antigen recognized and thus its interaction with the BCR may greatly determine the signaling strength of individual signaling routes downstream of the BCR. For example, differences in Ca^{2+} influx frequency and length are reflected by differences in activation patterns of NF-kB versus NFAT ((176-177)).

Heterogeneous response of CLL cells to BCR signaling

The skewed BCR repertoire in CLL suggests that chronic antigenic stimulation of the BCR drives leukemogenesis. As mentioned above, CLL cells generally express low levels of surface IgM (3), probably resulting from increased BCR internalization upon antigen binding. Accordingly, CLL B cells readily upregulate surface IgM expression *in vitro* (3) once they are isolated and thus deprived of their *in vivo* ligands. However, CLL B cells are often hampered in their response to α-IgM stimulation *in vitro* (reviewed in (178-179)) and only some CLL samples exhibit a physiological response regarding phosphorylation of Syk, PLCγ2 and Ca^{2+} influx (180-182). Although α-IgM stimulation may enhance B cell survival in responsive samples, it never induces full activation or proliferation of CLL B cells (183).

The reported anti- or pro-apoptotic effects of BCR stimulation of CLL B cells *in vitro* vary greatly between samples (184-188). This heterogeneity largely correlates with the mutational status of the BCR, and therefore also correlates to markers as CD38 and ZAP-70, which are differentially expressed by the two CLL subtypes (12, 189-191). Whereas U-CLL B cells often respond to α-IgM stimulation (albeit differently compared to wild-type B cells), M-CLL B cells are often unresponsive to BCR stimulation and go into apoptosis. This unresponsiveness of M-CLL B cells may not be due to an intrinsic defect to react to BCR stimulation, but may instead be caused by differences in the nature of the antigen recognized by the BCR (192). While stimulation of M-CLL B cells with soluble α-IgM does not improve B cell survival and does not lead to activation of downstream BCR signaling pathways, stimulation with immobilized α-IgM evokes a signaling response indistinguishable from U-CLL B cells (192).

Several factors other than the nature of the antigen may contribute to the different BCR signaling capacity in U-CLL versus M-CLL B-cells. One of these factors might be the more pronounced downregulation of IgM expression on M-CLL B cells (3), but this could merely reflect increased BCR engagement *in vivo*, rather than a constitutive feature of M-CLL B cells. Another marked difference between IgM expressed by U-CLL and M-CLL B cells is the difference in glycosylation patterns of IgM (193). A greater fraction of IgM in U-CLL B cells is mannosylated compared to M-CLL B cells. Mannosylated IgM is expressed by α-IgM stimulated B cells (193), and appears to be more signaling competent, possibly due to enhanced interactions with surrounding lectins, such as galectin, that impair BCR endocytosis (194). A third factor possibly contributing to altered expression and signaling of the BCR in CLL is the expression of a truncated form of Igβ/CD79b. This truncated form of Igβ, which lacks the extracellular Ig-like domain, is formed by alternative splicing of the Igβ transcript (195). Not only does it impair BCR signaling in CLL B cells (196), it also lowers BCR expression levels, which could be corrected in a CLL cell line by introducing normal Igβ (197).

Differences between BCR signaling in U-CLL and M-CLL are not only limited to changes in expression of the BCR and the associated Igα/β heterodimer, but they also extend to signaling pathways downstream of the BCR. Nevertheless, altered BCR expression or glycosylation patterns may have profound effects on downstream signaling events, since some studies demonstrated that in CLL samples unresponsive to *in vitro* α-IgM stimulation very early phosphorylation events fail to be triggered (186, 189).

It was recently reported that U-CLL cells are more prone to spontaneous apoptosis and subject to environmental pro-survival signals than M-CLL cells are (198). Interestingly, peripheral blood T cells could protect U-CLL cells from apoptosis – associated with recovery of NF-kB activity and Bcl-2 expression – by means of cell-to-cell contacts and soluble factors (198).

Proximal protein tyrosine kinases in CLL: their role and therapeutical options

The Scr-like kinase LYN. Phosphorylation of the Igα/β ITAMs by LYN is one of the earliest events in initiating BCR signaling. In CLL B cells LYN is overexpressed and constitutively phosphorylated and therefore LYN represents an attractive therapeutic target CLL (13). Importantly, multiple LYN-inhibitors can induce apoptosis in CLL B cells (13, 199). This finding encouraged further clinical studies on the effectiveness of LYN inhibitors in CLL patients, but the effects of dasatinib (Table 3), a non-specific inhibitor of SRC family kinases and ABL, proved to be modest, because only a fraction of CLL patients showed a partial remission upon treatment (200). The incomplete effectiveness of LYN inhibition cannot be explained by redundancy with other SRC family kinases (FYN, BLK or LCK), because these kinases should be targeted by dasatinib as well. Rather, it is conceivable that blocking LYN activity will not only inhibit its activating role in CLL B cells, but also its regulatory role. LYN also downregulates BCR signaling through phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) of inhibitory receptors, including CD22 and FcγRIIB (201-203).

The SYK/ZAP70 tyrosine kinases. Another logical target in CLL therapy is SYK, whose phosphorylating activity leads to assembly of the BCR micro-signalosome by connecting SLP65, BTK, and PLCγ2. Similar to LYN, also SYK appears to be elevated and constitutively activated in CLL (204-205). Importantly, inhibition of SYK by several selective inhibitors blocks BCR signaling and induces apoptosis of CLL cells (14-15, 204-205)). Not only could the SYK inhibitor fostamatinib disodium (R788) (Table 3) block tumor growth in a mouse model for CLL (206), it also proved to be effective in the majority of patients in a small clinical study (207). The actions of SYK inhibitors probably extend beyond dampening of BCR signaling itself, as BCR signaling via SYK also affects migration of CLL B cells by increasing expression of chemokines CCL3 and CCL4 and by increasing sensitivity to chemotaxis mediated by CXCR4 and CXCR5 (15, 204). In a recent phase 2 study in rheumatoid arthritis patients, SYK inhibition by R788 reduced disease activity (208), but substantial adverse events were observed, including diarrhea, hypertension, and neutropenia, some of which could be explained by off-target inhibition. Therefore, additional studies will be needed to further assess the safety and efficacy of Syk-inhibition therapy in CLL.

The SYK-related tyrosine kinase ZAP70, which is normally expressed in T cells where it signals downstream of T cell receptor (reviewed in (209)), can be expressed by activated human B cells and by malignant B cells including CLL (210-211). In fact, many homologous signaling proteins that were previously thought to be not expressed in the B cell lineage make important contributions to BCR or pre-BCR signal transduction in the mouse, as indicated by detailed analyses of compound knock-out mice. These proteins include next to ZAP70, also (i) the LAT/SLP76 adapter molecules, which enable pre-B cell differentiation in the absence of SLP65, (ii) the TEC kinase, which has the ability to partially compensate for BTK, and (iii) PLCg1 (212-214); (215).

In T cells ZAP70 is recruited to the T cell receptor by the ITAMs containing RhoH guanine nucleotide-binding protein. RhoH is overexpressed in CLL B cells and has been proven to contribute to CLL pathogenesis in a CLL mouse model (216). Moreover, ZAP70 is induced in normal B cells via activation of Toll-like receptor (TLR)-7 or TLR-9 in a MyD88-dependent manner (217). However, most of the CLL patients that manifested the activating L265P point mutation in MyD88 expressed low levels of ZAP70 and belonged to the M-CLL group (60), indicating that increased ZAP70 expression in CLL is not induced by MyD88 signaling.

Expression of ZAP70 in CLL correlates with aggressive disease development (218) and ZAP70 is specifically expressed by U-CLL cells that are responsive to BCR stimulation (12). ZAP70 associates with Igβ and becomes phosphorylated upon BCR ligation. ZAP70 directly enhances BCR signaling, because retroviral transfection of ZAP-70-negative CLL cells with a ZAP70-encoding construct could increase the effects of α-IgM stimulation (11, 219). Kinetic activity of ZAP70 was dispensable for enhancing BCR signaling in CLL B cells (220), indicating that ZAP70 functions in CLL B cells through assisting the activation of other upstream kinases in BCR signaling. Alternatively, it has been suggested that ZAP70 could divert regulatory signaling proteins from SYK (reviewed in (221)). Because blocking its kinase activity does not impair BCR signaling, ZAP70 does not directly represent an attractive target for new CLL therapies, despite the observed ZAP70 overexpression especially in U-CLL with an inferior prognosis.

The Tec-family kinase BTK. BTK is a direct substrate of SYK in BCR signaling, and given the promising results with SYK inhibitor treatment in CLL, targeting BTK may be equally effective in inducing CLL B cell apoptosis. It has already been demonstrated that BTK expression levels are increased in CLL by ~2-3 fold (reviewed in (222)). Overexpression of BTK in CLL is thought to be caused by dysregulation of a posttranscriptional mechanism, since the increase in BTK protein levels was not mirrored by an equal increase in BTK mRNA levels (18). This posttranscriptional mechanism might involve aberrant expression of miRNAs such as miR185, which has recently been shown to regulate BTK expression levels by binding to the 3' UTR of BTK mRNA (55). Recently, the first studies were published in which effects of the selective BTK inhibitor PCI-32765 (223) on CLL B cells were investigated (18, 224-225). Using an *in vivo* model with adoptive transfer of mouse CLL B cells to healthy recipient mice, PCI-32765 was shown to strongly suppress CLL development in these mice (224). *In vitro*, PCI-32765 was capable of inducing apoptosis in human CLL B cells through antagonizing soluble and stromal cell derived costimulatory factors and decreasing activation of AKT, ERK and NF-κB. (18, 224-225). Furthermore, treatment of CLL B cells with PCI-32765 reduced the production of CCL3 and CCL4 chemokines, and hampered CXCL12 and CXCL13 mediated migration as well as α4β1-integrin mediated adhesion of CLL B cells(224-225). Given the brief transient lymphocytosis and reduction of lymphadenopathy in CLL patients observed upon the start of PCI-32765 treatment, it is thought that inhibition of Btk disturbs the retention of CLL B cells in survival niches in lymph nodes and bone marrow, leading to a higher susceptibility to apoptosis of these cells (225). Recent findings in our lab affirm the critical role for BTK in CLL leukemogenesis, since BTK deficiency completely abrogated development of CLL in the IgH.TEμ mouse model (LPK and RWH, unpublished data), in which CLL spontaneously arises due to sporadic expression of the potent oncogene simian virus 40 (SV40) T antigen in mature B cells (226) (see below).

Interestingly, BTK inhibition by PCI-32765 (Table 3) leads to objective clinical responses in dogs with spontaneous non-Hodgkin's lymphoma (NHL)(227). This finding supports BTK inhibition as a therapeutic approach in the treatment of human B-lineage malignancies with inappropriate BCR activation, such as CLL and activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL). In a recent phase Ib/II follow-up trial in patients with relapsed or refractory CLL a response rate for PCI-32765 of 70% was observed (228).

Importantly, the recent discovery of GCI1746, a small-molecule BTK inhibitor chemotype with a new binding mode that stabilizes an inactive non-phosphorylated enzyme conformation, holds promise, because it inhibits BTK activity with ~1,000- to ~2,000-fold target selectivity over the next most sensitive

kinases, the TEC family members BMX and ITK (229). This signifies an exceptional selectivity, compared to other known BTK inhibitors, such as PCI-32765, for which these values are 1.6 and 21, respectively (230). Because of its excellent selectivity, it is expected that therapeutic application of GCI1746 will be associated with few adverse events. Although BTK is crucially involved in various signal transduction pathways, including Fc receptor and TLRs, in many cell types, the almost completely B-cell specific phenotype associated with defective BTK expression in human and mice (reviewed in (231)) does not make it very likely that BTK inhibition significantly affects other cells of the hematopoietic system.

