ESSENTIAL KINASES AND TRANSCRIPTIONAL REGULATORS AND THEIR ROLES IN AUTOIMMUNITY

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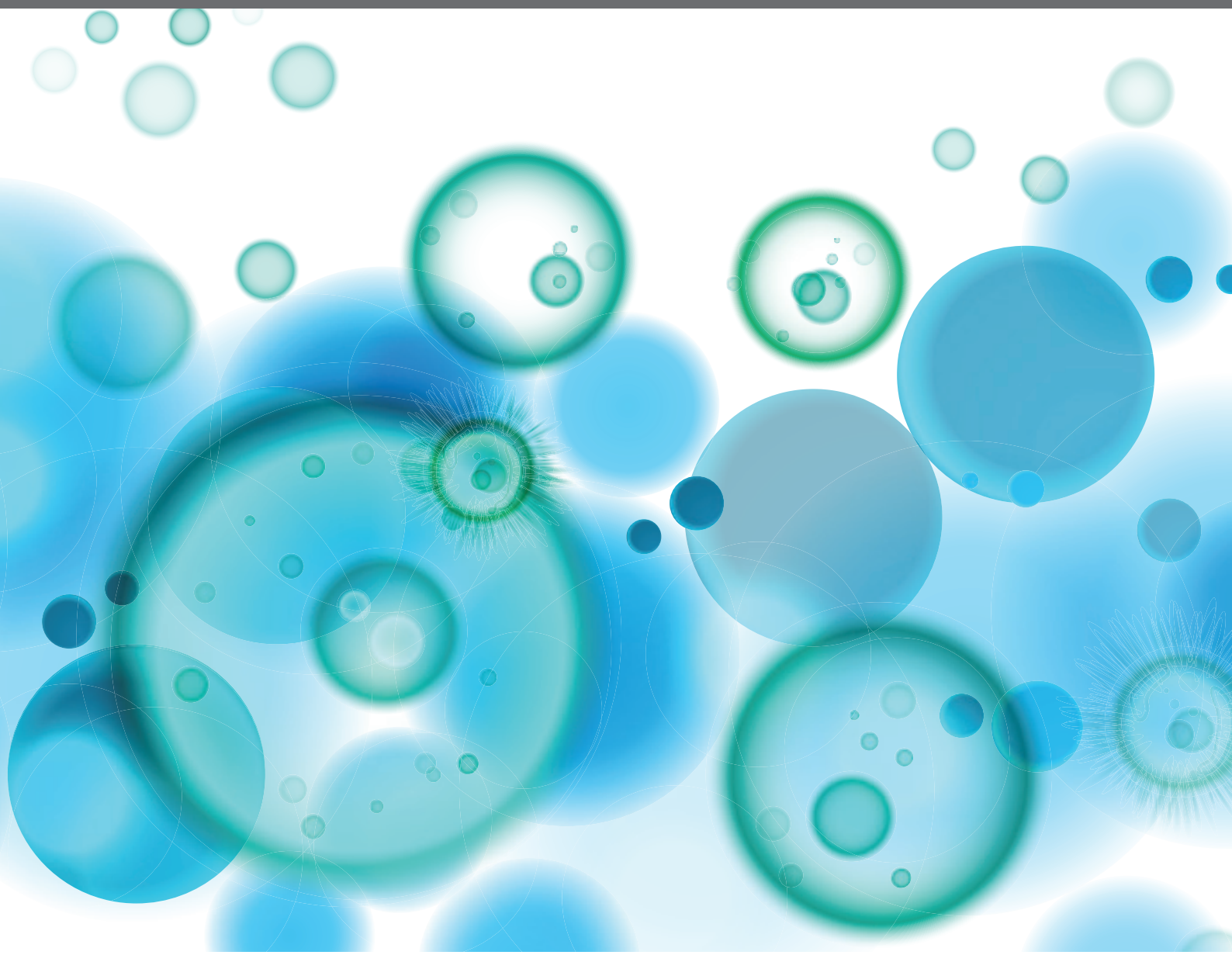

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ESSENTIAL KINASES AND TRANSCRIPTIONAL REGULATORS AND THEIR ROLES IN AUTOIMMUNITY

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Table of Contents

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Bruton's Tyrosine Kinase, a Component of B Cell Signaling Pathways, Has Multiple Roles in the Pathogenesis of Lupus

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the loss of adaptive immune tolerance to nucleic acid-containing antigens. The resulting autoantibodies form immune complexes that promote inflammation and tissue damage. Defining the signals that drive pathogenic autoantibody production is an important step in the development of more targeted therapeutic approaches for lupus, which is currently treated primarily with non-specific immunosuppression. Here, we review the contribution of Bruton's tyrosine kinase (Btk), a component of B and myeloid cell signaling pathways, to disease in murine lupus models. Both gain- and loss-of-function genetic studies have revealed that Btk plays multiple roles in the production of autoantibodies. These include promoting the activation, plasma cell differentiation, and class switching of autoreactive B cells. Small molecule inhibitors of Btk are effective at reducing autoantibody levels, B cell activation, and kidney damage in several lupus models. These studies suggest that Btk may promote end-organ damage both by facilitating the production of autoantibodies and by mediating the inflammatory response of myeloid cells to these immune complexes. While Btk has not been associated with SLE in GWAS studies, SLE B cells display signaling defects in components both upstream and downstream of Btk consistent with enhanced activation of Btk signaling pathways. Taken together, these observations indicate that limiting Btk activity is critical for maintaining B cell tolerance and preventing the development of autoimmune disease. Btk inhibitors, generally well-tolerated and approved to treat B cell malignancy, may thus be a useful therapeutic approach for SLE.

Keywords: Bruton's tyrosine kinase, lupus, autoantibody, B cell, plasma cell, Lyn

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INTRODUCTION

The development of a B cell repertoire capable of secreting antibodies against a wide range of foreign antigens is crucial for effective immune responses. However, the processes that generate this diversity also result in the production of self-reactive B cells that, if not kept in check, can be pathogenic and lead to autoimmune disease. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibodies against nuclear antigens. These autoantibodies promote disease

pathogenesis by forming immune complexes that deposit in and damage tissues and synergize with innate immune defects to sustain pro-inflammatory feed-forward loops (1). Autoantibodies arise prior to the development of overt clinical symptoms (2), suggesting that loss of B cell tolerance is an important initiating event in lupus. Understanding the signaling pathways that mediate autoantibody production may reveal new therapeutic targets for SLE, currently treated primarily by non-specific immunosuppression. Here, we review the contribution of Bruton's tyrosine kinase (Btk) to lupus, with a focus on its role in B cells.

Btk IN B CELL DEVELOPMENT AND ACTIVATION

Bruton's tyrosine kinase is a Tec family tyrosine kinase expressed in B and myeloid cells. It was first identified as the genetic defect in the primary immunodeficiency X-linked agammaglobulinemia (XLA) (3, 4). XLA patients have a block in B cell development at the pre-B stage and a paucity of circulating B cells and immunoglobulin (5). A mutation in the pleckstrin homology (PH) domain of Btk was subsequently found in X-linked immunodeficient (xid) mice (6, 7), which also have a B cell immunodeficiency, although milder than that of XLA patients. Btk-deficient mice phenocopy xid mice, with a block in B cell development at the immature stage, reduced peritoneal B1a cells, and impaired response to T-independent type II antigens (8–10).

Bruton's tyrosine kinase is an important proximal component of B cell receptor (BCR) signaling pathways. Upon BCR engagement, the Btk PH domain binds to PIP3, a signaling intermediate generated by PI3 kinase (PI3K), thus localizing Btk to the plasma membrane (11–14). This facilitates its phosphorylation and activation by Src kinases (12, 13, 15) and promotes access to its substrates. The most well described of these is PLCγ2, which is activated after phosphorylation by Btk (16, 17) leading to increased Ca^{++} flux (14, 16, 18) and activation of NF-κB (19–21). BCR-induced proliferation and survival are impaired in the absence of Btk (9, 22–24). Btk is also required for toll-like receptor (TLR)-induced IL-10 expression by B cells (25, 26), and for synergy between the BCR and TLRs in enhancing IL-6 expression (27). Integrin-mediated adhesion of B lineage cells (28) and their response to chemokines, such as SDF-1 (29, 30), are also controlled by Btk.

Btk AND MOUSE LUPUS MODELS

Btk Is Required for Autoantibody Production and Pathogenesis in Many Lupus Models

The xid mutation has long been known to reduce autoantibody levels in several murine lupus models, including NZB × NZW (31), BXSB (32), MRL.lpr (33), motheaten (34), and Gld (35). Renal disease was also prevented and survival improved by the xid mutation in NZB × NZW (31), BXSB (32), and MRL.lpr (33) mice.

The subsequent finding that Btk is a B cell signaling molecule suggested that enhanced B cell activation through Btk underlies autoantibody production in lupus models. This was tested using mice lacking B cell inhibitory signaling molecules. BCR signaling is normally limited by inhibitory receptors such as FcγRIIb, CD22, SiglecG, PIR-B, and CD72. The ITIMs of these receptors are phosphorylated by the tyrosine kinase Lyn, which results in the recruitment and activation of inhibitory phosphatases, such as SHIP and SHP-1 [reviewed in Ref. (36–38)]. B cell-specific deletion of Lyn, SHIP, or SHP-1 leads to B cell hyper-responsiveness and lupus-like autoimmune disease in mice (39–41). Mutations in inhibitory receptors result in milder autoimmunity, likely due to some degree of redundancy among them (42–47). Several of these inhibitory pathways target activating signals mediated by Btk (14, 16, 48).

Either the xid mutation (49) or Btk-deficiency (50–52) ameliorates the autoimmune phenotype of $Lyn^{-/-}$ mice. Similarly, Btk is required for autoantibodies in $FcγRIIb^{-/-}$.Yaa mice, which lack the inhibitory receptor FcγRIIb and also have enhanced TLR7 signaling (53). One caveat to these studies is that the reduction in mature B cells in xid and $Btk^{-/-}$ mice is exacerbated in the absence of Lyn. To circumvent this defect, a transgene expressing a low level of Btk in B cells (Btk^{lo}) (22) was crossed to $Lyn^{-/-}Btk^{-/-}$ mice (50–52). This normalized mature follicular B cell numbers to that of $Lyn^{-/-}$ mice. However, $Lyn^{-/-}Btk^{lo}$ mice failed to produce autoantibodies or develop kidney damage, indicating that Btk signaling in mature B cells, rather than simply effects of Btk on B cell development, is critical for autoimmunity.

The autoimmunity caused by loss of Lyn-dependent inhibitory signaling is likely mediated by heightened Btk responses, as it is mitigated by reducing Btk dosage. This is supported by gain-of-function studies in which either a constitutively active form of Btk [which carries a PH domain mutation that enhances Btk membrane localization (54)] or wild-type Btk were overexpressed in the B lineage (55–57). In both cases, autoimmunity ensued. Limiting Btk signal strength in B cells is thus critical to prevent the loss of B cell tolerance.

Multiple Functions of Btk Contribute to Autoantibody Production

Autoreactive B Cells Are Present in the Periphery in the Absence of Btk

Developing B cells are subjected to a central tolerance checkpoint at the immature B stage in the bone marrow. Those cells that express autoreactive receptors undergo receptor editing, rearranging a new Ig light chain to change their specificity. Cells that remain self-reactive after editing are deleted by apoptosis or rendered anergic. Autoreactive cells that escape are kept in check by peripheral tolerance mechanisms.

Taken together, the following observations suggest that Btk acts primarily in the periphery, rather than the bone marrow, to drive a loss of B cell tolerance. In $Btk^{-/-}$ mice carrying an anti-DNA Ig transgene, anti-DNA B cells are present in the periphery but do not produce antibodies *in vivo* (26). Single cell repertoire analysis of new emigrant B cells (recently arrived in the periphery from the bone marrow) from XLA patients revealed a higher frequency of autoreactive B cells than in healthy controls (58). This indicates that Btk signaling may actually promote central

tolerance, and that Btk-deficiency does not abrogate autoimmunity simply by preventing autoreactive B cells from reaching the periphery. Furthermore, immunoglobulin transgenic mouse models and analysis of XLA patient B cell repertoires have shown that receptor editing is independent of Btk (58–60). A role for Btk in the loss of peripheral B cell tolerance is highlighted by both loss-of-function and overexpression studies. Btk is required for autoimmunity in Lyn$^{-/-}$ mice (49–52), which have intact central tolerance but develop autoantibodies due to a breach of peripheral tolerance (61, 62). Mice overexpressing Btk in mature B cells and myeloid cells, but not at earlier stages of B cell development in the bone marrow, develop autoimmunity (56).