PLCγ$_2$. Although PLCγ$_2$ is clearly activated in CLL B cells following BCR stimulation (192) and ZAP70 has been demonstrated to enhance PLCγ$_2$ phosphorylation upon BCR stimulation (11, 219), to our knowledge there are no reports of potentially beneficial effects of PLCγ$_2$ inhibition in CLL (reviewed in (221)). Blocking PLCγ$_2$ activity may not be an optimal therapeutic option for CLL, since PLCγ$_2$ is abundantly expressed in many tissues and signals downstream of a broad array of receptors, making PLCγ$_2$-directed CLL therapies prone to systemic side effects or toxicity.

CD38. CD38 is a type II cell surface molecule that functions both as an membrane-anchored ecto-enzyme (ADP ribosyl cyclase; (232)) and as an activating receptor that can be triggered by its ligand CD31 (233). In mature B cells, it signals in supramolecular complexes with BCR co-receptor CD19 (234), whereby ligation of CD38 leads to CD19 phosphorylation and association with LYN and PI3K (235). Moreover, ligation of CD38 evokes phosphorylation of ZAP70, and levels of ZAP70 form a rate-limiting step in CD38 signaling ((236). CD38$^+$ CLL B cells have a greater BCR signaling capacity than CD38$^-$ CLL B cells, reflected by increased Ca^{2+} influx upon α-IgM stimulation (183) and by increased basal phophorylation levels of BCR downstream signaling molecules. CD38 expression in an unfavorable prognostic factor for CLL (183), and its expression often coincides with ZAP70 expression (237-240). In CD38$^+$ CLL samples CD38 is not expressed homogeneously by all CLL B cells, indicating that CD38$^+$ cells represent a proliferating subset of CLL B cells within the same CLL clone (236, 241-242). CD38$^+$ cells CLL B cells have a greater migratory capacity, since CD38 can reinforce CXCR4-mediated migration (243), and may thereby direct migration of these cells from the bloodstream into lymphoid organs. It has been speculated that blocking CD38 signaling may therefore lead to trapping of CLL B cells in the circulation, making it easier to expose these cells to high concentrations of chemotherapeutical agents (reviewed in (244)). It therefore appears that CD38 forms a global molecular bridge to the environment, promoting survival and proliferation over apoptosis (see recent review by Malavasi et al., (245)). Future studies should show if CD38 is an effective target for antibody-mediated therapy in CLL.

Downstream signaling events in CLL: their role and therapeutical options

Following α-IgM stimulation of wildtype B cells multiple downstream signaling pathways are normally activated, including robust NF-kB activation (246) (Figure 3). It has been shown that BCR stimulation of CLL B cells with immobilized α-IgM efficiently activates ERK and AKT, while JNK and NF-kB activation is incomplete (192). The effective induction of ERK but failure to activate JNK and NF-kB strongly mimics the α-IgM response of tolerant B cells (247), suggesting that chronic antigenic stimulation in CLL induces a signaling signature comparable to anergic B cells ((188); reviewed in (178)).

PI3K signaling to AKT in CLL. There is clear evidence for a role of constitutively activated PI3K

(particularly the PI3Kd isoform) in survival of CLL B cells (17, 248). It is therefore not surprising that inhibition of PI3K using inhibitor LY294002 leads to apoptosis of CLL B cells (17, 185, 249). An equally effective induction of apoptosis in CLL B cells could be established using the PI3Kd isoform-specific inhibitor CAL-101 (248). CAL-101 displays clinical activity in CLL, causing rapid lymph node shrinkage and a transient lymphocytosis (250). Next to inhibiting BCR- and chemokine-receptor-induced phosphorylated AKT and ERK, however, CAL-101 also downregulated chemokine secretion, thereby reducing interactions that retain CLL cells in protective tissue microenvironments (250).

Despite the undisputed increase of PI3K activation in CLL B cells, surprisingly more controversy exists concerning the activation of AKT, the primary signaling target of PI3K. While in some studies constitutive phosphorylation of AKT in CLL B cells could not be demonstrated (17, 188), in other studies increased AKT phosphorylation was found (249, 251). The primary AKT target in CLL that is responsible for anti-apoptotic effects seems to be MCL1 (251-252). Furthermore, TCL1, which is a co-activator of Akt (253-254), is a potent oncogene expressed in T prolymphocytic leukemia (255) and several B cell malignancies, including CLL (256). Importantly, CLL cases with high TCL1 expression are characterized by BCR hyperresponsiveness, and have an inferior prognosis (98, 257). AKT blockade with the specific inhibitor AiX induced apoptosis induction in U-CLL cells (258). AKT activation was enhanced by overexpressing TCL1 in CLL and decreasing TCL1 levels by small interfering RNA reduced AKT activation in a fludarabine-insensitive CLL cell line cells (258). Together with the finding that transgenic expression of TCL1 in B cells in mice leads to spontaneous U-CLL formation (259-260) (see below), these data reveal a significant role for the AKT-TCL axis in CLL survival.

PKCβ signaling to NF-kB in CLL. Activation of PKCβ by DAG generated by $PLC\gamma_2$ represents a critical step in the activation of NF-kB. PKCβ is overexpressed in a subset of CLL patients and correlates to poor prognosis (261). Multiple isoforms of PKCβ exist, and in CLL B cells particularly PKCβII seems to be overexpressed, whereas in normal B cells PKCβI expression outweighs PKCβII expression (262). Remarkably, overexpression of PKCβII correlated in CLL B cells with lower Ca^{2+} influx, and conversely treatment of CLL B cells with PKCβ inhibitors could increase BCR signaling. This unexpected finding could be explained by a dominant regulatory role instead of an activating role for PKCβII in BCR signaling in CLL B cells. PKCβ acts as a feedback loop inhibitor of BTK activation: treatment of B cell lines with PKCβ-inhibitors reduced the phosphorylation of BTK at position S180, which is required for terminating BTK signaling (263). Despite an important role for PKCβ in regulating Ca^{2+} influx in BCR-stimulated CLL cells, inhibition of PKCβ nevertheless could induce apoptosis in CLL B cells *in vitro* (262), possibly indicating that increased Ca^{2+} signaling in CLL B cells might lead to activation-induced cell death. Further evidence indicating a critical role for PKCβ in CLL pathogenesis was provided by studies in a CLL mouse model in which deletion of PRKCB could abrogate tumor formation (264). In the same study apoptosis could be induced in CLL cells *in vitro* using the non-specific PKCβ-inhibitor enzastaurin that also targets AKT (264).

Regarding this collective evidence for a role of PKCβ in CLL, aberrant activation of the downstream target NF-kB might play an important role in CLL pathogenesis. *In vitro* studies report that NF-kB activation upon BCR stimulation appears to be rather weak, judged by the incomplete degradation of IkB (192). Nevertheless, *in vivo* activation of NF-kB in CLL biopsies is clearly detectable (265)(266), and *in vitro* NF-kB activation could be enhanced by interaction of CLL B cells with vascular endothelial cells

(267). The significance of NF-kB activity in CLL pathogenesis is affirmed by the increased basal activation levels in CLL versus healthy B cells (268) and the inverse correlation between patient survival and NF-kB activation in CLL B cells (269). It should however be noted that it still remains unclear whether this NF-kB activation observed *in vivo* or in endothelial co-cultures is induced by BCR signaling or by other receptors that also activate NF-kB. Regardless of which receptor might induce NF-kB activation in CLL cells, inhibition of NF-kB seems to be an effective strategy to induce apoptosis. Indeed several inhibitors of Ikk were capable of inducing apoptosis of CLL B cells (268, 270-272).

Signaling of ERK and other MAP kinases in CLL. In line with the survival and proliferation-promoting role of ERK in normal B lymphocytes, ERK seems to be a key player that promotes CLL B cell survival. In a proportion of CLL samples, especially in samples unresponsive to α-IgM stimulation, constitutive ERK phosphorylation is observed (188). An anti-apoptotic role for ERK in CLL is further suggested by several observations of reduced ERK phosphorylation preceding apoptosis of CLL B cells induced by numerous pharmacological compounds, including the SYK inhibitor R406 (205), the Src-kinase inhibitor dasatinib (199), the cyclin-dependent kinase inhibitor flavopiridol (273), the VitD3 analog 1089 (274), or the PI3K-inhibitor CAL-101 (250). Despite this strong correlation between ERK activity and CLL B cell survival, introduction of constitutively active MEK mutants in primary CLL B cells did not significantly improve B cell survival (252), possibly indicating that ERK activation is dispensable for CLL B cell survival. Although in a recent study mutations in ERK have been demonstrated to occur in ~3% of CLL patients (24), the effects of these mutations on CLL formation still need to be studied.

The role of other MAP kinases in CLL pathogenesis is either much more disputed (in the case of p38) or largely unknown (in the case of JNK). Although some studies do demonstrate a critical role for p38 in CLL B cell survival (275), other studies indicate that their role in CLL B cell survival may be limited. In these studies, constitutive p38 or JNK phosphorylation seems to be weak in CLL B cells, and stimulation of CLL B cells with α-IgM does not markedly activate JNK and p38 signaling (192). In fact, there is much uncertainty whether p38 signaling may promote or inhibit apoptosis in CLL B cells, since some pharmacological compounds induce apoptosis associated with a decrease in p38 activity (dasatinib, (199)), while other compounds evoke an increase in p38 phosphorylation in apoptotic CLL B cells (flavopiridol, (273), and VitD3 analog 1089, (274)).

Mouse models for CLL

To date, several mouse models are valuable tools to study CLL and to investigate anti-tumor efficacy of novel compounds in vivo. The New Zealand Black (NZB) strain is a naturally occurring model of late-onset CLL and is characterized by B-cell hyperproliferation and autoimmunity early in life, followed by progression to CLL. A point mutation is present in the 3'-flanking sequence of miR16-1, which resulted in decreased levels of this microRNA, in addition to various DNA repair and telomerase defects (39) Recently, systemic *in vivo* lentiviral delivery of miR-15a/16 was shown to reduce malignancy in NZB mice (276).

Using transgenesis and gene targeting, various CLL-like mouse models have been generated to date. Bichi *et al.* (259) reported that overexpression of TCL1 under the control of a V_H promoter and IGH intronic enhancer (Em) in the B cell lineage leads to a murine disease with many of the characteristics

of human CLL. These animals spontaneously develop hyperplasia of the $CD5^+$ B cell lineage, initially in the peritoneal cavity and then in lymph nodes, spleen, bone marrow and blood, that becomes oligoclonal and eventually monoclonal with age (8–16 months). Yan et al. (260) extensively studied the mutational status of the BCR in the Em-TCL1 mouse model. The molecular features of the BCRs of these transgenic mice resembled those of human B-CLL patients with the more aggressive, unmutated form of the disease (U-CLL). In particular, Yan *et al* (260) observed biases in IGHV gene usage, whereby the Ig closely resembled antibodies to autoantigens and antibodies reactive with antigens found in bacterial cell membranes. Interestingly, using a different construct whereby the B29 minimal promoter was coupled to the IGH intronic enhancer Em, Hoyer et al. (277) generated a transgenic mouse with TCL1 overexpression which developed Burkitt-like lymphoma and DLBCL, pointing to an overlapping pathogenesis of these lymphomas and CLL. Also double transgenic mice overexpressing BCL2 and a dominant-negative form of the TNF receptor associated factor-2 in the lymphoid system (278) developed severe splenomegaly and often leukemia resembling human CLL. However, the underlying mechanism and interaction between TRAF1and BCL2 with respect CLL needs to be further investigated.

Transgenic mice expressing the TNF-like ligand APRIL (a proliferation-inducing ligand) under the T cell-specific LCK promoter develop lymphoid tumors that originate from expansion of the peritoneal B-1 B cell population (279). In these mice, APRIL levels were elevated in serum, which expanded the mature B-cell population and increased the survival time of these B-cells. A fraction (~40%) of APRIL transgenic mice had elevated level $CD5^+$ $B220^+$ B cells. Because in sera of CLL patients APRIL levels are increased, APRIL antagonism may provide a therapeutic strategy to treat CLL.

Transgene-mediated overexpression of IL-5 inhibited autoimmune disease in (NZBxNZW) F1 mice, but increased the risk of CLL. These mice exhibited a higher frequency of B-1 cells than non-transgenic littermates, beginning at 5 months of age, and therefore abnormalities in differentiation and proliferation of B-1 cells may provide a link between autoimmune disease and CLL (280).