Btk Contributes to Autoantibody Production beyond Its Role in Initial B Cell Activation

How does Btk signaling in the periphery drive autoantibody production? The role of Btk in the initial activation of BCR signals likely contributes, as residual B cells in Lyn$^{-/-}$ xid and Lyn$^{-/-}$Btk$^{-/-}$ mice proliferate poorly in response to anti-IgM (49, 50). However, Lyn$^{-/-}$Btk^{lo} B cells, like Lyn$^{-/-}$ B cells, have increased proliferative response to BCR engagement (50, 52), suggesting that in the absence of Lyn-mediated inhibitory signaling, low levels of Btk are able to transmit some aspects of BCR signals efficiently. However, Lyn$^{-/-}$Btk^{lo} mice do not develop autoantibodies or autoimmune disease (51, 52). Similarly, although Btk-deficient anti-DNA transgenic mice do not produce autoantibodies (26), Btk is not required for B cells from these mice or from AM14 rheumatoid factor (RF) transgenic mice to proliferate in response to nucleic acid-containing antigens (26, 63). Such autoantigens, common in lupus, activate B cells *via* both the BCR and nucleic acid-sensing TLRs (1). Thus, Btk has additional functions beyond transmitting proliferative signals from the BCR and TLRs that promote the loss of B cell tolerance.

Btk Drives Plasma Cell (PC) Accumulation

Accumulation of antibody-secreting PCs in the periphery is characteristic of SLE patients (64, 65) and murine lupus models, including Lyn$^{-/-}$ mice (51, 66–74). A subset of inactive SLE patients demonstrate a PC-focused gene expression profile in their B cells, indicating that some patients may have an intrinsic predisposition to inappropriate B cell terminal differentiation (75). Btk is required for PC accumulation, as the increased PC frequency observed in Lyn$^{-/-}$ mice is normalized in Lyn$^{-/-}$Btk^{lo} mice (51). This is likely due to enhanced Btk signaling in B cells, since B cell-specific overexpression of either constitutively active or wild-type Btk also results in elevated splenic PCs (55, 56).

Activating signals by Btk and inhibitory signals by Lyn converge on the transcription factor Ets1 (76). Ets1 is expressed in resting B cells and limits PC differentiation by inhibiting the activity of Blimp1 (77), a master PC transcription factor. Ets1$^{-/-}$ mice accumulate PCs and develop lupus-like autoimmunity, similar to Lyn$^{-/-}$ mice (71). Ets1 levels are significantly reduced in B cells from mice deficient in Lyn or the inhibitory signaling components SHP-1 or CD22 plus SiglecG, but are normalized in Lyn$^{-/-}$Btk^{lo} B cells (76). Restoration of Ets1 expression to Lyn$^{-/-}$ or SHP-1$^{-/-}$ B cells prevents excessive B cell differentiation *in vitro* (76). These observations indicate that autoreactive PCs accumulate in Lyn$^{-/-}$ mice at least in part because of excessive downregulation of Ets1 by Btk. This is likely an exacerbation of a normal process, as BCR signaling downregulates Ets1 in wild-type B cells in a Btk-dependent manner (76). TLR signaling also downregulates Ets1 in wild-type B cells, and synergizes with BCR signaling to do so (76). In contrast, failure to downregulate Ets1 in response to Btk signals results in decreased steady state PC levels, as demonstrated by the ability of Ets1-deficiency to rescue the reduction in IgM antibody-secreting cells that occurs in Btk$^{-/-}$ mice (78). Thus, a continuum of Btk signaling to Ets1 controls PC frequencies, and can result in autoimmunity, normal responses, or immunodeficiency depending on the signal strength (78).

Btk Promotes Class Switching of Autoreactive B Cells

Class switching to IgG is required for autoantibodies to be pathogenic (79). In Btk^{lo} mice carrying the 56R anti-DNA immunoglobulin transgene, anti-DNA IgM, but not IgG, is produced (26). Thus, Btk also promotes class switching of autoreactive B cells separate from its role in their initial activation and terminal differentiation.

Several functions of Btk contribute to this process. Expression of the class switching factors AID and T-bet is reduced in TLR-stimulated Btk$^{-/-}$ and Btk^{lo} B cells relative to wild-type cells (26). Btk likely also plays an indirect role in class switching. IL-6 is required for IgG autoantibodies and autoimmune disease in Lyn$^{-/-}$ mice (51, 70, 80). Lyn$^{-/-}$Btk^{lo} mice have decreased serum IL-6 levels and a reduced frequency of myeloid cells expressing IL-6 in response to LPS compared to Lyn$^{-/-}$ mice (51). B cell-derived IL-6 is also increased in Lyn$^{-/-}$ mice (70), and is required in other models for the formation of autoreactive germinal centers, in which class switching occurs (81, 82). Btk is required for the upregulation of IL-6 in B cells in response to synergistic BCR and TLR9 signaling (27), and B cells overexpressing Btk express more IL-6 (57). Btk also promotes expression of IL-21, a Tfh-derived cytokine, in Lyn$^{-/-}$ mice. This likely occurs *via* IL-6 as splenocytes from both Lyn$^{-/-}$Btk^{lo} and Lyn$^{-/-}$IL6$^{-/-}$ mice have reduced expression of IL-21 mRNA compared to Lyn$^{-/-}$ mice (80). Furthermore, Btk overexpression in B cells results in increased Tfh cells and IFNγ-producing T cells (57), which are important for autoreactive germinal centers and pathogenic autoantibodies (83–86).

Btk and Innate-Like B Cells

Bruton's tyrosine kinase is expressed in B1a and marginal zone (MZ) B cells. These innate-like B cells may have both pathogenic and protective roles in autoimmune disease. The relative importance of Btk in these specific roles is not clear.

B1a cells are found predominantly in the peritoneal cavity, have a repertoire enriched in polyreactivity (87, 88), and are increased in several lupus models (41, 42, 88–90). Whether they are elevated in Lyn$^{-/-}$ mice is controversial (52, 72, 91, 92). They secrete protective IgM autoantibodies (93) and the anti-inflammatory cytokine IL-10 (94, 95). In some cases they do not contribute to pathogenic autoantibodies (96), but they

can produce IgG autoantibodies and interact with T cells in a pro-inflammatory manner in some lupus models (88, 97, 98). B1a cells are reduced in $Lyn^{-/-}Btk^{lo}$ mice (52) and increased in mice expressing constitutively active Btk (55), and Btk is required for their expression of IL-10 (25).

Marginal zone B cells are also enriched in autoreactivity (99–101). Whether they contribute to pathogenic autoantibodies in lupus is model-dependent (71, 101–107), and may be modulated indirectly by alterations in splenic architecture in some strains. Btk is not required for MZ B cell development, but it controls the positive selection of particular B cell specificities into the MZ compartment (108). How this affects autoimmunity is not clear. Skewing of autoreactive B cells to the MZ is promoted by Btk in the 56R anti-DNA immunoglobulin transgenic model (26) and in NOD mice (109), a model of type I diabetes, but RF B cells carrying the xid mutation are enriched in the MZ relative to their wild-type counterparts (63).

Btk Inhibitors Are Effective in Mouse Lupus Models

The genetic evidence described above suggests that small molecule inhibitors of Btk could be an effective therapy for SLE. Preclinical studies with several inhibitors in multiple mouse models suggest that this may indeed be the case (**Table 1**) (110–117). Kidney damage was ameliorated in all cases and survival increased when measured. Btk inhibitors diminished B cell activity, as measured by reduced CD69 expression, PC frequencies, and/or germinal center B cell frequencies. Autoantibodies were also decreased, although in some cases not all specificities or isotypes were affected. Interestingly, kidney damage was prevented even in the few situations where IgG autoantibodies were not significantly reduced. This indicates that Btk has roles in lupus pathogenesis beyond its contribution to the loss of B cell tolerance. Btk inhibitors were effective in anti-GBM models of kidney disease, which measure only the effector phase of kidney inflammation and damage and do not depend on autoantibody production (113, 117). Btk inhibitors impair pro-inflammatory FcR responses of myeloid cells *in vitro* (111–113, 116–119), suggesting that Btk-dependent effector functions of myeloid cells may contribute to end-organ damage *in vivo*. However, Btk-deficiency in myeloid cells can have pro- or anti-inflammatory effects dependent on cell type and stimulus (120–135), and off-target effects of inhibitors cannot be ruled out (136). For instance, the Btk inhibitor ibrutinib also inhibits Itk (137), a related Tec kinase which has important functions in T cells. Further studies of the relative roles of B and myeloid cell-expressed Btk in lupus pathogenesis would be facilitated by the development of cell type-specific Btk knockout mice.

Btk IN HUMAN AUTOIMMUNITY

While polymorphisms in Btk have not been identified in GWAS studies of SLE or other autoimmune diseases, several lines of evidence suggest that increased Btk activity may be associated with autoantibody production in humans. Increased Btk expression and phosphorylation was observed in B cells from rheumatoid arthritis patients (138, 139), correlating with RF antibodies among RF-positive patients (139) and enriched in anti-citrullinated protein antibody-positive patients (138). Similarly, increased Btk expression and phosphorylation correlated with RF antibodies in Sjogren's syndrome patients (138). The frequency of Btk^{+} cells in the peripheral blood of SLE patients has been reported to correlate with disease activity, anti-dsDNA antibodies, proteinuria, and C3 levels (140), but whether this reflects changes in Btk signaling or cell subset distribution is unclear.

Several SLE-associated signaling defects and polymorphisms likely result in increased activity of Btk signaling pathways in B cells. Reduced expression of PTEN, which counteracts PI3K, has been observed in human lupus B cells (141). Btk activation and function require the binding of its PH domain to the product of PI3K in the plasma membrane (11–14), and PTEN haploinsufficiency enhances the efficiency of Btk signaling in mice (142).

TABLE 1 | Effects of Bruton's tyrosine kinase (Btk) inhibitors on B cell activation and end-organ damage in murine lupus models.

Model	Inhibitor	Autoantibodies	B cell activation	End-organ damage	Survival	Reference
MRL.lpr	PCI-32765 (ibrutinib)	IgG reduced but not significantly		Reduced (kidney)		(110)
MRL.lpr	HM71224	IgG reduced	Reduced (CD69)	Reduced (kidney, skin lesions)		(111)
NZBxNZW	HM71224	IgG reduced but not significantly	Reduced [CD69, plasma cells (PCs)]	Reduced (kidney)	Increased	(111)
NZBxNZW	RN486	IgM unchanged, IgG reduced	Reduced (CD69, PCs)	Reduced (kidney)		(112)
NZBxNZW	PF-06250112	IgG reduced	Reduced (PCs, GCs)	Reduced (kidney)		(113)
NZBxNZW	G-744	Total reduced	Reduced (GCs)	Reduced (kidney)	Increased	(114)
IFN-enhanced NZBxNZW	G-744	Total ANA reduced, anti-dsDNA unchanged	Reduced (proliferation, PCs, GCs)	Reduced (kidney)	Increased	(114)
Sle1.Sle3	PCI-32765 (ibrutinib)	IgM and IgG reduced	Reduced (CD69, PCs)	Reduced (kidney)		(115)
BXSB.Yaa	M7583	Total reduced	Reduced (CD69, PCs)	Reduced (kidney)	Increased	(116)
DBA/Pristane	M7583	Total reduced except anti-SmRNP	Reduced (PCs), increased (CD69)	Reduced (arthritis)		(116)
Anti-GBM	PF-06250112			Reduced (kidney)		(113)
Anti-GBM	BI-BTK-1			Reduced (kidney)		(117)

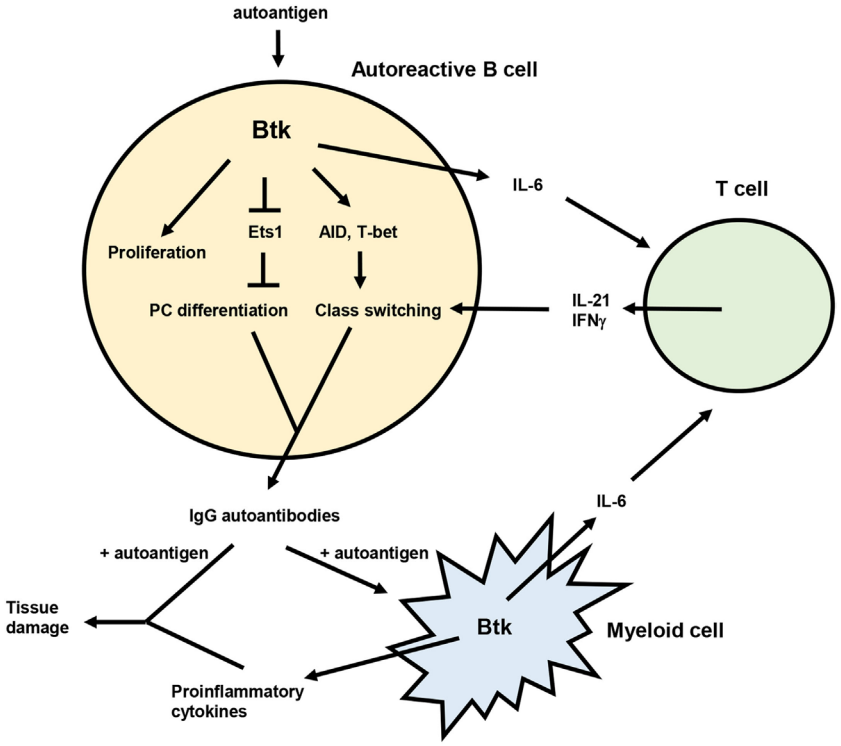

FIGURE 1 | Model for the role of Bruton's tyrosine kinase (Btk) in lupus pathogenesis. Btk acts in autoreactive B cells to promote proliferation, plasma cell (PC) differentiation, and class switching, resulting in the production of pathogenic IgG autoantibodies. IgG autoantibody production is also facilitated by the ability of Btk to enhance IL-6 expression from both B and myeloid cells. IL-6 then acts on T cells to promote differentiation of Tfh cells and IFNγ producing T cells, which in turn contribute to autoreactive B cell class switching *via* IL-21 and IFNγ. IgG autoantibodies produced in a Btk-dependent manner can then form immune complexes with autoantigen that deposit in tissues and induce inflammation and damage. These immune complexes can also activate myeloid cells, likely in a Btk-dependent manner, to produce inflammatory mediators that also damage tissues.