We generated a mouse model, *IgH.TE*m, based on sporadic expression of the simian virus (SV40) T antigen (226). SV40T is a potent oncogene able to transform many cell types and has been implicated in the etiology of various cancers, including B cell malignancies. We accomplished sporadic SV40T expression by introducing SV40T in the opposite orientation between the D and J genes in the IGH locus. Only in rare mature B cells in which the targeted allele has a germline configuration and thus still contains SV40T it can be expressed in circulating B cells. At 6-9 months of age most *IgH.TE*m mice showed accumulation of a monoclonal $CD5^+IgM^+IgD^{low}$ B cell population. IGHV sequence analysis revealed preferential usage of unmutated VH11.2 and non-stochastic usage of D and J genes, strikingly similar to those observed in Em-TCL1 mice (226). Because, two V_HJ558^+ leukemias manifested extensive SHM, these findings provide an animal model for both U-CLL and M-CLL and show that pathways activated by the SV40 T antigen play important roles in CLL pathogenesis.

Recently, Klein *et al* (40) demonstrated that deletion of the 13q14-minimal deleted region (MDR) encoding the DLEU2/miR-15a/16-1 cluster in mice initiates the development CLL-associated phenotypes observed in humans. A fraction of these mice developed NHL. Deletion of the MDR region accelerates proliferation of both human and mouse B cells by modulating the expression of genes controlling cell-cycle progression, rather than by influencing survival via regulation of BCL2. Interestingly, in mice

deleted for MDR or miR-15a/16-1 mice $CD5^+$ B cell tumors expressed unmutated IGHV genes, whereas the $CD5^-$ NHL mostly expressed somatically mutated IGHV genes.

Finally, the role of miR-29 and miR-181 was analyzed *in vivo*. Levels of TCL1 expression in B-CLL are regulated by these two miRNAs that target the 3' UTR of the TCL1 gene. Efanov et al. (281) showed by comparison of Em-TCL1 mouse models that did or did not contain the 3' and 5' UTR of the TCL1 gene that the presence of these UTR sequences delayed the onset of the disease. This could – at least in part – be explained by targeting of TCL1 expression by microRNAs. On the other hand, miR-29 is overexpressed in indolent CLL, when compared to aggressive CLL and normal $CD19^+$ B cells. To dissect the role of miR-29 in CLL, Santanam et al. (282) generated Em-miR-29 transgenic mice, which showed an increased population of $CD5^+CD19^+IgM^+$ B cells. Only ~20% of these mice developed frank leukemia, from which finding the authors concluded that miR-29 dysregulation can contribute to the pathogenesis of indolent CLL. Taken together, these diverse transgenic mouse models have improved our understanding of CLL pathogenesis and have increased our knowledge on several crucially involved genes. As CLL is a heterogeneous leukemia in human, it is likely that each of the described models will provide important information regarding the molecular mechanisms that mediate initiation, maintenance or progression of CLL.

Concluding remarks

It is becoming apparent that survival and proliferation of malignant CLL B cells crucially depends on BCR signals. Therefore, the next advances in the treatment of CLL involve specific blocking of BCR signal transduction, either as a single agent therapy or in combination with existing chemotherapy strategies. Fundamental investigation into BCR signaling pathways has now resulted in the development of several promising drugs that target proximal crucial signal transduction proteins, including LYN, SYK, BTK and PI3K, all of which are currently in clinical trials in patients with CLL to study their efficacy. Important recent progress includes the application of whole-genome sequencing in CLL cases to further identify molecular changes contributing to CLL pathogenesis. Interestingly, all of the four newly identified genes that were very recently found to be recurrently mutated in CLL are involved in signal transduction (60). In particular, the BTB-Kelch protein KLHL6 has been implicated in BCR signaling and GC formation, as in its absence BCR cross-linking was less sensitive, as measured by proliferation, Ca^{2+} response and PLCg activation (66). The comprehensive analysis of CLL using whole-genome sequencing therefore highlights the usefulness of this approach for the identification of clinically relevant mutations. It is conceivable that some of the genes identified by such genome-wide approaches represent attractive targets for the development of small molecule inhibitors, particularly if they are regulators of BCR signaling.

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Tables

Table 1. Most relevant recurrent chromosomal aberrations in CLL

Chromosomal aberration	*Gene(s) involved*	*Overall incidence (%)[a]*	*Incidence in refractory CLL (%)[b]*	*Overall survival (mo)[a]*	*Association with M-CLL or U-CLL*
13q14 deletion	miR-15a, miR-16-1	55	25	133	Mainly M-CLL
Trisomy 12	Unknown	16	12	114	U-CLL and M-CLL
11q23 deletion	ATM, + other?	18	25	79	U-CLL
17p13 deletion	TP53	7	31	32	U-CLL
No clear aberration	n.a.	~18	Unknown	111	n.a.

[a] Incidence and survival data from(20). [b] Incidence data from (1).

Table 2. Recurrent gene aberrations identified in CLL

Gene	*Location*	*Incidence (%)*	*Gene aberrations*	*Association with M-CLL or U-CLL*	*Reference*
BCL-6	3q27	24	5'UTR mutations, low-level expression	M-CLL	(59)
SF3B1	2q33.1	15.4	Mutated C-terminal PP2A repeat regions	Mainly in U-CLL	(24)
ATM	11q22.3	8.8-18.8	Various scattered mutations	Mixed	(23-24)
TP53	17p13.1	8.5-15.4	Mostly mutations of DNA binding domain	Mainly U-CLL	(24-25)
NOTCH1	9q34.3	4.4-12.2	PEST Domain Activating mutations	Mainly in U-CLL	(24, 60)
MYD88	3p22	2.9-9.9	TIR domain activating mutations	Mainly in M-CLL	(24, 60)
FBXW7	4q31.3	4.4	Various scattered mutations	Mixed	(24)
ZMYM3	Xq13.1	4.4	Various scattered mutations	U-CLL	(24)
DDX3X	Xp11.3-p11.23	3.3	Various scattered mutations	Mixed	(24)
ERK/MAPK1	22q11	3.3	Various mutations, mostly kinase domain	Mixed	(24)
XPO1	2p15	2.4	Inactivating mutation	U-CLL	(60)
KLHL6	3q27.3	1.8	Various Activating mutations	M-CLL	(60)
IRF4	6p25-p23	1.5	L116R mutation and higher expression	U-CLL	(67)

Table 3. Proximal BCR signaling molecules and small molecule inhibitors in CLL

Signaling molecule	*Inhibitor*	*Current clinical trials*	*CLL Trial number* [a)]	*Reference*
LYN	Dasatinib	CLL and many other tumors (dasatinib is a non-specific inhibitor of SRC family kinases and ABL)	NCT01173679	(199) (200) (283) (284) (285)
SYK	Fostamatinib (R788)	Rheumatoid Arthritis, T and B cell lymphoma	n.a.	(207) (208) (206)
BTK	PCI-32765	CLL Various lymphomas, including FL, MCL, BL, DLBCL	NCT01351935 NCT01217749 NCT01105247 NCT01109069 NCT01292135	(227) (18) (286)
	GCI1746	(efficacy in CLL not tested yet, but BTK selectivity of this inhibitor is superior compared to PCI-32765)		(229) (230)
	AVL-292	CLL NHL, Waldenstrom Macroglobulinemia	NCT01351935	(287)
PI3K	CAL-101	CLL Various lymphomas, including MCL, FL, HL Multiple myeloma, Acute Myeloid Leukemia	NCT01203930 NCT01088048 NCT00710528 NCT01090414	(288) (19) (18) (250) (289) (290)

[a)] See: registry of clinical trials at www.clinicaltrials.gov

10

General discussion

Parts of this chapter have been published in a review in International Reviews of Immunology: Aberrant B Cell Selection and Activation in Systemic Lupus Erythematosus

International Reviews of Immunology Aug 2013, Vol. 32, No. 4: 445–470

Antigen binding to the BCR represents one of the strongest activation signals a B cell can receive, and it critically determines B cell differentiation, selection, functioning and survival. Proper control of the magnitude and duration of BCR signals are therefore mandatory to prevent aberrant B cell responses to harmless self- or commensal antigens. Hyperresponsiveness of the BCR therefore may lead to a breakdown of B cell tolerance and initiate a pro-inflammatory inflammation loop involving other immune cells as well, eventually culminating in autoimmune disease. Alternatively, chronic activation of B cells through the BCR may predispose B cells to malignant transformation, resulting in B cell lymphoma or leukemia.

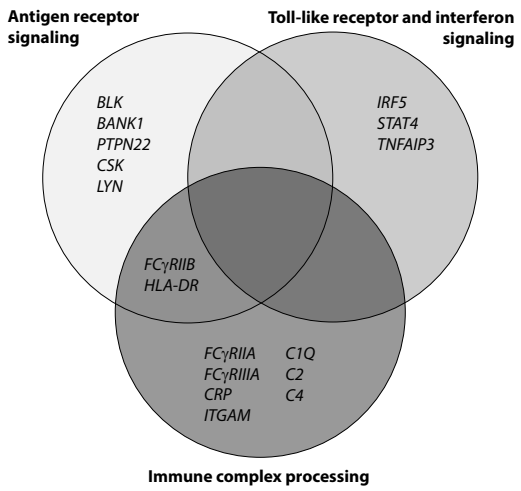
In this thesis we describe several surprising findings on how alterations in BCR signaling in patients and several murine disease models contribute to the development of rheumatic autoimmune disease or chronic lymphocytic leukemia. We found that the constitutive signaling capacity of the SLC when expressed in mature B cells does not breach but instead instructs B cell tolerance by imposing anergy. In line with this, constitutive BCR signaling established differently by mutant forms of Btk could not provoke systemic autoimmunity, but nevertheless programmed autoreactive IgM plasma cell formation. However, conditionally enhancing BCR signaling by overexpression of unmutated Btk spontaneously induces GC formation, apoptosis resistance of B cells and eventually a T-cell driven SLE-like disease. Importantly, though SLE patients do not invariably overexpress Btk, Btk expression is dysregulated in activated B cells in SLE, and in a fraction of patients Btk levels correlate with disease characteristics reminiscent of related autoimmune disorder APS.

Constitutively enhanced BCR signaling and B cell-driven autoimmunity

Either from a biological or clinical point of view, the role of BCR signaling in B cell development

and activation have been widely studied before. Despite our still expanding knowledge on the physiological function of BCR signaling in B cell differentiation and activation, far less is known about the perturbations in BCR signaling that cause or contribute to the development of rheumatic autoimmune disease in humans. Apart from limited functional studies characterizing BCR signaling in SLE patients that revealed aberrant Ca^{2+} influx upon BCR stimulation and enhanced phosphorylation of ERK, JNK and p38 (1-4), most evidence for a causative role for BCR signaling in human SLE is indirectly derived from genome-wide association studies identifying SLE-associated polymorphisms in BANK1 and BLK (5) (Figure 1). First reports on how certain polymorphisms affect the BCR signaling strength in SLE B cells now appear, including a recent study on proximal BCR signaling gene CSK showing that the intronic rs34933034[A] polymorphism increases BCR signaling through upregulation of CSK protein levels (6). In murine SLE, an extensive body of evidence has been generated for a causative role of defects in BCR signaling in SLE development (7). Nevertheless, in these models the genetic defects are diverse and so are the consequences of altered BCR signaling in the context of autoimmunity, ranging from mild auto-antibody production to destructive lethal disease.

Figure 1. SLE susceptibility genes identified in genetic association studies in SLE patients.