The autoimmune phenotype in Lyn$^{-/-}$ mice is mediated by excessive Btk activity in mice (49–52, 76, 80). Polymorphisms in Lyn are associated with SLE (143, 144), and reduced Lyn expression is observed in B cells from a subset of SLE patients (145–147). Expression of CSK, an inhibitor of Lyn, is increased by an SLE-associated polymorphism in the CSK gene. B cells carrying this SNP have reduced Lyn activity and increased responses to BCR signaling (148). Finally, several polymorphisms in Ets1 are associated with SLE, and Ets1 expression is reduced in PBMCs from SLE patients [reviewed in Ref. (149)]. Btk promotes the accumulation of autoreactive PCs in Lyn$^{-/-}$ mice by downregulating Ets1 (76).

CONCLUSION

Genetic studies demonstrate multiple roles for Btk in the development of murine lupus (**Figure 1**), including promoting the activation, differentiation, and class switching of autoreactive B cells. Btk inhibitors are effective at reducing autoantibodies and/or autoimmune symptoms in mouse lupus models and may act in both B and myeloid cells to exert these effects. In humans, several components of Btk signaling pathways are altered in B cells from lupus patients, and Btk expression and activation is elevated in B cells from other autoimmune diseases. Btk has dose-dependent effects on B cell activation and autoantibody production as illustrated by the phenotypes of Btk^{lo} and Btk-overexpressing mice. Such a rheostat effect of Btk (150) is supported by recent structural analysis indicating that Btk has graded degrees of activity (151), and suggests that partial inhibition of Btk may have significant functional consequences. Btk may thus be a useful therapeutic target for SLE. The Btk inhibitor ibrutinib is well tolerated and approved for treatment of several B cell malignancies (152, 153), and second generation, more specific inhibitors such as acalabrutinib are promising (154, 155). The use of these and other Btk inhibitors in B cell malignancy will be informative with respect to potential off-target and side effects (156, 157) that might be encountered in the context of autoimmune disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Signal Transducer and Activator of Transcription 3 Control of Human T and B Cell Responses

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Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated downstream of many key cytokine receptors expressed by lymphocytes. As such, it plays a critical role in regulating B cells as well as $CD4^+$ and $CD8^+$ T cells. Patients with clinically significant immunodeficiency and immune dysregulation resulting from loss-of-function or gain-of-function mutations in STAT3 have been described. These individuals provide insight into the critical role of this transcription factor in the regulation of immune responses and the balance between effective immune protection and autoimmunity.

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INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated downstream of a large range of cell surface receptors. It forms part of a family of proteins that also includes STAT1, 2, 4, 5A, 5B, and 6, which are activated in a similar manner downstream of surface receptors. Binding of their ligand by these receptors, leads to the activation of receptor-associated Janus activating kinases (JAKs). The activated JAKs then phosphorylate the receptor providing docking sites for STATs, which in turn become tyrosine phosphorylated. This leads to the formation of homodimers or heterodimers, followed by translocation to the nucleus where the dimers bind to DNA and induce transcription of a broad range of target genes (1, 2).

Importantly, many of the cytokine receptors that lead to STAT3 activation are expressed by lymphocytes including those for IL-6, IL-10, IL-21, IL-23, and IFNs. The critical role of STAT3 in lymphocyte biology was highlighted by the discovery that loss-of-function (LOF) mutations in *STAT3* cause the primary immunodeficiency autosomal dominant hyper IgE syndrome (AD-HIES), which is characterized by defects in both T and B cells (3, 4). More recently, gain-of-function (GOF) mutations in *STAT3* were also identified, this time in patients who presented with early onset autoimmunity as well as immunodeficiency (5–7). These diseases demonstrate that STAT3 plays a central role in regulation of immune responses.

IMMUNODEFICIENCY CAUSED BY *STAT3*^{LOF} MUTATIONS

Autosomal dominant hyper IgE syndrome was first described about 50 years ago, but it was not until 2007 that two groups demonstrated that it is caused by heterozygous LOF mutations in *STAT3* (3, 4). AD-HIES is characterized by a range of immunological manifestations including elevated IgE, eczema, chronic mucocutaneous candidiasis (CMC), recurrent staphylococcal infections, and pneumonias. Patients also display non-immunological manifestations such as joint hyperextensibility,

facial dysmorphism, and retention of primary teeth (8). Since the initial description over 89 disease-causing mutations in *STAT3* have been reported and are found distributed throughout the STAT3 molecule (9, 10). These mutations all lead to the same clinical phenotype, presumably because while different mutations impair signaling at different stages, they all impair the ability of STAT3 to bind to DNA and induce gene transcription (11). It should be noted that due to the dimerization step in the STAT3 signaling pathway these heterozygous mutations in *STAT3* work in a dominant negative manner. That is, in patient cells, 75% of STAT3 dimers would contain at least one LOF STAT3 molecule and thus be dysfuctional, leaving only 25% of dimers functioning normally (3, 4). Thus, AD-HIES results in severely compromised, but not completely ablated, STAT3 signaling. This 25% of residual STAT3 function is presumably critical for survival as germline deletion of *Stat3* in mice is embryonically lethal (12).

IMMUNE DYSREGULATION CAUSED BY *STAT3*^{GOF} MUTATIONS

More recently, patients with heterozygous GOF mutations in *STAT3* have also been described (5–7). These patients present with early onset autoimmunity and/or lymphoproliferation. The range of autoimmune manifestations is broad and includes cytopenias, type I diabetes, enteropathy, scleroderma, arthritis, and thyroid disease (5–7). However, many of these patients were also reported to suffer from recurrent or severe infections as well as hypogammaglobulinemia (6, 7) suggesting concurrent immunodeficiency. Overall, the clinical phenotype of the patients has been found to be quite variable and unaffected family members who carried STAT3^{GOF} mutations have also been identified suggesting there is incomplete disease penetrance and that other factors influence the pathogenicity of the mutations (7, 13).

The molecular mechanism that results in GOF from these germline mutations has not been extensively characterized; however, the varied patient phenotype suggests there may be more divergence in mechanism than is observed with LOF mutations. It has been observed that most disease causing GOF mutations do not alter phosphorylation; however, these mutations generally lead to increased transcriptional activity of STAT3 target genes in unstimulated and/or stimulated cells (6, 7). This in turn leads to upregulation of STAT3 target genes such as *SOCS3* (7). Interestingly, SOCS3 can regulate the activation of STAT family members, and cells from these patients were found to have reduced STAT5 phosphorylation in response to IL-2, and STAT1 phosphorylation in response to IFNγ (7). Moreover, some of the symptoms of STAT3^{GOF} patients are similar to those observed in STAT5b LOF patients (14) suggesting that reduced STAT5 activation may partially explain the phenotype (discussed below).

THE ROLE OF STAT3 IN B CELLS

Multiple findings in patients with dysregulated STAT3 function point to a role for STAT3 in regulating human B cells responses. For example, although patients with STAT3^{LOF} mutations have relatively normal levels of total serum IgM, IgG, and IgA, they have elevated levels of serum IgE, defects in antigen specific antibody responses and reduced memory B cells (8, 15–19). Further, the STAT3-activating cytokines IL-21, and to a lesser extent IL-10, are potent B cell activators. In combination with CD40L, IL-21 and IL-10 are capable of inducing the proliferation, class switching, and differentiation of human B cells (19, 20). Interestingly, some, but not all, of the actions of IL-21 and IL-10 were found to be disrupted in B cells from AD-HIES patients. Specifically, STAT3^{LOF} naïve B cells were unable to differentiate into antibody secreting cells in response to CD40L and IL-21 (19, 21) and failed to upregulate key transcriptional regulators of the plasma cell program such as BLIMP-1 and XBP-1 (19, 21). In contrast, IL-21 was able to induce normal levels of switching to IgG from naïve STAT3^{LOF} B cells and could stimulate increased levels of proliferation from these cells compared to cultures with CD40L alone, albeit lower than what was observed in naive B cells from healthy controls responding to CD40L and IL-21 (19). This decreased proliferation/expansion could at least partially be attributed to an increase in cell death (19). It must be remembered, however, that these patient cells retain 25% STAT3 activity so it unclear whether the responses to IL-21 that are preserved reflect STAT3-independent effects of IL-21 or the function of the residual STAT3. Some insight can be gained from mouse models of B cell specific deletion of *Stat3* in which all STAT3 function is ablated in these cells. These models showed relatively normal switching to IgG (22, 23) but decreased expansion and/or maintenance of B cells to a T cell-dependent antigen resulting in fewer germinal center B cells (23, 24). Further STAT3 deficiency also resulted in a defect in affinity maturation (23) although cells still underwent somatic hypermutation (19, 23). Together these studies reveal that STAT3 is required in naïve B cells to induce plasma cell formation, survival, and expansion of responding B cells but not for regulating switching to IgG. Interestingly, the small number of memory cells that do emerge in patients with STAT3^{LOF} can respond normally to IL-21 to form antibody-secreting cells (21). This demonstrates that naïve and memory B cells have differential requirements for STAT3, which may have important implications for attempts to target the STAT3 pathway therapeutically.

Confirmation of the critical role of IL-21 upstream of STAT3 was provided by the identification of patients with LOF mutation in *IL21R* and *IL21*. These patients displayed similar B cell defects to those with LOF *STAT3* mutations such as reduced memory B cells, poor responses to vaccination, and elevated levels of IgE (21, 25–28). This clearly demonstrates the importance of the IL-21/STAT3 signaling axis in human B cell function. However, the exact molecular mechanism that leads to decreased memory cells and increased IgE is still unclear. Interestingly, a study of a patient with somatic mosaicism of the *STAT3* mutation has shown that STAT3^{LOF} cells did not generate memory CD4^{+} or CD8^{+} T cells but the mutation was present in memory B cells, suggesting that there was an intrinsic requirement for STAT3 in T cells but not B cells for the generation of memory (29). Thus, it may be that the decrease in memory B cells is secondary to aberrant function of other cell types such as T follicular helper (Tfh) cells (discussed below).

B cells can influence immune responses through their role in antigen presentation and the production of cytokines. IL-10 produced by B cells has been implicated in a regulatory role in

immune responses (30). Interestingly, STAT3^{LOF} B cells have been shown to produce less IL-10 following stimulation, than normal controls (31) suggesting that this regulatory function of B cells may also be altered.

Given this critical role of IL-21/STAT3 in B cell differentiation, it might be predicted that STAT3^{GOF} patients would exhibit increased B cell activity. Surprisingly, however, some of these patients seem to display hallmarks of B cell dysfunction such as hypogammaglobulinemia and decreased switched memory B cells (6, 7). Conversely, many of these patients seem to display antibody-mediated autoimmunity suggesting that B cell tolerance is disturbed (5–7). Unfortunately, little functional work has been done on B cells from these patients so it remains unclear if there are B cell intrinsic effects of STAT3 over activation or whether this is secondary to defects in other cells such as Tfh cells or regulatory T cells (Tregs) (discussed below).

THE ROLE OF STAT3 IN CD4$^+$ T CELLS

Naive CD4$^+$ T cells are able differentiate into distinct effector subsets that play specific roles in the immune response. These subsets include Th1, Th2, Th9, Th17, Th22, Tfh cells, and Tregs. The differentiation of CD4$^+$ T cells is determined by the cytokine milieu at the time of activation and numerous STAT3-signaling cytokines have been implicated in this process (32, 33). Analysis of patients with *STAT3* mutations has provided key insights into the role of STAT3 in controlling these processes.

Th17

The differentiation of human Th17 cells is controlled by the action of several STAT3-activating cytokines including IL-6, IL-21, and IL-23 (34–36). Analysis of AD-HIES patients revealed a deficiency in Th17 cells in the blood of these patients as measured by expression of CCR6 and the production of IL-17A, IL-17F, and IL-22 (28, 37–40). Furthermore, STAT3^{LOF} naïve CD4$^+$ T cells from these patients fail to differentiate into IL-17-expressing cells *in vitro* (38, 41). Together, this not only demonstrates an essential requirement for STAT3 signaling in the generation of human Th17 cells but also provides an explanation for the CMC observed in AD-HIES patients as IL-17-mediated immunity is crucial for control of candida infections (42, 43). Interestingly, patients with GOF mutations in STAT1 also display defects in the generation of Th17 cells and susceptibility to candida infections demonstrating that balanced STAT1/STAT3 signaling is required for generation of these cells (41, 44, 45). On the other hand, patients with STAT3^{GOF} were not found to have increased IL-17-expressing CD4$^+$ T cells suggesting that, while STAT3 is required for the generation of these cells, over activation alone is not sufficient to drive Th17 differentiation (6, 7). However, more detailed analysis of these STAT3^{GOF} CD4$^+$ T cells may be required to definitively conclude this as some patients may have increased Th17 cells (46).

Th1/Th2

In contrast to Th17 cells, the generation of human Th1 and Th2 is thought to act primarily through IL-12/STAT4 and IL-4/STAT6 signaling, respectively (32, 33). Consistent with this, generation of these populations was found to be largely STAT3-independent, as shown by normal frequencies of CXCR3$^+$CCR6$^-$ and CXCR3$^-$CCR6$^-$ and IFNγ-producing and IL-4, IL-5, IL-13-producing cells, respectively, in AD-HIES patients (28, 37, 38). Similarly, naïve CD4$^+$ T cells from STAT3^{LOF} patients could differentiate into Th1 or Th2 cells when cultured under the relevant polarizing conditions (41, 47). Interestingly, IFNγ expression tended to be increased in STAT3-deficient CD4$^+$ T cells (28, 41), suggesting STAT3 signaling may inhibit Th1 cell differentiation.

Th9

Human Th9 cells develop in the presence of TGFβ and IL-4 (48, 49); however, they can also be induced by the addition of TGFβ to Th17 polarizing conditions (i.e., IL-1β/IL-6/IL-21/IL-23) (48), suggesting STAT3 may be involved in Th9 cell differentiation. Consistent with this, addition of IL-6, IL-10, or IL-21 to Th9 polarizing conditions enhanced IL-9 expression (50). In contrast, IL-27 partially suppressed TGFβ and IL-4-induced IL-9 expression (50). Since IL-6, IL-10, IL-21, and IL-27 are capable of activating both STAT1 and STAT3, further investigation is required to determine if the regulation of IL-9 production by these cytokines results from both STATs, or whether one STAT has a dominant function in regulating IL-9 production. However, one paper found that IL-9 production in responses to candida antigens was decreased in patients with STAT3^{LOF} mutations (51) suggesting that STAT3 may be important, at least under some conditions, for the induction of IL-9.

Tfh

Like Th17 differentiation, the generation of human Tfh cells is driven by numerous STAT3-ativating cytokines, namely IL-6, IL-12, IL-21, and IL-27. Consistent with a requirement for STAT3 to induce this differentiation program, patients with STAT3^{LOF} have a reduction in circulating CXCR5$^+$ Tfh cells (28, 52, 53), and naïve STAT3^{LOF} CD4$^+$ T cells failed to differentiate *in vitro* into IL-21-producing Tfh-like cells (41, 52, 54). The role of STAT3 in differentiation and/or function of Tfh cells has also been demonstrated in mouse studies of Stat3-deficient T cells (55–59). However, the degree to which Stat3 is required seems to be dependent on the presence of other signals such as STAT1 and type 1 IFNs as well as the site of the immune response (57, 59, 60). This defect in Tfh cells in AD-HIES would contribute to impaired humoral immunity in these patients and potentially to the deficiency in memory B cells. *In vitro*, IL-12 was found to be the main driver of IL-21-producing cells (61, 62), but since patients with defects in IL-12R signaling do not present with impaired humoral immunity (63–65), it is likely that the other STAT3-dependent cytokines IL-6, IL-21, and/or IL-27 plays a redundant role in this process *in vivo*. Consistent with this, IL-6 and IL-21 were found to induce ICOS expression in cord blood CD4$^+$ T cells in a STAT3-dependent manner (53, 66), and patients with mutations in IL21/IL21R show defects in Tfh cell development and function (28, 41).

REGULATORY T CELL

Studies in mice have suggested that IL-6/STAT3 signaling inhibits Treg differentiation inasmuch as Stat3$^{-/-}$ CD4$^+$ T cells stimulated under Th17 conditions (TGF-β$^+$ IL-6 in mice) showed decreased Th17 differentiation and increased Treg differentiation (67–69). However, normal frequencies of Tregs (defined as CD25^{hi}CD127^{lo} or FoxP3$^+$) were reported in AD-HIES patients (28, 38, 47) and Tregs from these patients displayed normal suppressive behavior *in vitro* (47), suggesting STAT3 was somewhat redundant in controlling the generation of human Tregs. On the other hand, a recent study demonstrated that naïve CD4$^+$ T cells from STAT3^{LOF} patients showed an increased propensity to develop into iTregs in culture (70). CD4$^+$ T cells from these patients have also been reported to display a defect in IL-10 production (28, 38, 41) and DCs from STAT3-deficient patients failed to induce Tregs from naïve CD4$^+$ T cells (47). Thus, it may be that in these patients the increased propensity of STAT3^{LOF} CD4$^+$ to form induced Tregs is compensated for by the reduced ability of STAT3^{LOF} dendritic cells to induce them.

Indeed, patients with STAT3^{GOF} mutations much more clearly demonstrate a role for STAT3 in the regulation of Tregs. As discussed above, these patients display early onset autoimmunity that is reminiscent of patients with Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is caused by mutations in *FoxP3* leading to a loss of Treg function (71). These overlapping clinical phenotypes suggested that STAT3^{GOF} patients also have dysfunctional Tregs. Consistent with this, they were found to have lower percentage of FoxP3$^+$ cells in their blood and lower CD25 expression on their Tregs (7). This is thought to be due to increased SOCS3 levels that inhibit the activation of STAT5 downstream of IL-2 (7).

Studies in mice have now demonstrated that there are different populations of Tregs that seem to be specialized for inhibiting particular T-helper populations (72). Thus, a population of Treg cells that express CCR6 and are specialized for suppressing Th17 cells has been described. These "Treg17" cells, like the Th17 cells they suppress, were found to be dependent on STAT3 signaling (73). AD-HIES patients were also shown to have decreased CCR6$^+$ Tregs suggesting that human "Treg17" cells may also exist and be dependent on STAT3 (74).

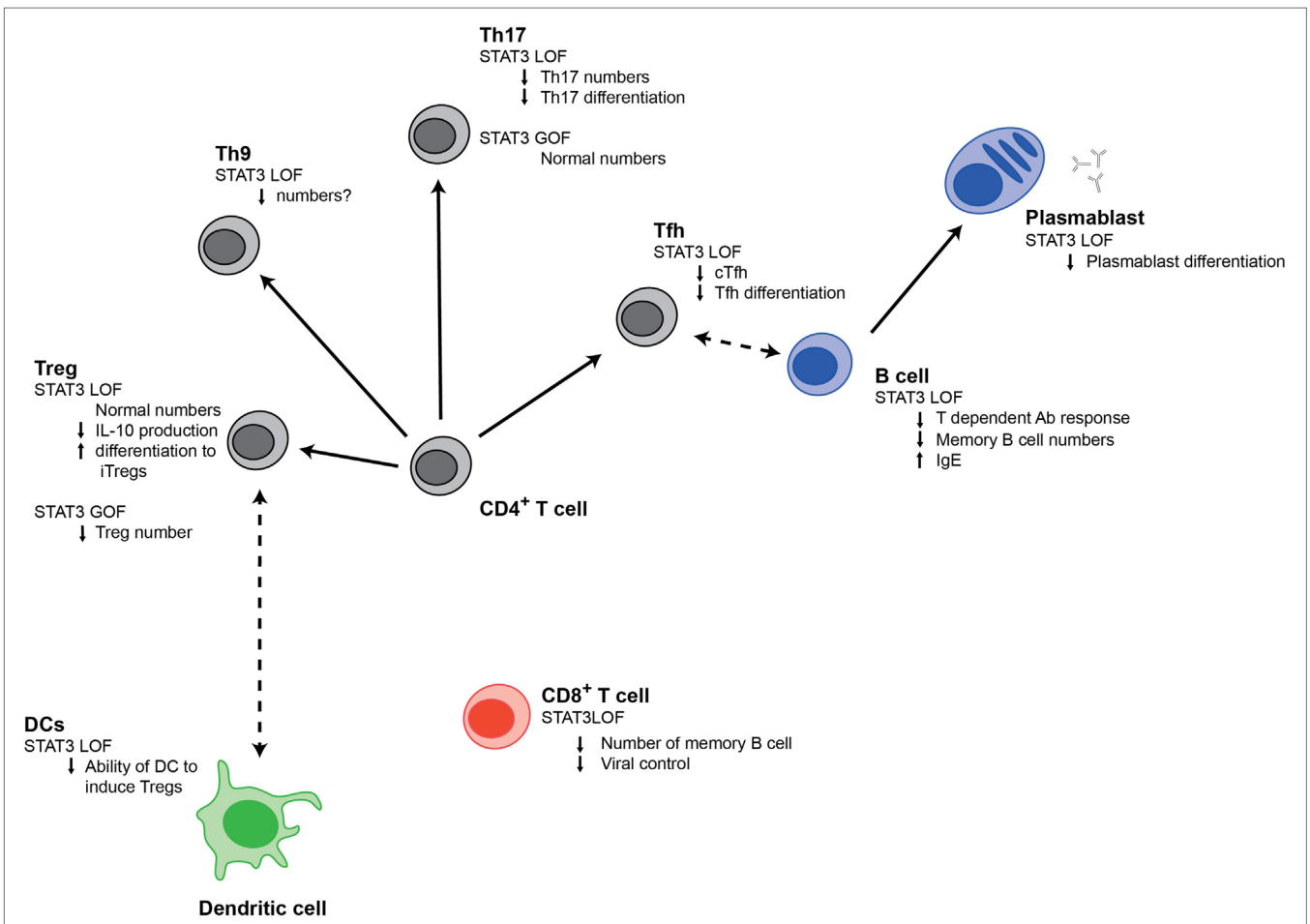

FIGURE 1 | Signal transducer and activator of transcription 3 (STAT3) regulates multiple populations of immune cells. Changes in different immune cell populations that are observed in STAT3^{LOF} or STAT3^{GOF} patients are shown.

Taken together, these data suggest STAT3 plays a complex role in the regulation of Treg responses and care should be taken in targeting this pathway as a means of regulating Treg responses.

THE ROLE OF STAT3 IN CD8$^+$ T CELLS

STAT3 activating cytokines such as IL-21 also play a role in regulating CD8$^+$ T cells. Studies on STAT3^{LOF} CD8$^+$ T cells showed they had impaired induction of perforin and granzyme B in response to IL-21; however, this could be rescued by strong TCR ligation (75). In contrast, proliferation induced by IL-21 was not affected in naïve STAT3^{LOF} CD8$^+$ T cells (75). STAT3 deficiency, however, did result in reduced memory CD8$^+$ T cells (29, 75), an effect that was shown to be cell intrinsic (29). IL-21R patients also showed decreases in memory CD8$^+$ T cell populations suggesting that IL-21 may be a cytokine upstream of STAT3 that contributes to the maintenance of memory cells (75). Patients with AD-HIES also show increased susceptibility to reactivation of viruses such as EBV and VZV (29) indicating that STAT3^{LOF} CD8$^+$ T cells may be defective in their ability to control these chronic infections.

STAT3 IN AUTOIMMUNITY

STAT3^{GOF} patients provide a clear demonstration that STAT3 plays an important role in controlling autoimmunity. However, previous evidence from other disease states had already indicated that STAT3 played an important role in the regulation of autoimmunity. In particular, multiple studies have associated polymorphisms in *STAT3* with various autoimmune conditions including Crohn's disease, ulcerative colitis, psoriasis, and Behcet's disease (76–78). Furthermore, many of the cell populations that STAT3 can induce (**Figure 1**) have been implicated in driving autoimmunity; this includes Tfh cells and B cells, which support autoantibody production and Th9 and Th17 cells, can produce potentially damaging cytokines (79, 80). Conversely, STAT3 can inhibit Tregs, which act to restrain destructive immune responses.

Interestingly, somatic STAT3^{GOF} mutations have been reported in large granular lymphocytic (LGL) leukemia (81, 82) and are associated with higher rates of autoimmune complications such as rheumatoid arthritis and autoimmune cytopenias (81–83). As these LGL leukemias are of either CD8$^+$ T cell or NK cell origin, this suggests that STAT3 overactivation in CD8$^+$ T cells or NK cells alone may be sufficient to drive autoimmunity, possibly by inducing the production of cytokines/inflammatory mediators (84).

Taken together, these data are consistent with a role for STAT3 in promoting autoimmunity; however, STAT3 is also likely to play a role in inhibiting damaging responses downstream of IL-10. Indeed, germline LOF mutations in the genes coding for IL-10 or IL-10R are a major cause of early onset inflammatory bowel disease (85), demonstrating a critical requirement for IL-10 signaling (presumably through STAT3) to maintain tolerance in the bowel.

It remains unclear which of these many roles of STAT3 in multiple cell types underlies the phenotype in STAT3^{GOF} patients. Certainly, the similarities with IPEX (71, 86) point to a clear role of Tregs in the phenotype; however, contributions from other pathways are also likely to make contributions. Further research will help clarify this and in turn pave the way for targeted treatments for both rare patients with STAT3^{GOF} mutations as well as patients who suffer from autoimmunity more generally.