Schematic overview of genes linked to SLE susceptibility identified in genome-wide association studies in SLE patients or other genetic screening studies. SLE risk genes are clustered based on the inflammatory signaling pathways in which they are implicated. *BANK1*, B-cell scaffold protein with ankyrin repeats 1; *BLK*, B lymphoid tyrosine kinase; *C1Q*, complement component 1, q subcomponent; *C2*, complement component 2; *C4*, complement component 4; *CRP*, C-reactive protein; *CSK*, c-src tyrosine kinase; *FCgRIIA*, Fc fragment of IgG, low affinity IIa, receptor; *FCgRIIB*, Fc fragment of IgG, low affinity IIb, receptor; *FCgRIIIA*, Fc fragment of IgG, low affinity IIIa, receptor; *HLA-DR*, human leukocyte antigen DR; *IRF5*, interferon regulatory factor 5; *ITGAM*, integrin, alpha M (complement component 3 receptor 3 subunit); *LYN*, v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; *PTPN22*, protein tyrosine phosphatase, non-receptor type 22; *STAT4*, signal transducer and activator of transcription 4; *TNFAIP3*, Tumor necrosis factor, alpha-induced protein 3. Adapted from Harley et al., Nature Reviews Genetics, 2009 (56).

The requirement of a certain window of BCR signaling for counterselection of autoreactive B cells during B cell differentiation can be deduced from the relatively increased emergence of autoreactive B cells in BCR signaling-deficient mice and patients. For example, increased frequencies of self-reactive B cells or increased susceptibility to autoimmune disease are observed in XLA patients and healthy subjects carrying PTPN22 polymorphisms that decrease the BCR signaling strength (8-10), and deficiency for the SLC in mice induces anti-nuclear autoantibody production (11). Conversely, enhanced BCR signaling during early B cell development does not promote the emergence of increased numbers of selfreactive B cells, but instead enforces the silencing of supposedly selfreactive B cells mainly through clonal deletion and receptor editing. Results supporting this view are extensive, and include the reduced numbers of peripheral B cells in strains of LMP2A-transgenic mice (12), mice overexpressing (human) CD19 (13), mice expressing high levels of constitutively signaling Btk-mutants (14, 15) or high levels of the autonomously signaling SLC (16). As BCR signaling in these models strongly interferes with normal B cell development, the effects of constitutively enhanced BCR signaling on the development of autoimmunity may be masked. In studies presented in this thesis, we therefore investigated whether constitutively enhanced BCR signaling in a lower dose may not obstruct normal B cell development but instead promote the activation of peripheral B cells with self-reactive BCRs.

We here demonstrate that even low-level expression of BCR signaling molecules with constitutive signaling capacity surprisingly cannot induce systemic autoimmune disease. In mice expressing near- or sub-physiological levels of active Btk mutants (Btk^{E41K} and $Btk^{E41K\text{-}Y223F}$) in B cells do not develop IgG anti-nuclear autoantibodies and systemic disease (**Chapter 3**). Although the observed production of non-pathogenic IgM autoantibodies in mutant Btk-transgenic mice demonstrates that lowering the levels of constitutive BCR signaling now no longer absolutely blocks the emergence selfreactive B cells expressing selfreactive BCRs, apparently other or additional activation requirements exist to promote IgG autoantibody production. Likewise, in SLC-transgenic mice we observed that lowering the dose of SLC-expression does alleviate clonal deletion and thereby normalizes peripheral B cell numbers, even allowing the production of IgG autoantibody production on autoimmune-prone genetic backgrounds (**Chapter 2**). Surprisingly however, we observed that rather than promoting autoimmunity, low-dose SLC expression in peripheral B cells ameliorated induced autoimmune arthritis.

The incapability of B cells in SLC-tansgenic mice to induce autoimmunity likely indicates that low-level, continuous BCR signaling results in a rewiring or even unresponsiveness of downstream BCR signaling pathways, reflecting anergy. In agreement with this, we observed that ectopic SLC expression dose-dependently diminished BCR-induced Ca^{2+} influx and upregulation of activation markers. Despite the many observed similarities between mutant-Btk transgenic mice and SLC-transgenic mice regarding B cell deletion, IgM plasma cell formation (16), and the inability to induce fulminant autoimmune disease, B cell anergy unlikely represents a shared mechanism in models with continuous BCR signaling that prevents activation of autoreactive B cells in IgG autoantibody responses, since we observed that Ca^{2+} influx upon BCR triggering is even exaggerated in mutant-Btk mice. While this BCR hyperresponsiveness contradicts the induction of an anergic phenotype in mutant-Btk expressing cells, functionally these cells still share characteristics with anergic cells in vivo given the limited antigen-specific antibody production after immunization with T-independent and T-dependent antigens. Further research should

explore how increased Btk signaling in mutant-Btk transgenic mice paralyzes antigen-specific antibody responses while exhaustion of BCR signaling as observed in anergic cells does not occur. It is noteworthy however that regarding the molecular basis of anergy, different adaptations of proximal BCR signaling have been described (17, 18), and that mouse models to study B cell anergy often exhibit contrasting phenotypes regarding B cell differentiation, mature B cell longevity, activation marker upregulation and in vivo antibody responses (19).

Conditionally enhanced BCR signaling and B cell-driven autoimmunity

The reason why continuous B cell activation by constitutively signaling BCR components as the SLC or mutant forms of Btk does not induce fulminant autoimmune disease may lie in insufficient T cell engagement and subsequently the production of high-affinity auto-antibodies in GC reactions. Firstly, it has been shown that prolonged BCR signaling induced by continuous BCR stimulation in vitro does not effectively prime B cells for T-dependent responses based on the relatively low expression of chemokines as CCL3 and CCL4 and chemokine receptor CCR7 involved in initiating B-T interactions (20). Secondly, increasing the binding affinity between hen egg lysozyme (HEL) and HEL-specific transgenic BCRs in vivo reduced germinal center formation and programmed B cells for extrafollicular antibody responses (21), a decision process likely guided by chemokine receptor EBI2 whose up- or downregulation depends on BCR signals (22). Thirdly, in many murine models in which BCR signaling has not been constitutively enhanced but instead conditionally by blocking BCR inhibitory pathways full-blown systemic autoimmunity develops characterized by IgG autoantibody production (23-27). We therefore investigated how conditionally increased Btk-mediated BCR signaling may contribute to the development of systemic autoimmunity.

In **Chapter 4** we describe that overexpression of unmutated Btk in B cells by a CD19-hBtk transgene is sufficient to enhance BCR-mediated B cell activation, leading to spontaneous GC formation, IgG autoantibody formation and SLE-like disease in mice. The finding that autoantibody production could be elicited in the airways of Btk-overexpressing mice upon influenza infection demonstrated a negative selection defect of autoreactive B cells, presumably occurring in virus-induced iBALT structures. Further evidence for a defect in the selection of Btk-overexpressing GC B cells was derived from the in vitro resistance to Fas receptor signals selectively following BCR stimulation of Btk-overexpressing B cells. These results, strongly point to a defect in T cell-mediated control of autoreactive GC B cells. Further affirmation of the role of T cells in Btk-driven autoimmunity is described in **Chapter 5** where we found that, despite the selective genetic targeting of B cells in CD19-hBtk mice, autoimmunity in these mice is fully T cell dependent. CD40L deficiency not only abrogates IgG plasma cell formation and autoantibody production in these mice, but in return also corrects T_{FH} formation and T cell activation.

The role of dysregulated GC reactions in autoimmunity has been well known (28), but the mechanisms by which selective enhancement of BCR signaling may thwart GC B cell selection are still largely unclear. In fact, the role of BCR signaling in physiological GC reactions has even been seriously challenged. First of all, the role of BCR signaling in GC B cell selection was found to be less decisive based on the observation that bypassing BCR-mediated antigen uptake still allowed proper positive selection of GC B cells (29, 30). Secondly, BCR signaling in GC B cells proved to be heavily attenuated by increased

phosphatase activity, only allowing BCR signals during G2-phase (31). Thirdly, asymmetric division of GC B cells proved to render a large fraction of GC B cell progeny BCR-negative with only a limited number of GC B cells inheriting BCR molecules and thus retaining the capacity to continuously respond to present antigen (32). The finding that BCR signaling capacity was restricted to GC B cells in G2 phase (31) seems to marginalize a role for BCR signaling in GC B cells even further. Firstly, these cells in G2 phase exclusively reside in the GC dark zone were antigen is virtually absent, and secondly dark zone B cells express lower BCR levels, thereby further challenging BCR signaling in GC B cells (29, 33).

Collectively, a new GC B cell selection model can be proposed based on these findings in which the light zone simply seems to facilitate B cell selection by antigen presentation to T cells and not necessarily by BCR signals, and in which the dark zone truly forms a region for proliferative expansion. The capacity however of dark zone B cells to respond to antigenic stimulation while antigen is locally absent seems conflicting, but the observation that GC B cells can capture antigen at the cell membrane for more than 72 hours in GCs may nevertheless enable BCR signaling in dark zone GC B cells (32). Assuming that BCR signaling can still intermittently occur in GC B cells, intrinsic B cell signals may not largely affect direct T cell-dependent B cell selection but instead facilitate prolonged B cell survival that may bridge the time window needed for the induction of plasma cell differentiation, or directly induce a plasma cell transcriptional program. In line with this view, high affinity GC B cells were shown to be selectively programmed to exit the GC by differentiating into plasma cells (34).

In **Chapter 6**, we describe that through deep sequencing analysis of total mRNA we identified a limited set of 115 genes that is differentially regulated upon BCR stimulation in Btk-overexpressing B cells compared to WT B cells. One of these genes which was more strongly downregulated by enhanced Btk-mediated BCR signaling proved to be Bcl6, a key transcription factor preventing Blimp1 induction and thus plasma cell differentiation (35). Although the enhanced downregulation of Bcl6 may facilitate the exit of GC B cells as plasma cells, this downregulation may also strongly interfere with GC B cell functioning since Bcl6 is indispensable for GC formation (36-38). To reveal whether enhanced BCR signaling not only plays a role in the initiation of T-dependent auto-antibody responses, but whether it also directly affects GC B cell functioning and differentiation more studies are required that not only characterize BCR signaling in sorted GC B cells, but also examine the kinetic expression of transcription factors orchestrating B cell survival and differentiation in these GCs in the context of autoimmunity. The focus of further experiments on signaling alterations of the BCR and not of other receptors in which Btk has been implicated (39) is justified by the finding that differentially expressed genes between wildtype and Btk-overexpressing B cells in vivo almost exclusively belonged to the gene signature induced by BCR signaling in wildtype B cells.

B cell depleting therapies in SLE

After the initial identification of B cells and the ANAs they produce as the critical factor in SLE pathogenesis (40), SLE research focused on uncovering B cell aberrancies in murine lupus models and SLE patients. This indeed yielded substantial insight into intrinsic defects in B cell selection and activation that facilitate B cell tolerance breakdown, fuelling the exploration of B cell depleting therapies in diseases as SLE. In agreement with auto-antibody independent roles of B cells in SLE, B cell depleting treatments in

SLE mouse models could halt disease progression while serum auto-antibody levels were not declining (41). But despite this success of B cell depleting agents in murine SLE models, complex and sometimes disappointing results have been yielded in clinical studies of the efficacy of B cell depletion therapies, including chimeric monoclonal anti-CD20 antibody Rituximab (42-46).

Though puzzling as the limited success of B cell depletion trials in SLE patients may seem given the canonical role of B cells in SLE pathogenesis, several appealing explanations for this failing therapy have been postulated. One comprises the incomplete depletion of B cells observed in SLE patients and murine SLE models, in which the clearance of targeted B cells is possibly compromised by defects in antibody-mediated cell death, apoptosis, or Fc-receptor mediated clearance of opsonized cells (47). Another compelling explanation is based on the observation that deleting the entire B cell repertoire in an SLE mouse model also removes B cells with immunosuppressive capacities, e.g. IL-10 producing "B10" cells, which normally ameliorate SLE-like disease (48). Indeed, such B cells have been found in human subjects, and their numbers in SLE patients are correlated with prognosis, are variably reduced upon Rituximab treatment, and seem predictive of Rituximab response (46). Furthermore, depleting the entire B cell population by Rituximab may relieve negative selection thresholds of repopulating B cells after treatment. The reported rise in serum levels of the B cell survival cytokine BAFF during and after Rituximab treatment (49-52) may induce clinical relapses, since BAFF overexpression in mice is sufficient to induce systemic autoimmune disease resembling SLE and Sjögrens syndrome (SS) (53).

The seemingly conflicting findings in SLE of overt pathogenic roles of B cells versus the doubtful success of B cell depleting regimens in SLE patients clearly demonstrate that multiple B cell subsets may have opposing effects in SLE pathogenesis. This should not discourage the lupus community to continue to explore new strategies to target B cells; it should instead urge to more specifically target pathogenic B cell subsets and their activity in disease. Affirming this view, a major breakthrough has recently been achieved with the successful treatment of SLE patients with Belumimab, a human monoclonal antibody that does not deplete B cells but instead blocks BAFF (B-cell activating factor) (54, 55).

Although blockage of BAFF is directly only affecting B cell survival and to lesser extent B cell differentiation, the effectiveness of this treatment can likely be attributed to its effects several amplification loops between multiple immune cell types that eventually culminate in B cell tolerance breakdown, ANA production and then overt disease. Therefore the (partial) failure of B cell depleting therapies in SLE versus the success of targeting a B cell survival cytokine may establish a paradigm shift in our immunologic view on SLE development, from a strongly B-cell centered pathogenesis model with underestimation of the immunoregulatory functions of certain B cell subsets. The crucial contribution of these amplification loops including NET-driven inflammation, the IFN type I signature, and disturbed GC reactions is affirmed by the identification of SLE risk genes that support these pro-inflammatory loops, such as genes involved in TLR signaling, BCR signaling, complement and immune complex induced signaling, B-T cell interactions and B cell-dendritic cell interactions (5, 56, 57) (Figure 1).

Btk-inhibitors in SLE: all that glitters is gold?

With the identification of SLE risk genes that collaborate in remarkably few pro-inflammatory signaling pathways, future studies should further explore how these pathways are interconnected and how

crosstalk between these pathways can be interrupted. This will ultimately reveal new molecules as therapeutic targets that may not be directly affected by hardwired genetic mutations in SLE, but that represent molecular bridges that interconnect pro-inflammatory pathways. In our studies, we have identified Btk as such a bridging molecule whose expression levels can be increased by several NF-kB activating pathways including BCR and TLR signaling, thereby establishing a detrimental overexpression loop in B cells that in mice can cause SLE (58). Moreover, since small molecule Btk inhibitors were shown to block BCR-dependent B cell proliferation as well as FcR-induced production of pro-inflammatory cytokines in macrophages and monocytes (59-61), Btk inhibition may not only block crosstalk of pro-inflammatory pathways in a single cell type, but also between different immune cells. Notably, Btk inhibition can strongly dampen FcR signaling since the proximal signaling machinery of Fc receptors display a very strong homology with the BCR microsignalosome (62).

As promising as treatment of autoimmune disease with Btk inhibitors may seem, future research should also focus on effects of off-target inhibition of related Tec kinases. Though Btk itself is not expressed in T cells, T cells do express Tec family kinases as Itk and Tec that exhibit close homology to Btk (63-65). Indeed, Btk-inhibitor Ibrutinib/PCI-32675 could not only block Ca^{2+} signaling upon antigen receptor stimulation in Ramos cells but also modestly dampened Ca^{2+} influx in Jurkat cells (66). Furthermore, a first report has been published in which Itk was shown to be effectively inhibited in human and murine T helper 2 (T_H2) cells, leading to selective skewing of T_H cell responses to a T_H1 profile in CLL patients treated with Ibrutinib (67). Although data from murine models simply show a great effectivity of Ibrutinib in the treatment of murine SLE and no disease aggravation or alteration based on presumed T_H cell skewing is observed (68, 69), reprogramming of T cell responses in human lupus may have unwanted effects on the course of disease that cannot be fully appreciated from these data in murine models.

Apart from effects of Itk inhibition in T cells, effects of Tec inhibition in both B and T cells can be anticipated based on the extensive homology between Btk and Tec. Although Tec does not play a major role in T cell development, it has been demonstrated that in the absence of Tec signaling murine T_H cells are more easily programmed upon in vitro stimulation with cognate antigen to produce IL-17 (70), a cytokine whose overexpression by T cells in SLE correlates with disease severity (71, 72). In B cells, so far only a complementing role for Tec has been described in Btk-mediated signaling of the BCR (73) since the partial block in pre-B cell development in Btk-deficient mice was dramatically more pronounced when simultaneously Tec expression was genetically ablated. Nevertheless the function of Tec signaling in mature B cells is still unclear as Tec-deficient mice do no exhibit clear defects in B cell differentiation (73). However, we recently observed that mature Tec-deficient B cells stimulated in vitro through the BCR displayed an enhanced upregulation of several activation markers and increased proliferative expansion (L.K. and R.H., unpublished results). Fitting with a possibly regulatory role of Tec signaling downstream of the BCR, it has been demonstrated that Src kinase Lyn, whose signaling dampens BCR signaling after its initiation (74, 75), can phosphorylate Tec (76), suggesting that Tec may act downstream of Lyn in mature B cells. If Tec truly exerts regulatory functions in BCR and TCR signaling, off-target inhibition of Tec in both B and T cells by Btk-inhibitors may unwantedly jeopardize immune tolerance. Reassuringly, newly developed Btk-inhibitor CGI1746 has been shown to exhibit no cross-reactivity with Tec and minimal affinity for other Tec family members as Itk and Bmx (59, 62).

Apart from the quest for new therapeutic targets, another future challenge in the treatment of SLE is the categorization of SLE patients in disease subphenotypes which may predict different responses to newly developed treatments. In this context a large step forward has been made by the differential clustering of SLE risk alleles with three SLE subphenotypes has been reported (77). Apart from associating hardwired mutations with disease subphenotypes, detailed investigation on the function and biology of proposed new molecular treatment targets in SLE is required. As we set out to explore BTK expression levels in B lymphocytes in SLE patients, we observed a marked heterogeneity in BTK protein levels (**Chapter 7**). While this heterogeneity complicated the detection of significant BTK expression differences between healthy control subjects and SLE patients, it did reveal that BTK expression levels may be associated with a clinical SLE subphenotype overlapping with anti-phospholipid syndrome (APS) based on the association between anti-cardiolipin auto-antibodies and increased BTK expression levels(78). As BTK levels did not correlate to treatment characteristics, and since BTK upregulation could not be a phenomenon secondary to acute inflammation since patients experiencing flares were excluded from the study, increased BTK levels and thus enhanced BTK signaling in B cells may represent a constitutive B cell feature driving disease pathogenesis in SLE-patients with APS-like disease.

Future research should address whether APS represents a disease entity that may be particularly responsive to Btk-inhibitor treatment. In addition, this association of BTK overexpression with APS-like disease indicates that future studies on BTK expression in autoimmune disease should not selectively focus on SLE, but instead should include other rheumatic diseases as well including SS and rheumatoid arthritis (RA). Importantly, it may even be worthwhile to study the role of BTK signaling in the pathogenesis of organ-specific immune diseases as idiopathic pulmonary fibrosis (IPF) in which enhanced BCR signaling has been shown to aggravate fibrosis development (79).

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11 |

Summary

B lymphocytes provide protection against invading pathogens by producing pathogen-specific antibodies, by presenting pathogen-derived antigens to T lymphocytes, and by activating other immune cells through the production of cytokines. To exert these functions, antigen-specific activation of B lymphocytes critically depends on antigen recognition via the B cell receptor (BCR) that is produced and selected in a stepwise manner during B cell differentiation. While the stochastic assembly of BCR components ensures an unlimited variety in antigen specificities of mature B cells to enable the recognition of all possible pathogens, this stochastic assembly also inevitably results in the generation of BCRs that react to self-antigens. If self-reactive B cells are inappropriately controlled, activation of such cells would induce devastating autoimmune disease. Therefore many checkpoints to assess the autoreactivity of the BCR are implemented during B cell differentiation and activation, and signals from the BCR orchestrate the silencing of autoreactive B cells during these checkpoints. Aberrancies in BCR signaling may thus thwart the counterselection or activation of selfreactive B cells, and importantly many genetic defects in BCR signaling molecules have been linked to the development of rheumatic autoimmune diseases as systemic lupus erythematosus (SLE). In addition, chronic activation of B cells through continuous BCR signaling may propel the malignant transformation of B cells leading to lymphoma or leukemia.

In this thesis, we investigated how changes in the signaling capacity or expression of BCR signaling molecules, in particular Bruton's tyrosine kinase (Btk), may predispose to the development of systemic autoimmune disease or B cell leukemia. The previous finding of ectopic expression of the autonomously signaling surrogate light chain (SLC) in B cells that infiltrate inflamed joints in rheumatoid arthritis patients made us investigate in **Chapter 2** whether SLC expression in mature B cells may drive or dampen autoimmune disease. We here describe that in a dose-dependent fashion transgenic SLC expression in mature B cells instructs clonal deletion of immature and transitional B cell and imposes anergy on residual mature B cells. This more stringent silencing of autoreactive B cells by SLC signaling likely explains the relative resistance of SLC-transgenic mice to autoimmune arthritis.

In **Chapter 3** we describe that while constitutively signaling Btk mutants (Btk^{E41K} and $Btk^{E41K\text{-}Y223F}$) induce deletion of developing B cells, constitutively active Btk inflicts a GC-independent differentiation of residual peripheral B cells into autoreactive IgM plasma cells, leading to IgM anti-nucleosome autoantibody production but not overt systemic autoimmune disease. As the strong amplification of BCR signaling by these Btk point mutations interferes with the development of high-affinity germinal

center(GC)-dependent (auto-)antibody responses, we studied alternative ways by which more subtly increased Btk-mediated signaling in B cells could provoke autoimmunity. In **Chapter 4** we found that Btk protein levels are dynamically upregulated upon B cell activation via the BCR, toll-like receptors (TLRs) or CD40, prompting is to study the effects of Btk overexpression on B cell activation and selection. We describe that mild transgenic Btk overexpression in B cells suffices to enhance BCR-mediated B cell activation, GC and IgG plasma cell formation, and anti-nuclear autoantibody formation culminating in SLE-like autoimmune disease. The increased BCR-mediated activation of Btk-overexpressing cells enhanced NF-κB activation, counteracted B cell apoptosis even when competitive pro-apoptotic Fas receptor signals were provided, and permitted the emergence of autoreactive B cells during T cell-dependent antibody responses against influenza.

In **Chapter 5** we studied whether T cells are accessory or necessary cells in the autoimmune disease induced by enhanced Btk signaling in B cells. We found that in Btk-transgenic mice pathogenic follicular T helper (T_{FH}) cells expand with lupus development in aging mice, during development of induced autoimmune arthritis, and in GC reactions provoked by immunization with exogenous antigen. This enhanced T_{FH} formation was paralleled by increased induction of ICOS expression on T helper (T_H) cells, which probably selectively relied on enhanced BCR signaling as increased ICOS expression could be recapitulated *in vitro* only when naive T_H cells were co-cultured with BCR-stimulated Btk-overexpressing B cells. Importantly, we found that autoimmunity in Btk-transgenic mice fully relies on T cell co-stimulation since Cd40l-deficiency not only abrogates GC formation and autoimmunity but also T_{FH} expansion in these mice. As Btk signals in B cells downstream multiple receptors including the BCR, TLRs and chemokine receptors, we assessed the contribution of enhanced BCR signaling to autoimmunity in Btk-transgenic mice by comparing gene expression profiles in BCR-stimulated and Btk-transgenic B cells. In **Chapter 6**, using a total mRNA deep sequencing analysis we show that differentially expressed genes in Btk-transgenic B cells *in vivo* almost completely overlap with BCR-induced genes in non-transgenic B cells, implying that Btk overexpression selectively amplifies BCR signaling. Furthermore, enhanced Btk signaling downstream the BCR allowed quicker Blimp-1 induction by more stringent Bcl6 downregulation, providing a mechanism to promote *in vivo* plasma cell formation.