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PTEN-Regulated AID Transcription in Germinal Center B Cells Is Essential for the Class-Switch Recombination and IgG Antibody Responses

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Class-switch recombination (CSR) and somatic hypermutation (SHM) occur during the differentiation of germinal center B cells (GCBs). Activation-induced cytidine deaminase (AID) is responsible for both CSR and SHM in GCBs. Here, we show that ablation of PTEN through the Cγ1-Cre mediated recombination significantly influences the CSR and SHM responses. The GCs fail to produce the IgG1 B cells, the high affinity antibodies and nearly lost the dark zone (DZ) in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice after immunization, suggesting the impaired GC structure. Further mechanistic investigations show that LPS- and interleukin-4 stimulation induced the transcription of Cγ1 in IgM-BCR expressing B cells, which efficiently disrupts PTEN transcription, results in the hyperphosphorylated AKT and FoxO1 and in turn the suppression of AID transcription. Additionally, the reduced transcription of PTEN and AID is also validated by investigating the IgM-BCR expressing GCBs from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon immunization. In conclusion, PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody responses.

Keywords: PTEN, germinal center, IgG1$^+$ B cells, class-switch recombination, somatic hypermutation

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INTRODUCTION

The germinal center (GC) is the region where antigen-activated B cells undergo proliferation and differentiation responses to differentiate into either plasma cells or memory B cells. Somatic hypermutation (SHM) and class-switch recombination (CSR) occur during the proliferation of GC B cells (GCBs) (1–3). SHM and CSR account for the generation of high affinity and class-switched B cells in humoral immunity (4). There are eight sets of C_H exons at the *Igh* locus in mice, which are constituted as 5′-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′. During the CSR, the assembled V(D)J exons from Cμ encoded IgM-expressing B cells is juxtaposed next to one of the sets of the downstream C_H exons, converting IgM-expressing B cells to different IgH sub-classes (e.g., IgG3, IgG1, and IgG2b), which are, respectively, encoded by different C_H genes (e.g., Cγ3, Cγ1, and Cγ2b) (5). Activation-induced cytidine deaminase (AID), as the B cell-specific factor, is required for the CSR (6). During GC responses, AID produces C:G to U:G and even C:G to A:T mismatches (7), which then triggers the mismatch and base-excision repairs. Furthermore, the generation of DNA double-strand breaks (DSBs) at switch regions between Sμ and a downstream S region leads to a rearranged C_H locus and the deletion of the intervening sequence (8, 9). The repair of the AID induced DSBs

via nonhomologous end-joining (NHEJ) eventually completes the CSR by rejoining the two broken S regions (10, 11).

Previous studies suggested that the phosphatidylinositol-3-kinase (PI3K) and AKT signaling can both regulate the *Ig* gene rearrangement during B cell development and the CSR during GC responses (12–18). Phosphatase and tension homolog (PTEN) is known to negatively regulate PI3K-mediated growth, survival, proliferation and cellular metabolism of B cells (16, 17, 19–22). Thus PTEN deficiency alters B1, marginal zone B (MZB) and follicular B (FOB) cell subsets in *Pten*$^{fl/fl}$ *CD19-Cre* mice (16, 17). Further study revealed that imbalanced PTEN and PI3K signaling impaired the μHC recombination in pro-B cells in *Pten*$^{fl/fl}$ *mb1-Cre* mice (12). Recently, emerging efforts have been placed to investigate the molecular mechanism of PTEN- and PI3K-tuned AKT signaling in regulating the strength of GC responses (14, 15, 23). B cell specific deficiency of PTEN in *Pten*$^{fl/fl}$ *mb1*$^{Cre/+}$ mice leads to the severe defects of B cell development at the bone marrow stage due to failed VJD recombination (12). The loss of the mature naïve B cell population in *Pten*$^{fl/fl}$ *mb1*$^{Cre/+}$ mice prevented the assessment of the function of PTEN in GCB-mediated CSR and antibody responses. As a solution, PTEN was recently knocked out in mature B cells in *Pten*$^{fl/fl}$ *hCD20Tam*$^{Cre/+}$ mice, which demonstrated the importance of PTEN in regulating GC responses (23). Although mature B cell specific deficiency of PTEN in *Pten*$^{fl/fl}$ *hCD20Tam*$^{Cre/+}$ mice excluded the B developmental defects as in the case of *Pten*$^{fl/fl}$ *mb1*$^{Cre/+}$ mice, the usage of *Pten*$^{fl/fl}$ *hCD20Tam*$^{Cre/+}$ mice cannot explicitly separates the function of PTEN in mature B cell activation and proliferation upon antigen stimulation versus that in GC responses since GCBs were differentiated from activated mature naïve B cells after antigen stimulation. Here, to precisely assess the function of PTEN in GCB-mediated humoral responses *in vivo,* we used a mouse model with a PTEN deletion only in specific subsets of GCBs. Our results reveal that PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody responses.

MATERIALS AND METHODS

Mice, Cell Culture

C57BL/6J (B6) background *Pten*$^{fl/fl}$ mice (a kind gift from Dr. Wei Guo, Tsinghua University) were mated to *Cγ1-Cre* transgenic mice (a kind gift from Dr. Tomohiro Kurosaki, Osaka University and Dr. Klaus Rajewsky, Max Delbrück Center) in which expression of Cre is controlled by the endogenous promoter of the B cell-specific gene Cγ1. Offspring carrying *Cγ1-Cre* and two copies of the floxed *Pten* allele or *Cγ1-Cre* plus two copies of the WT *Pten* allele were used in the analyses as homozygous mutant (*Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$) or WT (*Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$) mice, respectively. All mice were maintained under specific pathogen-free conditions and used in accordance of governmental and institutional guidelines for animal welfare. Primary B cells were negatively isolated from the spleen of *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ or *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice as previously reported (24). Single cell suspensions were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 μM β-mercaptoethanol (Sigma-Aldrich), penicillin/streptomycin antibiotics (Invitrogen) and Non-Essential Amino Acids (Invitrogen). B cells were stimulated for 4 days using 10 μg/mL LPS (Sigma) alone or LPS plus 50 ng/mL interleukin-4 (IL-4) (R&D) or 1 μg/mL anti-CD40 (eBioscience) alone or anti-CD40 plus 50 ng/mL IL-4 (R&D) in order to drive primary B cells class-switch *in vitro.*

Immunization, ELISA Assay, and Immunohistochemistry

For mice GC Flow cytometry analysis, 6-week-old *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were injected intraperitoneally with 1×10^9 sheep red blood cells (SRBCs, Bioren, China) or emulsified BSA in Alum adjuvant then analysis at day 7 after the immunization. Qβ virus-like particles (VLPs) were expressed in *E. coli* strain JM109 with exogenous expression plasmid pQ10 and then purified. The CpG contained VLPs were obtained by packaging VLPs with CpG ODN G10 *in vitro* as described (25). 6-week-old *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were injected intraperitoneally with 10 μg VLP in 400 μL PBS for the immunization. Mice were analyzed at day 7 or day 14 after immunization. For NP-antigen specific T-cell-dependent immunization, 6-week-old *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were injected on footpad with 10 μg NP_{33}-KLH in 20 μL PBS and boost at day 35. Mice were bled at the indicated days after immunization.

To detect VLP or NP-specific IgM, IgG, IgG1, IgG2b, IgG2c, and IgG3 in immunized mice (6 *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and 6 *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$), 2 μg/mL VLP, 5 μg/mL NP_8-BSA or 5 μg/mL NP_{30}-BSA were coated on maxisorb plates (Nunc) and incubated overnight at 4°C. All these plates were blocked with 0.3% gelatin in PBS buffer (2 h at 37°C), followed by addition of pre-diluted mice serum into each well and incubated at 37°C for 1 h. 1:10,000 diluted HRP conjugated goat anti-mouse IgM, IgG, IgG1, IgG2b, IgG2c, and IgG3 were used to detect VLP or NP-specific antibodies also the innate immune antibodies. Then followed the protocol as published before for the final readout results (26).

For immunohistochemistry 6-week-old *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were injected intraperitoneally with 1×10^9 SRBC in PBS. Spleen sections were frozen from day 7 after immunization in OCT compound (Sakura Finetek). After that spleen sections were stained with Alexa Fluor 488 anti-mouse/human CD45R/B220 (#103225, BioLegend), PE anti-mouse FAS (#152608, BioLegend), and APC anti-mouse CD3ε (#100312, BioLegend) by following the protocol described by Sandrine Sander (14).

Western Blotting

Primary splenic B cells or cultured cells were lysed by 2× Lysis buffer. Proteins were extracted by 10% Bis-Tris PAGE (Life technologies) with the cocktail of proteinase inhibitors (Life technologies) in it, transferred to polyvinylidene fluoride (PVDF). The electroblotted membranes were blocked by TBST containing 5% non-fat milk (BD) and were probed with anti-PTEN, anti-β-actin, anti-p-AKT 473, or anti-p-FoxO1/3a primary antibodies overnight at 4°C then immunoblotted with HRP-conjugated secondary antibodies (Dako). PTEN (138G6) Rabbit antibody (#9559), Phospho-AKT (Ser473) rabbit antibody (#4060), phospho-FoxO1 (Thr24)/FoxO3a (Thr32) rabbit antibody (#9464), and β-actin (13E5) rabbit antibody (#4970) were purchased from

Cell Signaling Technology. AID rabbit antibody was a kind gift from Dr. Feilong Meng (Shanghai Institute of Biochemistry and Cell Biology, China).

RT-PCR

Pure *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ splenic B cells were stimulated for 4 days with 10 μg/ml LPS and 50 ng/mL IL-4. Pre-incubated IgM-BCR expressing B cells were sorted by using FACSAria III Cell Sorter (BD). SRBC immunized *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice IgM-BCR expressing GCBs were first enriched by CL-7 marker (GL-7 monoclonal antibody, Biotin, eBioscience; streptavidin microbeads, Miltenyi Biotec) by using the LS columns (Miltenyi Biotec). On the basis of enrich, cells were sorted by using FACSAria III Cell Sorter (BD) after labeling with IgM-BCR expressing GCBs dyes (anti-CD19-APC, anti-FAS-PE, Alexa Fluor 488 goat Fab anti mouse IgM μ chain and ef450-streptavidin for GL-7).

Total RNA was extracted using TRIzol (GIBCO) according to the manufacturer's instructions and was subjected to the reverse transcription to make cDNA. For PCR of post-switch Cγ1, transcript was amplified using the following primers pair: (Cγ1, 429 bp) 5′-GGC CCT TCC AGA TCT TTG AG-3′ (Cγ1 forward), 5′-GGA TCC AGA GTT CCA GGT CAC T-3′ (Cγ1 reverse). For amplification of the PTEN (387 bp) transcript, the primer pair of 5′-TTG AAG ACC ATA ACC CAC CAC AG-3′ (PTEN forward) and 5′-GGC AGA CCA CAA ACT GAG GAT TG-3′ (PTEN reverse) was used. Forward primer for AID (349 bp): 5′-GAG GGA GTC AAG AAA GTC ACG CTG GA-3′, reverse primer for AID: 5′-GGC TGA GGT TAG GGT TCC ATC TCA G-3′. Forward primer for control GAPDH (566 bp): 5′-TGT GTC CGT CGT GGA TCT GA-3′ and reverse primer for GAPDH: 5′-TTG CTG TTG AAG TCG CAG GAG-3′. PCR conditions were 94°C for 3 min, 94°C for 1 min, 60°C for 45 s, 72°C for 45 s, 30 cycles (from step 2 to step 4), and 72°C for 10 min for final extension.

Flow Cytometry

Cells were preincubated with FcBlock (anti-CD16/32, eBiosence) to minimize nonspecific staining. Cells were then stained with cocktails of various mAbs (anti-CD19, -B220, -IgG1, -IgM, -IgD, -CD43, -CD93, -CD21, -CD23, -CD5, -FAS, -CL-7, -CD3ε, -CXCR4, or anti-CD86) conjugated with different fluoresce proteins. All antibodies were purchased from BD, eBioscience or BioLegend. Labeled cells were detected by 5 lasers Fortessa (BD), all data were analyzed with FlowJo software. Cell sorting was performed by using FACSAria III Cell Sorter (BD), following the protocols provided by BD.

Microscopy Instruments

Confocal images were captured using Olympus FV-1000 microscope equipped with 10 × objective lens and a 405, a 473, a 549, and a 635 nm laser. The acquisition was controlled by FV10-ASW4.0 software.

Image Process and Statistical Analyses

Images were analyzed by Image Pro Plus (Media Cybernetics) software following our previous protocols (27). Statistical tests were performed with Prism 5.0 (GraphPad). Two-tailed *t* tests were used to compare end-point means of different groups. Statistical significant results (p) are indicated as: $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Normal Development and Homeostasis of B Cells in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ Mice

To detect the PTEN function in GCBs, we generated the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice by breeding *Pten*$^{fl/fl}$ mice to *Cγ1-Cre* mice. In *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice, PTEN would only be knocked out in B cells with Cγ1 transcription, which are ideally the IgG1-BCR expressing B cells (**Figure 1A**). We avoided to breed *Pten*$^{fl/f}$ mice with *Aicda*$^{Cre/+}$ mice since the *Pten*$^{fl/fl}$ *Aicda*$^{Cre/+}$ mice have been reported to develop severe submandibular hair loss, skin thickening and the manifestation of squamous papillomas (28).