Finally, to assess whether enhanced BTK expression in B cells may contribute to the development of SLE in human, we determined BTK expression in B cells from SLE patients. We observed in **Chapter 7** that BTK expression is dysregulated in SLE B cells based on reduced BTK upregulation in BCR-stimulated B cells *in vitro,* and on similar average BTK expression levels in naive $CD27^-$ and antigen-experienced $CD27^+$ B cells *in vivo*. Although BTK protein expression was not significantly increased in any B cell subset from SLE patients, in particular naive B cells exhibited a marked heterogeneity in BTK expression levels which correlated to clinical parameters as anti-cardiolipin autoantibody production, a feature more commonly associated with the related rheumatic disorder anti-phospholipid syndrome (APS).

Apart from a pathogenic role for Btk signaling in autoimmune disease, we also addressed the contribution of increased Btk expression to the susceptibility to chronic lymphocytic leukemia (CLL) since BTK protein levels were reported to be increased in established CLL in human. By studying disease progression in CLL-developing IgH.ETμ mice that were either Btk-transgenic or Btk-deficient, we show in **Chapter 8** that in the absence of Btk CLL does not develop, and that Btk overexpression accelerates CLL

development and increases mortality. Furthermore, increased Btk signaling facilitated the selection of B cells carrying non-stereotypic BCRs into malignant CLL clones. In **Chapter 9**, we further elaborate on the identified aberrancies in BCR signaling that contribute to disease formation or disease progression. In addition, we discuss the advances and pitfalls of new drugs targeting BCR signaling molecules in CLL.

In **Chapter 10**, the findings of the research presented in this thesis are discussed in the context of new insights in BCR-driven autoimmunity, and new directions for future studies and clinical implications are provided. Our studies have demonstrated the pathogenic potential of Btk signaling in the development of B cell driven autoimmune diseases and chronic lymphocytic leukemia. The finding that increased Btk signaling selectively through the BCR enhances B cell as well as T cell activation not only confirms the crucial contribution of altered BCR signaling to SLE and CLL, but additionally shows that not hardwired mutations, but instead small modulations of protein expression of BCR signaling molecules may be key to disease susceptibility. Our studies identify Btk as a promising therapeutic target in the treatment of SLE-like autoimmune disease and CLL, and while the efficacy of Btk-inhibitors is currently evaluated in B cell malignancies, the studies in this thesis strongly plead for the clinical testing of Btk-inhibitors in the treatment of rheumatic autoimmune diseases.

Nederlandse samenvatting

Ons lichaam wordt doorlopend blootgesteld aan ziekteverwekkende (micro-)organismen, variërend van virussen en bacteriën tot schimmels en parasieten. Zonder een adequaat reagerend afweersysteem zouden we kunnen bezwijken aan infecties van deze ziekteverfwekkers. Om deze uiteenlopende ziekteverwekkers te kunnen bestrijden bestaat ons afweersysteem uit diverse typen witte bloedcellen (leukocyten) die elk specifieke taken uitvoeren. Onder deze leukocyten hebben lymfocyten het vermogen om na infectie geheugen op te bouwen tegen deze ziekteverwekkers om nieuwe infecties met dezelfde ziekteverwekker te voorkomen.

B lymfocyten (B cellen) bieden bescherming tegen ziekteverwekkers door het maken van antistoffen gericht tegen de ziekteverwekker, door het presenteren van moleculen (antigenen) van de ziekteverwekker aan andere witte bloedcellen, en door het produceren van afweerhormonen (cytokines). Om deze taken uit te kunnen voeren is een B cel afhankelijk van specifieke herkenning van antigenen via de B cel receptor (BCR), de cel-gebonden vorm van de antistof die de B cel kan gaan uitscheiden. De BCR wordt in elke B cel stapsgewijs en willekeurig samengesteld uit diverse BCR gensegmenten waardoor rijpe B cellen een onbegrensde hoeveelheid verschillende BCRs tot expressie brengen waarmee alle potentiële ziektewekkers herkend kunnen worden. De onbegrensde variatie in BCRs echter leidt ook tot de aanwezigheid van B cellen die lichaamseigen antigenen kunnen herkennen. Als zulke autoreactieve B cellen niet worden geremd of geëlimineerd leidt dit tot destructieve auto-immuunziektes waarbij het immuunsysteem eigen weefsels aanvalt.

Voor het beteugelen van deze autoreactieve B cellen zijn er gedurende B cel ontwikkeling en B cel activatie diverse autoreactiviteitscheckpoints geïmplementeerd, waarbij signalen van de BCR het uitschakelen van autoreactive B cellen dirigeren. Afwijkingen in BCR signalering kunnen dus het uitschakelen van autoreactieve B cellen verstoren, en vele genetische afwijkingen in BCR signaleringsmoleculen zijn dan ook gekoppeld aan de ontwikkeling van reumatische auto-immuunziekten zoals systemische lupus eryhtematosus (SLE). Bij deze auto-immuunziekte, die tot wel 1 op de 2000 mensen treft en vooral bij vrouwen voorkomt, leidt de verstoorde eliminatie van autoreactieve B cellen tot massale productie van antistoffen tegen antigenen uit de celkern, waarbij circulerende antistoffen in organen als de nieren, huid, gewrichten en longen neerslaan en lokaal voor ontstekingen zorgen. Een andere consequentie van afwijkende BCR signalering kan zijn dat de B cellen door chronische activatie kwaadaardig ontsporen, leidend tot chronische lymfatische leukemie (CLL). Bij deze vorm van leukemie, die vooral optreedt bij ouderen en ieder jaar bij zo'n 20.000 mensen in Europa wordt vastgesteld, treedt er niet zozeer woekering van geactiveerde B cellen op, maar stapelen de B cellen door een defect in apoptose (geprogammeerde celdood).

In dit proefschrift hebben we onderzocht hoe veranderingen in de signaleringssterkte of eiwitniveaus van BCR signaleringsmoleculen, in het bijzonder Bruton's tyrosine kinase (Btk), kunnen bijdragen tot de ontwikkeling van auto-immuunziekten of B cel leukemie. De eerdere bevindingen dat in ontstoken gewrichten van patiënten met reumatoïde arthritis de B cellen expressie vertonen van de surrogaat lichte keten (SLC), een BCR signaleringscomponent dat normaliter alleen in precursor-B cellen aangemaakt wordt, zette ons ertoe aan om in **Hoofdstuk 2** te onderzoeken of de productie van deze SLC in rijpe B cellen actief kan bijdragen aan het ontwikkelen van auto-immuunziekten. We

hebben ontdekt dat de SLC in rijpe B cellen op een dosisafhankelijke manier eliminatie van onrijpe B cellen instrueert en rijpe B cellen minder responsief maakt bij antigene stimulatie. Deze meer stringente repressie van autoreactieve B cellen door SLC signalering verklaart waarschijnlijk waarom muizen met overexpressie van SLC in rijpe B cellen minder zware arthritis kunnen ontwikkelen.

In **Hoofdstuk 3** beschrijven we hoe auto-immuniteit kan ontwikkelen wanneer de signaleringscapaciteit van Btk doorlopend versterkt wordt door in Btk activerende puntmutaties aan te brengen. We hebben gevonden dat hoewel deze versterkte Btk signalering de eliminatie van potentieel autoreactieve onrijpe B cellen versterkt, dat de resterende rijpe B cellen spontaan uitgroeien tot autoreactieve antistofproducerende cellen die desondanks geen volledige auto-immuunziekte veroorzaken, doordat de antistoffen van het relatief onschadelijke type IgM zijn en omdat deze B cel responsen optreden zonder hulp van T lymfocyten en buiten kiemcentra (germinal centers, GCs) waar B cellen met hulp van T cellen hun BCR/antistof verder kunnen specialiseren.

Omdat deze wijze van versterking van Btk signalering dus juist lijkt te interfereren met de vorming van hoog-affiene antistoffen die noodzakelijk zijn voor het ontwikkelen van reumatische auto-immuunziekte, hebben we bekeken of een subtielere wijze van versterkte Btk signalering wel kan leiden tot auto-immuniteit. In **Hoofdstuk 4** hebben we gevonden dat Btk eiwitniveaus niet constant zijn in B cellen en toenemen bij activatie, waardoor we zijn gaan onderzoeken of verhoging van de Btk eiwitspiegels het risico op auto-immuniteit verhoogt door veranderingen in B cel activatie en B cel selectie. We hebben in muizen aangetoond dat lichte (transgene) overexpressie van Btk in B cellen voldoende is om door versterkte BCR signalering B cel activatie, GC vorming en autoantistofproductie te veroorzaken, leidend tot een SLE-achtige auto-immuunziekte. De door Btk versterkte BCR signalering zorgde voor een resistentie tegen apoptose waardoor geactiveerde autoreactieve B cellen signalen voor eliminatie afkomstig van T cellen kunnen negeren.

In **Hoofdstuk 5** hebben we bestudeerd of T cellen ondersteunend of noodzakelijk zijn in dit auto-immuunsyndroom veroorzaakt door hogere Btk eiwitspiegels in Btk-transgene muizen. We hebben in deze muizen ontdekt dat gespecialiseerde GC T cellen (folliculaire T helper cellen; TFHs) toenemen bij de spontane vorming van SLE, bij opgewekte arthritis, en bij immunisaties tegen synthetische modelantigenen. Deze toegenomen vorming van TFHs werd vergezeld door een toegenomen expressie van de activerende receptor ICOS op T cellen, en ICOS kon selectief versterkt tot expressie worden gebracht op T cellen wanneer deze werden gekweekt met BCR-gestimuleerde Btk-transgene B cellen. De auto-immuniteit in Btk-transgene muizen bleek volledig afhankelijk te zijn van T cellen aangezien het uitschakelen van molecuul Cd40l, dat essentieel is voor B cel activatie door T cellen, voldoende was om auto-immuniteit, GC vorming en uitgroei van TFHs te corrigeren.

Hoewel Btk vooral een rol speelt in de signalering van de BCR, is Btk ook betrokken bij de signalering van andere receptoren op B cellen. Om te bepalen of verhoogde Btk eiwitspiegels selectief het signaleren van de BCR versterken, hebben we gekeken welke genen worden gebruikt in cellen met normale of verhoogde Btk expressie, met of zonder stimulatie van de BCR. In **Hoofdstuk 6** hebben we gevonden dat het gengebruik van ongestimuleerde Btk-transgene B cellen een zeer sterke overlap vertoont met BCR-gestimuleerde niet-transgene B cellen, duidend op een selectieve versterking van BCR signalering door transgene Btk overexpressie. Verder vonden we dat versterkte Btk signalering het uitrijpen van B

cellen tot antistofproducerende cellen kon versterken door de genactiviteit te beïnvloeden van Blimp-1 en Bcl6, twee DNA-regulerende factoren die de vorming van antistofproducerende cellen sturen.

Uiteindelijk hebben we onderzocht of veranderde Btk niveaus in menselijke B cellen mogelijk bijdragen aan de ontwikkeling van SLE. In **Hoofdstuk 7** hebben we aangetoond dat de regulatie van Btk spiegels in B cellen van SLE patiënten verstoord is, ten eerste omdat Btk spiegels afwijkend bleken na *in vitro* activatie van B cellen die waren geïsoleerd uit het bloed van SLE patiënten, ten tweede omdat Btk spiegels in de SLE patiënten al hoger bleken te zijn in B cellen die nog niet eerder geactiveerd waren geweest, en ten derde omdat geheugen B cellen in SLE patiënten geen hogere Btk spiegels hadden dan niet-geactiveerde B cellen. Verder vonden we een sterke spreiding in soms verhoogde Btk spiegels in B cellen van SLE patiënten, waardoor we niet konden concluderen dat Btk spiegels structureel verhoogd zijn in SLE patiënten. Wel bleek uit onze analyses dat hogere Btk spiegels correleerden met specifieke klinische afwijkingen zoals de productie van anti-cardiolipine autoantistoffen, die sterk geassocieerd zijn met het aan SLE verwante ziektebeeld anti-fosfolipiden syndroom (AFS).