We first examined the development and homeostasis of B cells in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice and confirmed that PTEN deletion in IgG1-BCR expressing B cells did not affect B cell development in the bone marrow and peripheral lymphoid organs (**Figure 1B**). Further flow cytometry analysis of the splenic IgM and IgG1-BCR expressing B cells showed comparable amounts of surface IgM or IgG1 BCRs in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ versus *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ control mice (**Figure 1C**).

Impaired Antibody Responses in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ Mice

Pten$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice showed B cell normal development that allowed us to examine the humoral responses upon the immunization with either the T cell-dependent (TD) antigen NP_{33}-KLH or the Qβ VLPs as reported (25). Age and gender matched *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were undergone footpad injection with 10 μg NP_{33}-KLH in 20 μL PBS and boost at day 35 for the TD antigen immunization (**Figure 2A**). For Qβ virus immunization, 6-week-old *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice were immunized intraperitoneally with 10 μg VLP in 400 μL PBS (**Figure 2B**). ELISA analyses showed that the IgM antibody responses upon the induction by both NP-KLH and VLP were significantly higher in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice compared to the control *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice (**Figures 2A,B**). However, the production of not only IgG1 but also IgG2b and IgG3 were significantly blunted in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice (**Figures 2A,B**). We hypothesized that the decreased IgG antibody responses may be due to a damaged CSR reaction within the GC of *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice.

Damaged CSR in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ Mice

To test whether or not PTEN deletion in IgG1^{+} B cells will damage the CSR within the GC reaction, GCs were induced by the immunization of SRBC in both *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ control mice. Flow cytometry analyses of the splenic B cells at day 7 after SRBC injection unexpectedly demonstrated an increased but not decreased levels of GCBs in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice than the control *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice even though the size of the spleen of both types of mice was comparable (**Figures 3A,B**). Remarkably, further analyses showed that the IgM-BCR expressing GCBs were obviously increased while the class-switched IgG1-BCR

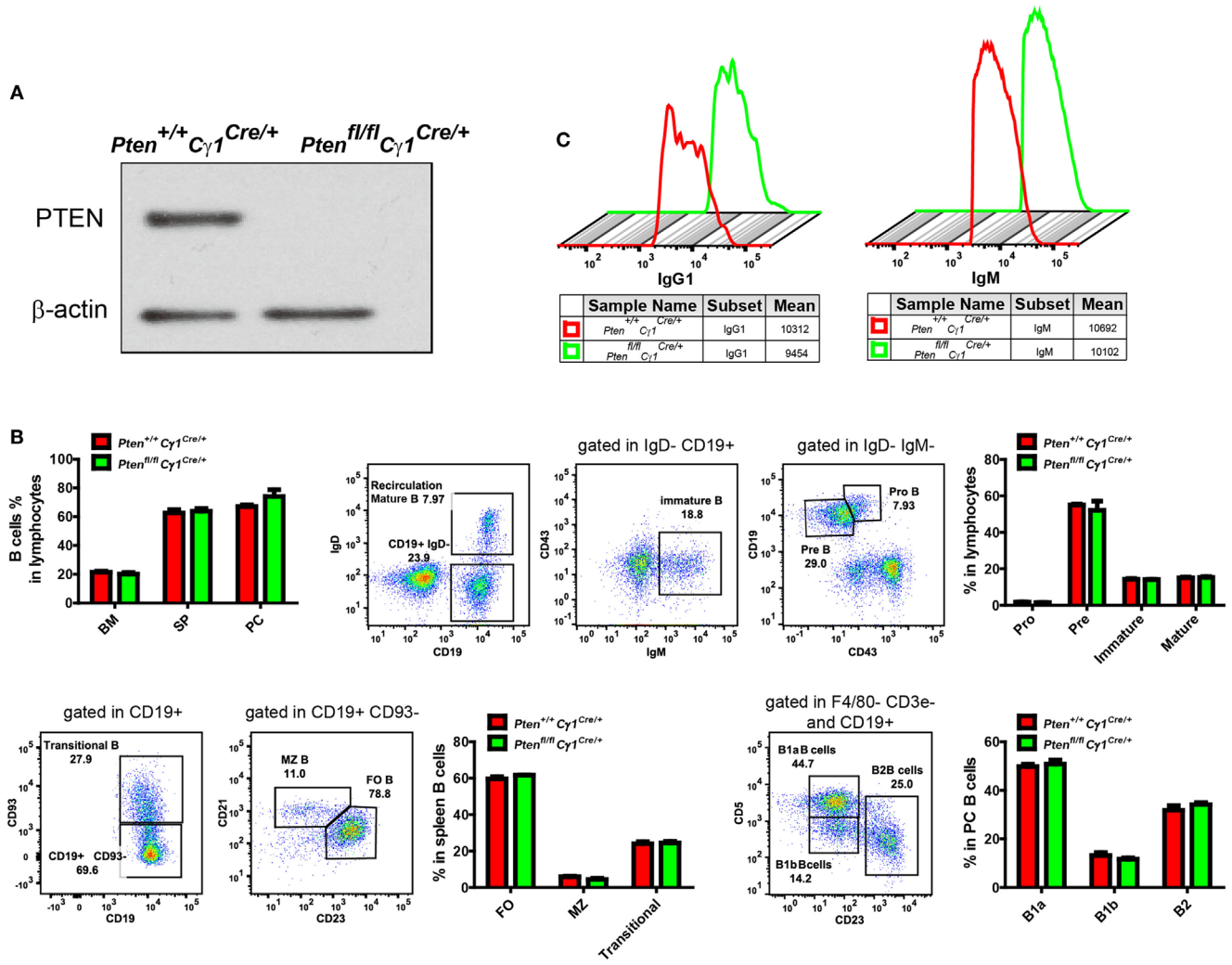

FIGURE 1 | Normal development and homeostasis of B cells in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** Expression of PTEN in IgG1-BCR expressing splenic B cells from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$. Data were given from one representative of at least three independent experiments. **(B)** Comparable B cell percentage in bone marrow (BM), spleen (SP), and peritoneal cavity (PC) (left, top pattern). B cell development in BM (right, top pattern), SP and PC (bottom pattern). MZ, marginal zone; FO, follicular; PC, peritoneal cavity; SP, spleen. The data represent the mean ± SEM of four mice per group in three independent experiments. **(C)** Comparable amount of surface IgG1- (left) or IgM- (right) BCR level in the indicated type of B cells from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/}$mice. Data were given from one representative of at least three independent experiments.

expressing GCBs were almost lost in SRBC immunized *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice GC (**Figures 3C,D**). Similar results were also obtained in the immunized *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice by the utilization of alum adjuvant-precipitate BSA (Figures S1A,B in Supplementary Material). These results suggested that PTEN deletion in IgG1^{+} B cells significantly damaged the CSR within GCs.

To further verify the above observation of the impaired CSR within the GC of *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice, we purified the splenic B cells and performed an *in vitro* CSR assay following the published protocols (13, 16). Clearly, B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice failed to undergo CSR to form IgG1-BCR expressing B cells in the presence of lipopolysaccharide (LPS) alone or LPS plus IL-4 after 4 days of stimulation, respectively (Figures S2A,B in Supplementary Material). Similar results were acquired in the presence of other CSR-driven reagents of anti-CD40 antibodies or anti-CD40 plus IL-4 (Figures S2A,B in Supplementary Material).

Abnormal GC Structure and SHM in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ Mice

It is known that GC contains the light zone (LZ) and the dark zone (DZ). GCBs undergo consecutive and cyclic phases of proliferation and SHM in the DZ, followed by the migration to the LZ, where they capture and internalize antigen for the acquisition of survival signals from follicular helper T cells (3, 29, 30). We thus quantified the formation of LZ and DZ within the GC and found that DZ and LZ compartmentalization was severely disturbed in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon the immunization by both SRBC and VLP (**Figures 4A–C**). Moreover, we observed that the number of

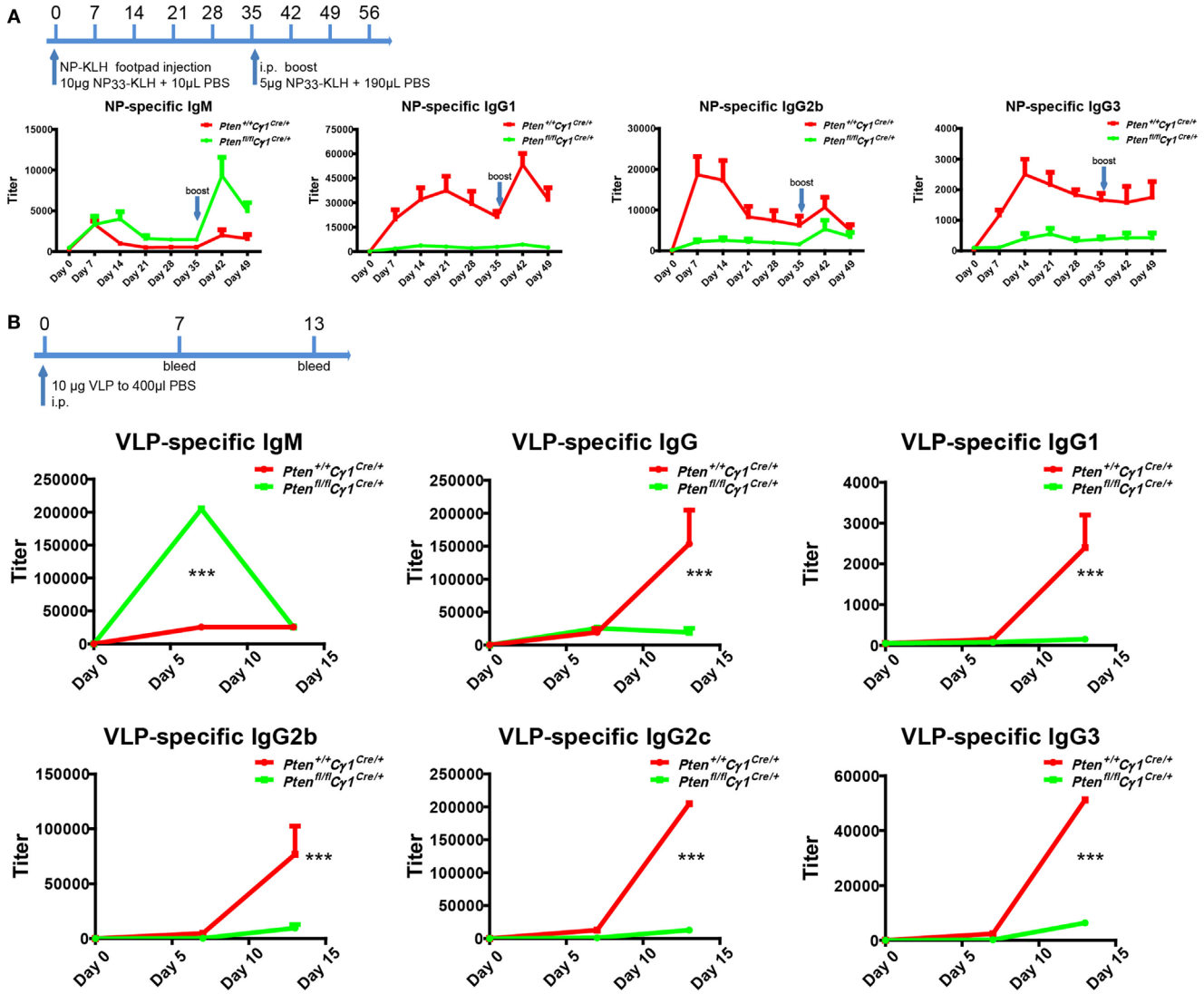

FIGURE 2 | Impaired antibody responses in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** Titers of NP-specific antibodies in the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon immunization with 10 μg NP_{33}-KLH at day 0. NP-specific antibody titers in IgM, IgG1, IgG2b, and IgG3 isotypes were examined by ELISA using NP_8-BSA (5 μg/ml) as the coating Ag. The data represent the median ± interquartile range from three independent experiments with six mice per group at the indicated time point and were analyzed with Kruskal–Wallis test. **(B)** The production of virus-like particle (VLP)-specific antibodies in *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon the immunization (i.p.) with 10 μg VLP at day 0, day 7, and day 14. VLP specific antibody titers in IgM, IgG and IgG1, IgG2b, IgG2c, or IgG3 isotype were examined by ELISA experiment by using VLP (2 μg/ml) as the coating Ag. The data represent the median ± interquartile range from three independent experiments with six mice per group at the indicated time point and were analyzed with Kruskal–Wallis test. ***$p < 0.001$.

DZ B cells in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice GCs was dramatically decreased, consistent with a recent study showing that upon FoxO1 ablation or induction of PI3K activity, GCs lost their DZ, owing at least partly to downregulation of the chemokine receptor CXCR4 (14, 15). Since an essential step in the selection of high affinity GCBs is the recruitment of LZ GCBs into the GC DZ (31, 32), it is not a surprise that the SHM in GCBs was significantly damaged in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon the immunization by TD antigen NP_{33}-KLH (**Figure 4D**). Lastly, it should be noted that even though the GC DZ formation was impaired in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice, the size of the GCs was normal in the spleen section as detected by immunofluorescence and the number of GCs per spleen section was even higher in these PTEN KO mice (**Figures 5A,B**), which are consistent with the results that *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice exhibited an increased levels of GCBs than the control *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice upon immunization (**Figure 3A**; Figure S1B in Supplementary Material).

Repression of AID Induction in GCBs from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ Mice

Our observations suggested that Cγ1 transcription-mediated PTEN KO impairs antibody responses, CSR activity, as well as the DZ and LZ compartmentalization and SHM within the GC. We next investigated the molecular mechanism accounting for

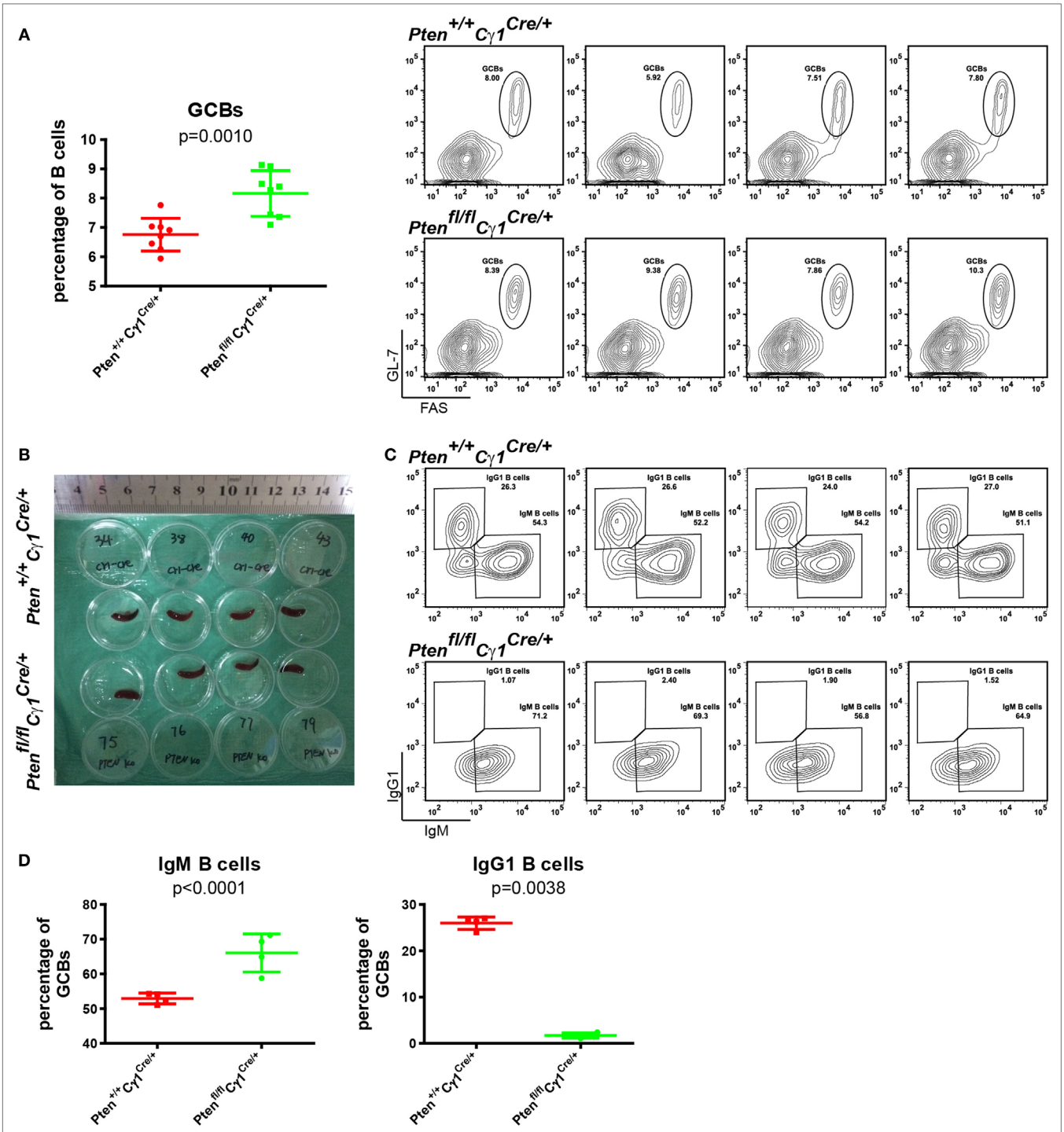

FIGURE 3 | Damaged class-switch recombination in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** The statistical comparison of the percentage of germinal center B cells (GCBs) from splenocytes at day 7 after sheep red blood cell (SRBC) immunization (left). Each symbol represents an individual animal ($n = 8$ for each group). The data represent the mean ± SD from at least three independent experiments. Two-tailed *t*-tests were performed for statistical comparisons. Flow cytometry analysis the increased GCBs number in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice spleen (right). Values indicated the percentage of cells in the gated population. **(B)** Macroscopic appearance of spleen from day 7 after SRBC immunization of *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. Data were given from one representative of at least two independent experiments. **(C)** Representative flow cytometry analysis of IgM, IgG1-BCR expressing GCBs from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice at day 7 after SRBC immunization. Data were given from one representative of at least two independent experiments (n = 4 for each group). The cells were pregated in GCBs. **(D)** The statistical quantification of the flow cytometry data of IgM (left) and IgG1 (right) as shown in **(C)**. Each symbol represents an individual animal (four mice for each group). The data represent the mean ± SD. Two-tailed *t*-tests were performed for statistical comparisons.

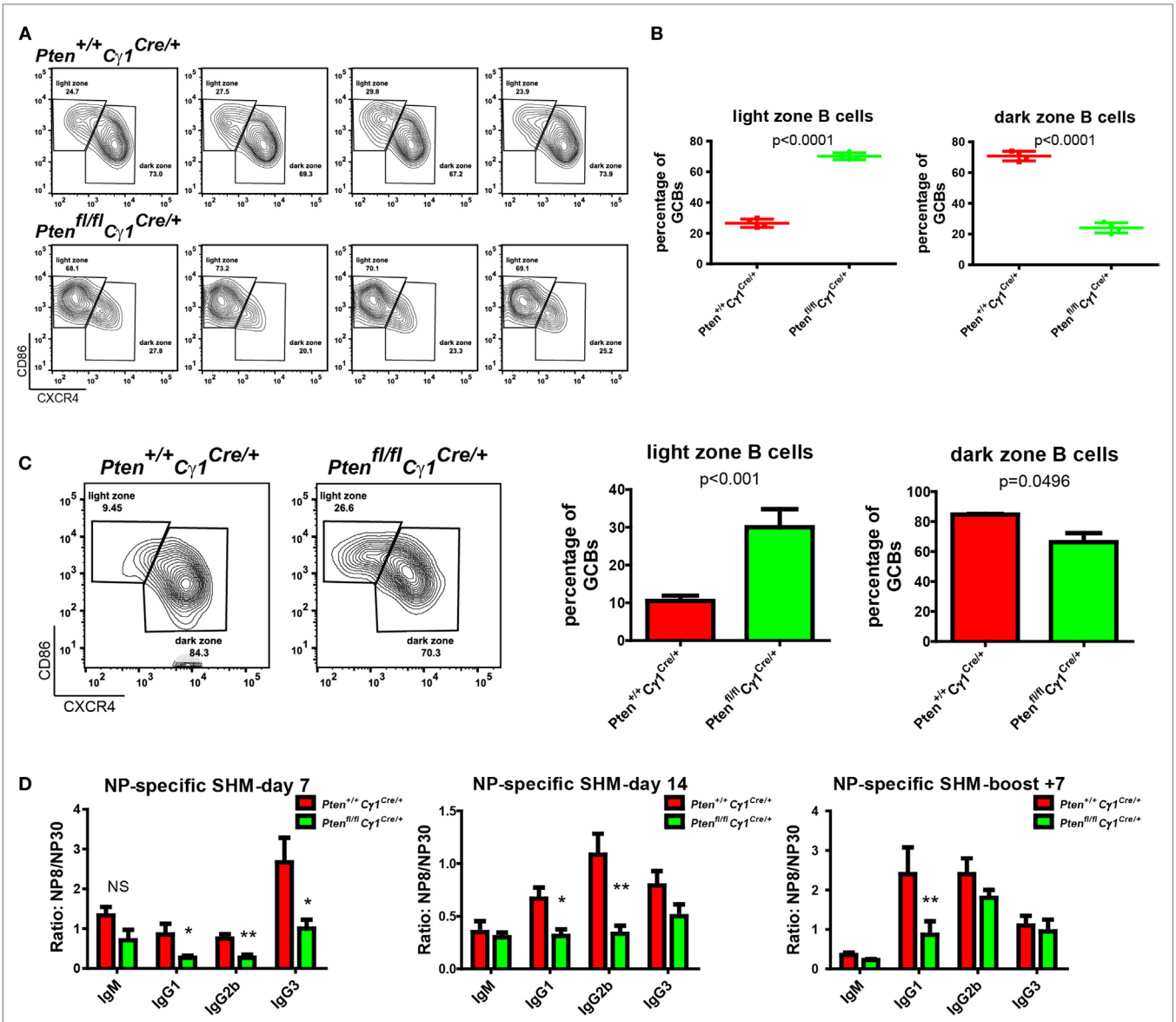

FIGURE 4 | Abnormal germinal center (GC) structure and somatic hypermutation (SHM) in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** Representative flow cytometry analysis of GC light zone (LZ) and dark zone (DZ) splenocytes from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice at day 7 after SRBC immunization. Data were given from one representative of at least two independent experiments ($n = 4$ for each group). The cells were pregated in GCBs. **(B)** The statistical quantification of the flow cytometry data of LZ (left) and DZ (right) splenocytes as shown in **(A)**. Each symbol represents an individual animal (four mice for each group). The data represent the mean ± SD. Two-tailed *t*-tests were performed for statistical comparisons. **(C)** Representative flow cytometry analysis of GC LZ and DZ from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice at day 14 after VLP injection (left). Statistical comparison for LZ (middle) and DZ (right) cells percentage of total GCBs was also shown. The data represent the mean ± SD of six mice per group in three independent experiments. Two-tailed *t* tests were performed for statistical comparisons. **(D)** The affinity maturation of the NP-specific IgM, IgG1, IgG2b, and IgG3 antibodies in *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ versus *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice as determined by the ratio NP8/NP30. The data represent the median ± interquartile range from three independent experiments with six mice per group at the indicated time point and were analyzed with Kruskal–Wallis test. *$p < 0.05$; **$p < 0.01$.

the lack of CSR and SHM in the B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. It is well known that AID, which is specifically induced in the GCBs, is a crucial enzyme responsible for both CSR and SHM (6). Thus, we hypothesized that the PTEN expression level in these *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice shall be influenced before the CSR reaction to drive the switch of IgM-BCR to IgG-BCR expressing B cells, which might subsequently impair the function of AID. To test this hypothesis, splenic B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice were stimulated with LPS and IL-4 to induce CSR *in vitro*. To specifically examine the B cells without effective CSR, we sorted the IgM-BCR expressing B cells from the LPS- and IL-4 stimulated splenic B cells (**Figure 6A**). WB of these stimulated IgM-BCR expressing B cells detected the reduced PTEN and AID protein expression in the cells derived from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice compared to the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ control mice (**Figure 6B**). Meanwhile, these IgM-BCR expressing B cells

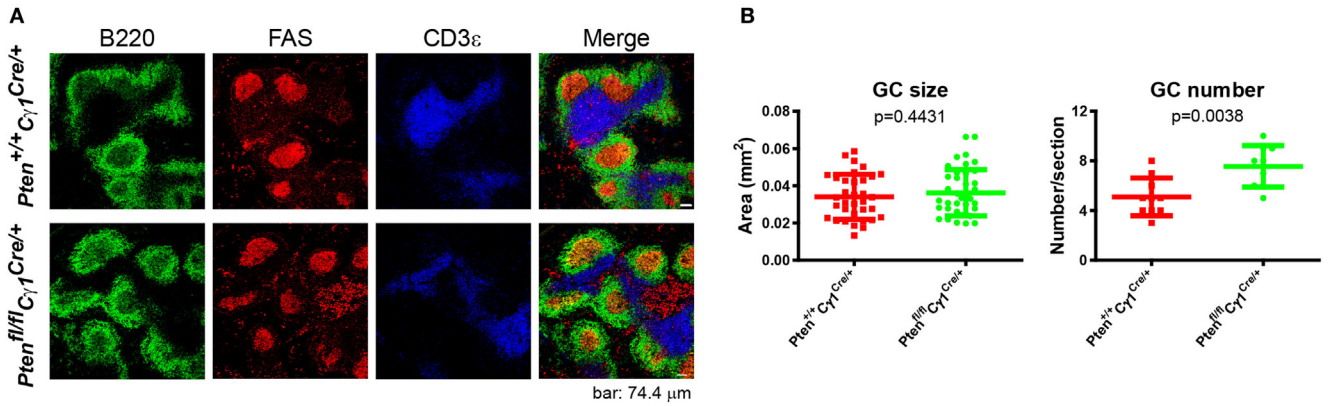

FIGURE 5 | Normal germinal center (GC) size, but abnormal GC number in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** Immunofluorescence analysis of spleen sections from immunized *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice at day 7 after sheep red blood cell immunization. Antibodies detecting B220 (expressed on B cells), FAS (expressed by GCBs), and CD3ε (expressed on T cells) were utilized. Data were given from one representative of at least three independent experiments. The scale bar represents 74.4 μm. **(B)** The statistical quantification of the GC size (left) measured in terms of area or the GC number (right) measured in terms of the number of GCs per each spleen section in *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice as described in **(A)**. The bars represent the mean ± SD from one representative of at least three independent experiments. Two-tailed *t* tests were performed for statistical comparisons.