Naast de rol van Btk signalering in het ontwikkelen van SLE-achtige auto-immuunziekten hebben we ook bekeken of versterkte Btk signalering de vatbaarheid voor CLL kan beïnvloeden omdat verhoogde Btk niveaus waren vastgesteld in kwaadaardige B cellen in CLL patiënten. Door de Btk spiegels te variëren in een spontaan muizenmodel voor CLL hebben we in **Hoofdstuk 8** ontdekt dat in de afwezigheid van Btk CLL niet kan ontstaan, en dat verhoogde niveaus van Btk in B cellen de ontwikkeling van en sterfte door CLL versterkt. Verhoogde expressie van Btk bleek de drempel voor kwaadaardige ontsporing te verlagen van B cellen die een BCR maken die niet typisch geassocieerd is met CLL. In **Hoofdstuk 9** geven we een overzicht welke afwijkingen in BCR signalering bijdragen aan de ontwikkeling van CLL. Daarnaast bespreken we de voordelen van en kanttekeningen bij nieuwe medicijnen die aangrijpen op BCR signaleringsmoleculen.

In **Hoofdstuk 10** plaatsen we de nieuwe bevindingen in dit proefschrift in het kader van nieuwe inzichten in BCR-gedreven auto-immuniteit, en geven we nieuwe richtingen aan voor vervolgonderzoek en bespreken we klinische implicaties van ons onderzoek. Onze studies hebben de bijdrage van Btk signalering aan de ontwikkeling van reumatische auto-immuunziekten en chronische lymfatische leukemie gedemonstreerd. Deze bevindingen bevestigen niet alleen de bijdrage van afwijkingen in BCR signalering aan het ontstaan van SLE en CLL, maar toont ook aan dat in plaats van DNA mutaties juist kleine veranderingen in de eiwitniveaus van BCR signaleringsmoleculen al doorslaggevend kunnen zijn voor de ontvankelijkheid van deze ziekten. Onze studies hebben Btk geïdentificeerd als veelbelovend moleculair aangrijpingspunt voor de behandeling van SLE-achtige auto-immuunziekten en CLL. Terwijl nu in de kliniek de efficiëntie van Btk-inhibitors als Ibrutinib in de behandeling van B cell leukemieën en lymfomen wordt aangetoond, pleiten de studies in dit proefschrift voor het klinisch testen van Btk-inhibitors in de behandeling van reumatische auto-immuunziekten.

List of abbreviations

ACA	anti-cardiolipin autoantibody
Akt	v-akt murine thymoma viral oncogene homolog
ANA	anti-nuclear autoantibody
APS	anti-phospholipid syndrome
BCR	B cell receptor
BM	bone marrow
Bcl6	B cell lymphoma 6
Blimp-1	B lymphocyte-induced maturation protein 1
Btk	Bruton's tyrosine kinase
C4	complement component 4
CIA	collagen-induced arthritis
CDR	complementarity determining region
CLL	chronic lymphocytic leukemia
CpG	cytosine / guanine triphosphate deoxynucleotides phosphodiester
c-rel	reticuloendotheliosis oncogene
CSR	class switch recombination
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
FcγRIIB	Fc receptor, IgG, low affinity IIb
GC	germinal center
HAI	hemagglutinin inhibition
ICOS	inducible co-stimulator
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
Igκ	immunoglobulin kappa light chain
Igλ	immunoglobulin lambda light chain
IgL	immunoglobulin light chain
IP3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
λ5	immunoglobulin lambda chain 5
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CLL	mutated CLL
MFI	mean fluorescence intensity
MHCII	major histocompatibility complex II
MZ	marginal zone
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
PI3K	phosphatidylinositol-3 kinase
PIP	phosphatidylinositol phosphate
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 1,4,5-triphosphate
PKC	protein kinase C

PLCγ2	phospholipase Cγ2
PNA	peanut agglutinin
Poly I:C	polyinosinic:polycytidylic acid
pre-BCR	precursor B cell receptor
RA	rheumatoid arthritis
SHM	somatic hypermutation
SLC	surrogate light chain
SLE	systemic lupus erythematosus
SLP65	SH2-domain leukocyte protein of 65 kD
SS	Sjögren's syndrome
Syk	spleen tyrosine kinase
TCR	T cell receptor
TI-II	T-independent type II
TD	T-dependent
TLR	Toll-like receptor
U-CLL	unmutated CLL
VpreB	pre-B lymphocyte gene
Xid	x-linked immunodeficiency

Dankwoord

Promoveren is een hele klus, en zonder hulp en steun van mensen om mij heen had dit boekje er een stuk minder mooi uitgezien. Niet alleen was het boekje anders minder compleet geworden, maar waren de afgelopen jaren ook veel minder leuk geweest, en had ik mijzelf niet zo kunnen verliezen in het doen van immunologisch onderzoek. Iedereen bedankt voor alle gezelligheid, adviezen, inspiratie en schouderklopjes!

Beste Rudi, enorm bedankt voor de mooie en intensieve samenwerking, je bezielde begeleiding, je flexibiliteit en je goede leiderschap, waarbij je altijd de balans weet te bewaren tussen wetenschappelijke vrijheid en persoonlijke aansturing. Je bent een enorm voorbeeld voor me en ik heb heel veel van je geleerd.

Prof.dr. Henk Hoogsteden, het was een voorrecht om nogal fundamenteel onderzoek te mogen verrichten naar onderwerpen als auto-immuniteit en leukemie, die niet of maar heel zijdelings met longziekten te maken hebben. Bedankt voor het steunen en faciliteren van zulk ongewoon onderzoek, en bedankt voor de belangstelling voor mijn onderzoek.

De leden van de kleine commissie wil ik niet alleen bedanken voor het beoordelen van het manuscript, maar ook voor andere waardevolle bijdragen aan dit proefschrift. Prof.dr. Bart Lambrecht, bedankt voor alle interessante wetenschappelijke discussies en scherpe adviezen tijdens de gezellige GREAT meetings. Prof.dr. Frans Kroese, zonder uw hulp was de studie naar BTK in SLE patiënten nooit van de grond gekomen. Prof.dr. Erik Lubberts, heel erg bedankt voor alle bijdragen aan de vele CIA-experimenten en input voor vervolgexperimenten.

Van buiten het Erasmus MC heb ik de nodige hulp gekregen om experimenten op te zetten, te voltooien of zelfs uit te besteden. Prof.dr. Friedrich Thaiss, many thanks for the proteinuria analyses that completed the Blood paper. Prof.dr. Dirk Elewaut en dr. Jens van Praet, hartelijk dank voor de semi-kwantitatieve autoantistofscreenings in de Btk-transgene muizen. Dr. Roelof Flierman, bedankt voor de eerste antinucleosoom autoantistofanalyses en proteinuria testen waardoor we op het spoor van SLE-achtige autoimmuniteit kwamen in de Btk-transgene muizen. Dr. Marcel Spaargaren en Annemieke Kuil, heel erg bedankt voor de mooie B cell migratie-assays. Dr. Melissa Dullaers, dank je wel voor het meedenken met het opzetten van humane B cell kleuringen voor BTK-analyses. Prof.dr. Hendrika Bootsma, bedankt voor uw hulp bij de humane studie naar BTK in SLE patiënten.

Ook binnen het Erasmus hebben een heleboel mensen hun steen(tje) bijgedragen aan mijn promotieonderzoek. Heel veel hulp (en gezelligheid!) heb ik gekregen van de mensen bij de Reumatologie. Odilia, mede-MSc student, mede-AIO, en mede-promovendus, wat fijn dat je de afgelopen jaren een klankbord bent geweest, het was heerlijk om dan weer eens te brainstormen over B en T cellen, dan weer eens samen te experimenteren, en soms ook eens frustratie te ventileren over alle hobbels die we tijdens het promoveren tegen zijn gekomen. Patrick, je bent een rots in de branding,

dank je voor al je harde werk voor die talloze, soms te strak geplande CIA-proeven. Jan-Piet, bedankt voor alle gezelligheid en input, soms van wetenschappelijke aard en soms van heel andere aard. En al die andere (oud-)collega's van de Reumatologie die incidenteel of bijna structureel hebben geholpen met kolossale proeven of met bijvoorbeeld die zware bieravonden bij Boudewijn: bedankt Anne-Marie, Ferry, Franka, Kim, Maarten, Nadine, Sandra, Sonja, en Wendy!

Zonder de hulp van de afdeling Immunologie en in het bijzonder onze EMCLL-groep had ik de publicaties over CLL niet kunnen voltooien. Alice, BCR-signaleringsmaatje, heel erg bedankt voor alle wetenschappelijke discussies en alle gezelligheid! Dr. Ton Langerak, bedankt voor de ideeën voor experimenten en voor het meelezen en –schrijven aan de CLL-manuscripten. Het Center for Biomics en in het bijzonder dr. Wilfred van IJcken wil ik bedanken voor het vakkundige mRNA sequencen. De hulp van dierverzorgers (Yara, Miriam, Calinda, Hans, Miranda, Sandra, Patrick, Henk, Vincent, en alle andere verzorgers) en proefdierdeskundigen van het EDC is van enorme waarde geweest bij alle experimenten: bedankt voor alle alerte, goede zorgen voor de muisjes en voor alle behulpzaamheid.

Verder wil ik dr. Stefan, medestudent bij Geneeskunde en de MSc, bedanken voor de interessante Zeeuws-Brabantse collaboratie op het gebied van X-chromosoom inactivatie.

Binnen het Erasmus wil ik uiteraard ook de mensen van de MSc Molecular Medicine opleiding bedanken die met het opzetten van deze mooie opleiding het mogelijk maken voor geneeskundestudenten als ik toch moleculair onderzoek te gaan doen. Prof. dr. Anton Grootegoed, Benno Arentsen, prof. dr. Elaine Dzierzak, prof.dr. Claire Wyman, dr. Dik van Gent, dr. Ruud Delwel, en alle andere betrokken docenten, hartelijk dank, ook voor de goede 'nazorg' van promovendi afkomstif uit het MSc Molecular Medicine programma! Verder wil ik ook Frank van Vliet, Joris Lenstra en de andere leden van de AIO-commissie bedanken voor de mooie tijd bij de postgraduate Molecular Medicine school, met wie ik een paar mooie edities van de Molecular Medicine Day heb mogen helpen organiseren.

Met de verdediging van mijn proefschrift ben ik heel blij dat ik word bijgestaan door twee heel bijzondere mensen die me de afgelopen jaren op heel verschillende manier hebben gesteund.

Marjolein, wat heb jij een enorm aandeel in dit boekje met je harde werk, je flexibiliteit, de hulp bij het begeleiden van vele studenten, en je kritische meedenken bij proeven en manuscripten. Met bewondering heb ik gezien hoe je je als B celdeskundige ontpopt, en het schrijven van dat Tec-manuscript gaat dan ook helemaal goedkomen. Heel erg bedankt voor alles, ook voor alle gezellige onderonsjes en humor, en heel veel succes met je ontwikkeling en werk op de afdeling!

Vincent, je bent meer voor me dan een broer. Wat was het heerlijk om tussen alle drukte door samen concerten te bezoeken, uit eten te gaan, slap en onzinnig te ouwehoeren, biertjes en wijnen te proeven, tot tranen toe te lachen, op stedentrips te gaan, de bossen in te trekken, en vooral heel veel muziek te maken. Heel erg bedankt voor je luisterend oor en je heerlijke relativeringsvermogen!

Mijn eerste stappen in het moleculaire immunologische onderzoek heb ik gezet onder de vakkundige begeleiding van een aantal mensen van 'Lab 853'. Rogier, bedankt voor je geduldige en enthousiasmerende introductie in de wereld van Btk en B cellen. Gemma, bedankt voor het bijbrengen van de fijne kneepjes van het pipetteren. Pieter Fokko, ik bewonder je scherpzinnige geest, dankzij jouw

persoonlijke en toegewijde begeleiding heb ik me het wetenschappelijk denken echt eigen kunnen maken, iets waar ik ook na mijn promotie nog heel veel plezier aan ga beleven. Bedankt!

In de afgelopen 5 jaar heb ik fijn samengewerkt en veel plezier gehad met een heleboel collega's van de afdelingen Longziekten en daarvoor Immunologie. Heel erg bedankt Alex (behulpzame nestor van het lab), Annemieke (heel gezellig om met jou samen door de MSc en geneeskunde te rollen), Anouk, Bart, Bernt, Bobby, Bregje, Caroline, Claudia (thanks for the 'gezelligheid'), Corine, Cynthia, Debbie, Femke, Heleen, Hoyan, Ingrid (PCR-lotgenootje in de kweek; samen PCRen was nog best wel leuk zo!), Ismé, Jacqueline, Joachim, João (fellow metalhead), Joey, Joost (je bent altijd erg lief geweest voor de studenten die ik begeleid heb), Joris (onze Paul McCartney – of toch John Lennon? – bij afdelingsfeesten), Karolina, Koen (en dan is alles wat je hoort, het lied van …), Leonie, Lisanne, Maarten (hoop in bange dagen bij monsterexperimenten), Margaretha (wat fijn om af en toe met zo'n muziekadept te kunnen kletsen, ook als het niet over muziek gaat), Marlies (bedankt voor alle gezellige koffiepauzes en af en toe een concertje), Marthe, Herr Menno (niet alleen bedankt voor alle hulp bij proeven, maar vooral ook voor je absurdistische humor, filosofische uiteenzettingen, en artistieke uitspattingen), Mirjam (bedankt voor alle gezelligheid en voor je scherpe ideeën over auto-immuniteit), Nanda, Niken, Peter, Peter Ng, Petra, Robin, Roel (gezellig om samen met Annemieke zo'n vergelijkbaar traject af te leggen), Sanne, Saravanan (thanks for the nice collaboration on CLL, and I'm glad that you are still continuing some Btk research), Sinem, Tamara, Tanja, Timothy, Van (wat was het altijd gezellig om met jou als B celmaatje in de late uurtjes op het lab te kunnen kletsen, soms over wetenschap, soms over andere dingen), Wilma (mijn gezellige eerste kamergenootje op het lab) and Yelvi.

Andere hele speciale labgenootjes die ik wil bedanken zijn de vier studenten die ik heb mogen begeleiden; ik hoop dat jullie net zoveel van mij hebben geleerd als ik van jullie. Nteleah, it was wonderful to see your scientific eagerness and to see how you enthusiastically dived into the difficult subject of CLL. Jennifer, ik heb ervan genoten hoe snel je in je stage groeide van leergierige student naar een slimme, zelfstandige, productieve en leuke collega, en wat is het leuk dat je nog steeds op het lab bent! Bedankt voor je waardevolle hulp met de CLL-experimenten! Arndt, thanks for your great help in finishing the required experiments for the SLC manuscript, and of course for the German treats you prepared for me and other colleagues. You were an extremely bright colleague, your future as a scientist must be bright as well, good luck! Ilke, with all this traveling you haven't chosen the easiest road to become a scientist, but your hard-working and friendly attitude will get you wherever you want to be; good luck in Swiss!

Buiten het lab heb ik de afgelopen jaren heel veel steun gehad aan een paar hele bijzondere vrienden. Pim en Wout, zonder al die zaterdagen dat ik me met jullie in repetitiehokken kon verliezen in (muzikale) waanzin, zonder knallende optredens met de band, zonder concerten meepikken en zonder af en toe een avondje lomp bier drinken was het promoveren een veel taaiere klus geweest. Bedankt voor jullie luisterend oor en ontnuchterende humor. Ok bye.

Pa en ma, jullie bijdrage aan dit boekje is enorm, en bijna niet meer te vangen in woorden. Altijd hebben jullie me gesteund in wat ik doe en in de keuzes die ik heb gemaakt, steeds hebben jullie (met succes) geprobeerd om te begrijpen wat ik nu precies allemaal onderzoek aan Btk, altijd leefden jullie mee met succesjes en tegenslagen, als ik het nodig had luisterden jullie, en als het nodig was dachten jullie met me mee. Bedankt voor alle goede zorgen op alle mogelijke manieren, zonder jullie was ik nu niet waar ik nu ben.

Laura, mijn liefste Lau, wat een immens geluk dat jij bij me bent. Ik was al een eindje op weg met promoveren toen jij in mijn leven stapte, maar jouw steun en hulp in de eindsprint van het promoveren zijn van onschatbare waarde geweest. Alles stelde je in dienst van mijn promotie, je hebt jezelf teveel weggecijferd de afgelopen maanden. Excuses voor al die avonden, nachten en weekenden dat ik me met een laptop afzonderde, ik hoop dat ik dat goed kan gaan maken en dat ik je nu kan gaan geven wat je verdient. Je bent alles voor me. Ik hou van je.

Laurens

About the author

The author of this thesis was born in Bergen op Zoom, the Netherlands, on 29 August 1984. At R.K. Gymnasium Juvenaat H. Hart in Bergen op Zoom he attended secondary school (Gymnasium) and graduated cum laude in 2003. In that year he started his study in Medicine at the Erasmus University Rotterdam. Starting in 2004, the author combined his Medicine study with the MSc Molecular Medicine program. In 2007, after obtaining his doctoral degree in Medicine, he started his MSc research project "Studies on the role of Bruton's tyrosine kinase in terminal B lymphocyte differentiation" at the departments of Immunology and Pulmonary Medicine at the Erasmus MC under supervision of prof.dr. R.W. Hendriks and dr. P.F. van Loo. After obtaining his MSc degree in 2008, the continuation of his research on B cell receptor signaling in autoimmune disease and leukemia at the department of Pulmonary Medicine under supervision of prof.dr. R.W. Hendriks has resulted in this thesis.

List of publications

van Loo PF, **Kil LP**, Hendriks RW. Role of surrogate light chain expressing B cells in the formation of self-reactive antibodies. Biochemistry and Histocytochemistry Research Development (book chapter). 2010. ISBN: 978-1-61668-932-2

Kersseboom R, **Kil L**, Flierman R, van der Zee M, Dingjan GM, Middendorp S, Maas A, Hendriks RW. Constitutive activation of Bruton's tyrosine kinase induces the formation of autoreactive IgM plasma cells. Eur J Immunol. 2010 Sep;40(9):2643-54.

Kil LP, de Bruijn MJ, van Nimwegen M, Corneth OB, van Hamburg JP, Dingjan GM, Thaiss F, Rimmelzwaan GF, Elewaut D, Delsing D, van Loo PF, Hendriks RW. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. Blood. 2012 Apr 19;119(16):3744-56.

Kil LP, Yuvaraj S, Langerak AW, Hendriks RW. The role of B cell receptor stimulation in CLL pathogenesis. Curr Pharm Des. 2012;18(23):3335-55.

Kil LP, de Bruijn MJ, van Hulst JA, Langerak AW, Yuvaraj S, Hendriks RW. Bruton's tyrosine kinase mediated signaling enhances leukemogenesis in a mouse model for chronic lymphocytic leukemia. Am J Blood Res. 2013;3(1):71-83.

Kil LP, Hendriks RW. Aberrant B cell selection and activation in systemic lupus erythematosus. Int Rev Immunol. 2013;32(4):445-70.

PhD portfolio: summary of PhD training and teaching

L.P. (Laurens-Paul) Kil
Pulmonary Medicine

Research school: Postgraduate Molecular Medicine
PhD period: October 2008 – October 2013
Promotor: Prof. dr. R.W. Hendriks

General courses

2007 Laboratory animal science (art. 9)

In-depth courses

2008 Workshop on bioinformatic analysis, tools and services (BATS)
2008 Course Biomedical Research Techniques
2008 Course Molecular Immunology
2007 Workshop Browsing genes and genomes with Ensembl

(Inter)national conferences

2013 Keystone symposia, Advances in the Knowledge and Treatment of Autoimmunity, Whistler (CAN) – 4-9 April
2013 11th B cell forum, Schluchsee (GER) – 25-27 February
2012 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 19-20 December
2012 Regulators of the Humoral Immune Response, Erlangen (GER) – 5-7 October
2012 Najaarsdagen Reumatologie, annual meeting of the Dutch Society for Rheumatology, Arnhem (NL) – 27-28 September
2011 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 14-15 December
2011 2nd International Conference on Immune Tolerance, Amsterdam (NL) – 23-25 October
2011 9th B cell forum, Bad Sooden (GER) –1-3 April
2010 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 18-19 December
2010 8th B cell forum, Dresden (GER) – 26-28 April
2010 Keystone Symposia "Tolerance and Autoimmunity", Taos (USA) – 21-26 February
2009 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 17-18 December
2009 European Congress of Immunology (ECI), Berlin (GER) – 13-16 September
2008 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 17-18 December

2007 Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI), Noordwijkerhout (NL) – 20-21 December

Seminars and workshops

2013 4th Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting, 24 April
2013 17th Molecular Medicine Day, 13 February
2012 16th Molecular Medicine Day, 29 February
2011 Mucosal Immunology IV, 26-27 May
2011 15th Molecular Medicine Day, 3 February
2011 2nd Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting, 13 January
2010 1st Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting, 21 April
2010 14th Molecular Medicine Day, 4 March
2008 Mucosal Immunology III, 2-3 October
2008 12th Molecular Medicine Day, 6 February
2007 11th Molecular Medicine Day, 2 February

Presentations

2013 Enhanced Btk signaling drives autoimmunity through corrupting T cell homeostasis. "4th Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting", 24 April
2012 Btk inhibition in rheumatic diseases: a new therapeutic entity. "Najaarsdagen Reumatologie", annual meeting of the Dutch Society for Rheumatology, Conference center Papendal, Arnhem (NL) – 27 September
2011 Expression levels of Btk determine the susceptibility for chronic lymphocytic leukemia. "Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI)", Noordwijkerhout (NL) – 15 December
2011 Btk levels set the threshold for activation and negative selection of autoreactive B cells. "2nd International Conference on Immune Tolerance", Amsterdam (NL) – 24 October
2011 Enhanced B cell receptor (BCR) signaling through Btk impairs negative selection of autoreactive B cells. "9th B cell forum", Bad Sooden (GER) –2 April
2011 Enhanced B cell receptor (BCR) signaling through Btk impairs negative selection of autoreactive B cells. "2nd Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting", 13 January
2010 Overexpression of Bruton's tyrosine kinase (Btk) in B lymphocytes drives autoimmunity. "1st Gent-Rotterdam Exchange on Airway Topics (GREAT) meeting", 21 April
2010 Overexpression of Bruton's tyrosine kinase (Btk) in B lymphocytes drives autoimmunity. "14th Molecular Medicine Day", Erasmus MC Postgraduate School Molecular Medicine, 4 March
2009 Overexpression of Bruton's tyrosine kinase (Btk) in B lymphocytes drives autoimmunity. "European Congress of Immunology (ECI)", Berlin (GER) – 15 September
2008 Overexpression of Bruton's tyrosine kinase (Btk) drives spontaneous germinal center formation. "Annual Meeting / Winter School of the Dutch Society for Immunology (NVVI)",

Noordwijkerhout (NL) – 18 December

2008 The Btk Y223F mutant induces spontaneous germinal center formation and auto-immunity. "12th Molecular Medicine Day", Erasmus MC Postgraduate School Molecular Medicine, 6 February

Other

2010 Oral presentation award, "14th Molecular Medicine Day", Erasmus MC Postgraduate School Molecular Medicine, 4 March

2013 Publication award (2nd prize), "17th Molecular Medicine Day", Erasmus MC Postgraduate School Molecular Medicine, 13 February

2010-2013 Member of the PhD committee Postgraduate School Molecular Medicine. Involved in the organization of the 15th (2011), 16th (2012) and 17th (2013) Molecular Medicine Days.

Teaching

2011-2013 Lectures for the "Proefstuderen" program for Dutch secondary school (VWO) students

2012-2013 Supervising the MSc thesis project of MSc Infection and Immunity student Ilke Ilgaz

2012 Supervising a 3-month summer project of MSc Biology student Arndt Krause

2011-2012 Supervising the BSc thesis project of BSc Biology and Medical Laboratory Research student Jennifer van Hulst

2010-2011 Supervising the MSc thesis project of MSc Molecular Medicine student Nteleah Valeri