also showed hyper-phosphorylated AKT and FoxO1 (**Figure 6B**). These results suggested that the AID transcription was affected by the hyper-phosphorylation of AKT and FoxO1 since AKT are known to inhibit the expression and function of AID (16, 23). Indeed, RT-PCR assay demonstrated that the level of PTEN and AID mRNA was markedly reduced in the stimulated IgM-BCR expressing B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice than those B cells from the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ control mice (**Figure 6C**, top and **Figure 6D**). The transcription of Cγ1 was also detected in both the murine control and KO IgM-BCR expressing B cells, which readily explained the Cγ1-mediated PTEN deletion in the IgM-BCR expressing B cells in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice (**Figure 6C**, top and **Figure 6D**). We future validated these conclusions by utilizing purified GBCs *in vivo* from SRBC immunized *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. We sorted the IgM-BCR expressing GCBs at day 7 after SRBC immunization (Figure S3 in Supplementary Material). RT-PCR of IgM-BCR expressing GCBs from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice also detected the significantly reduced transcription of PTEN and markedly reduced AID (**Figure 6C**, bottom and **Figure 6D**). The transcription of Cγ1 was also detected in IgM-BCR expressing GCBs from both *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice (**Figure 6C**, bottom and **Figure 6D**), which readily explained the Cγ1-mediated PTEN deletion in the IgM-BCR expressing GCBs. All these results demonstrated that the PTEN expression level in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice was significantly impaired in GCBs. Thus, PTEN regulated AID transcription through PI3K-AKT signaling pathway in GCBs controls the CSR, IgG antibody response, and SHM.

DISCUSSION

We investigate the function of PTEN in regulating the strength of GC responses by employing a mouse model with the ablation of PTEN through Cγ1-Cre mediated recombination. Upon immunization, we found significantly higher IgM antibody responses and drastically lower IgG1 antibody responses in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice compared to the control *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ mice. Mechanistically, we found that the ablation of PTEN leads to the abnormal GC responses as demonstrated by: (i) severely disturbed compartmentalization of DZ and LZ; (ii) significantly decreased amount of IgG1-BCR expressing B cells; and (iii) the SHM in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice than the control *Pten*$^{+/+}$ *Cγ1*$^{Cre/}$mice. Moreover, an *in vitro* CSR assay for the purified splenic B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice also indicates the blunted CSR under a variety of differentiation-promoting conditions. Interestingly, the results of the impaired GC function in this report are different from the PTEN deletion in *Pten*$^{fl/fl}$ *CD19*$^{Cre/+}$, *Pten*$^{fl/fl}$ *mb1*$^{Cre/+}$ or *Pten*$^{fl/fl}$ hCD20TamCre mice in the published studies (13, 16, 23), the deletion of *Pten*$^{fl/fl}$ loci in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice shall only be effective upon the transcription of *Cγ1-cre* gene, which shall be ideally only available in the IgG1-BCR expressing B cells. Thus, theoretically, only the IgG1 antibody responses shall be affected in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice upon immunization. However, an unexpected observation in this report is that the production of not only IgG1 but also IgG2b and IgG3 were significantly blunted in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. All these intriguing results were explained by the further mechanistic investigations show that LPS- and IL-4 stimulation robustly induced the transcription of Cγ1 in IgM-BCR expressing B cells, which efficiently disrupt the transcription of PTEN and AID in the stimulated splenic IgM-BCR expressing B cells from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. There are eight sets of C_H exons organized as 5′-VDJ-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′ at the *Igh* locus in mice. Upon the CSR, the assembled V(D)J exons from Cμ encoded IgM-expressing naïve B cells is juxtaposed next to one of the sets of downstream C_H exons, allowing the production of different IgH classes (e.g., IgG3, IgG1, and IgG2b) (5). Thus, the transcription of Cγ1 in IgM-BCR expressing B cells and the subsequent disruption of PTEN and AID expression explained the poor production of not only IgG1 but also IgG2b and IgG3 in the *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice.

The observation of the pre-transcription of Cγ1 in IgM-BCR expressing B cells is consistent with the examination of the Cγ1 reporter mice, which reported the Cγ1 reporter gene expression in 85–95% of the GCB fraction 10–14 days after immunization with SRBC (33). Not a surprise, hyper-phosphorylated AKT and FoxO1 are observed as a result of the drastically reduced

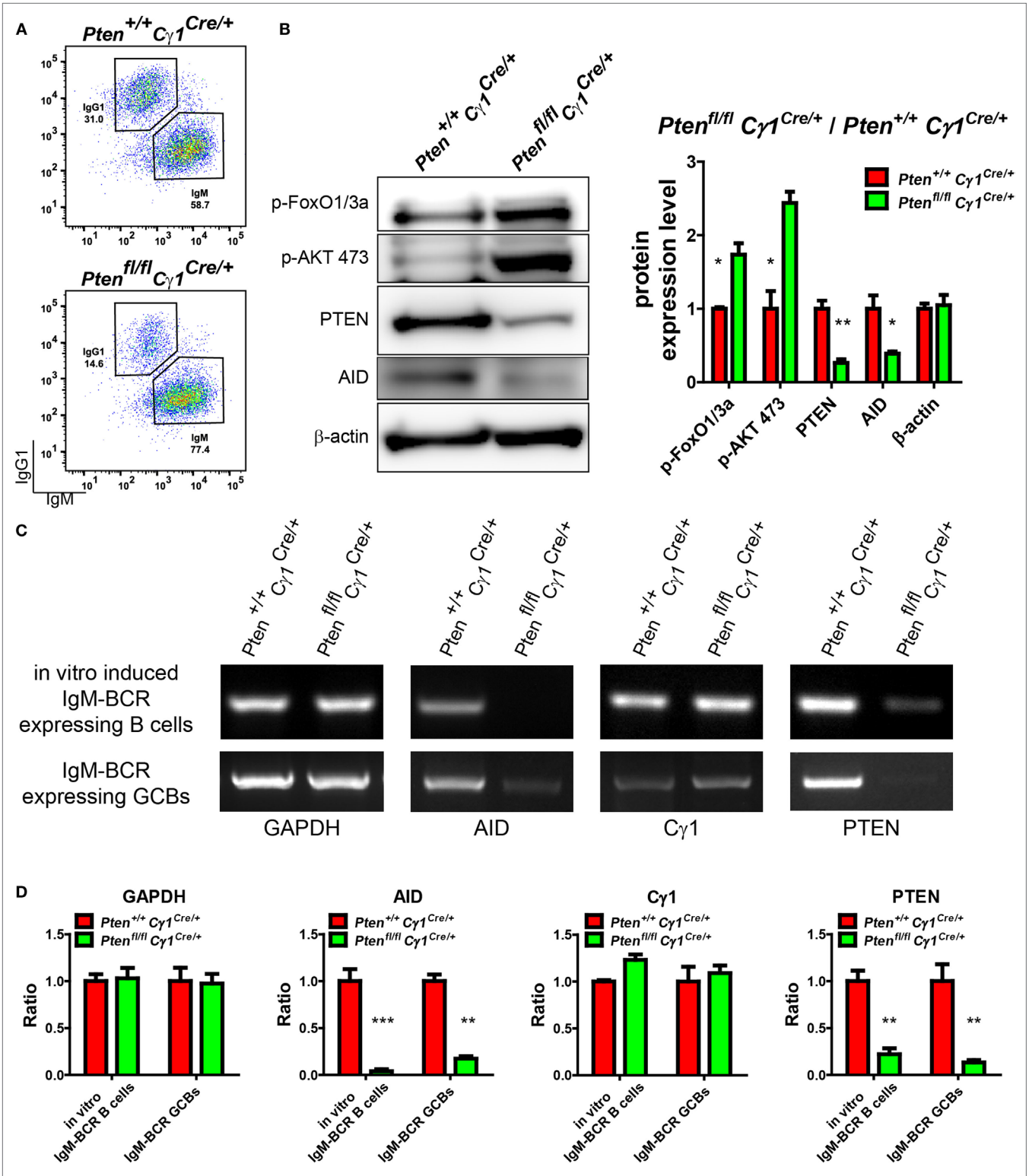

FIGURE 6 | Continued

FIGURE 6 | Repression of activation-induced cytidine deaminase (AID) induction in germinal center B cells (GCBs) from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. **(A)** Flow cytometry sorting of *in vitro* induced IgM-BCR expressing splenic B cells from *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. Pure splenic B cells from control and KO mice (three mice for each group) were stimulated with LPS plus interleukin-4 (IL-4) for 4 days before sorting. **(B)** The expression levels of PTEN, p-AKT, p-FoxO1/3a, and AID were determined in *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ and *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells (left). Reduced PTEN, AID expression and enhanced p-AKT and p-FoxO1/3a expression were observed in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells. The *in vitro* induced IgM-BCR expressing B cells were isolated by the FACSAria III Cell Sorter as described in **(A)**. Statistical comparison of protein expression level was also shown in right. Ratio values of different proteins from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells were normalized to that of the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells. Data were given from one representative of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. **(C)** The mRNA levels of AID, Cγ1, and PTEN were analyzed by RT-PCR. The Cγ1 mRNA was detected in both *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing splenic B cells that was induced by LPS and IL-4 *in vitro* and the sheep red blood cell immunized *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing GCBs. The PTEN mRNA level was reduced and the AID mRNA expression level was nearly undetectable in *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. The *in vitro* induced IgM-BCR expressing B cells were isolated by the FACSAria III Cell Sorter as described in **(A)**, and the IgM-BCR expressing GCBs were isolated by the FACSAria III Cell Sorter as described in (Figure S3 in Supplementary Material). Data were given from one representative of at least three independent experiments. **(D)** Statistical comparison of GAPDH, AID, Cγ1, and PTEN transcription level. Given were the ratio values of different molecules from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells to that of the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing B cells or from *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing GCBs to that of the *Pten*$^{+/+}$ *Cγ1*$^{Cre/+}$ IgM-BCR expressing GCBs. Data were given from one representative of at least three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

expression of PTEN. The reduced transcription of PTEN and AID are also confirmed by investigating the IgM-BCR expressing GCB cells *in vivo* from SRBC immunized *Pten*$^{fl/fl}$ *Cγ1*$^{Cre/+}$ mice. The hyper-phosphorylated AKT and FoxO1 in turn influence the AID expression in IgM-BCR expressing GCBs and reduce the differentiation of DZ GCBs partially through downregulation of the chemokine receptor CXCR4 (14, 15). However, whether or not the reduced expression of AID can directly contribute to the decrease in the DZ GCBs deserves further investigation. In the literature, the important functions of PI3K-AKT pathway on the regulation of cell growth, survival, proliferation, cell cycle and cellular metabolism were also reported (16, 19–22, 34, 35). Thus, it is also of interest to investigate how these events can influence the formation of GC structures.

In conclusion, our research provides an alternative mechanistic explanation for the significantly impaired CSR in PTEN deficient GCBs in addition to the recent published studies showing that constitutive PI3K activation or ablation of FOXO1 impairs AID targeting to particular switch regions. which leads to the partly lost CSR (14, 15). Our results demonstrate that PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody response, and SHM.

ETHICS STATEMENT

All animal protocols used in this study are approved by the IACUC (Institutional Animal Care and Use Committee) of Tsinghua University and performed in accordance with guidelines of the IACUC. The laboratory animal facility has been accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). The assurance identification number is 15-LWL2 and was issued by Dr. Zai Chang, the vice chair of IACUC of Tsinghua University, Beijing, China.

AUTHOR CONTRIBUTIONS

WL and JW conceived, designed, and drafted the article; JW and SL performed experiments and laboratorial analysis; BH and MY supported the materials; JW and WL wrote the manuscript; WL, HQ, ZD, BH, MY, and JW reviewed and approved the manuscript final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00371/full#supplementary-material.

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REVIEW
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Lyn, Lupus, and (B) Lymphocytes, a Lesson on the Critical Balance of Kinase Signaling in Immunity

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Systemic lupus erythematosus (SLE) is a progressive autoimmune disease characterized by increased sensitivity to self-antigens, auto-antibody production, and systemic inflammation. B cells have been implicated in disease progression and as such represent an attractive therapeutic target. Lyn is a Src family tyrosine kinase that plays a major role in regulating signaling pathways within B cells as well as other hematopoietic cells. Its role in initiating negative signaling cascades is especially critical as exemplified by Lyn$^{-/-}$ mice developing an SLE-like disease with plasma cell hyperplasia, underscoring the importance of tightly regulating signaling within B cells. This review highlights recent advances in our understanding of the function of the Src family tyrosine kinase Lyn in B lymphocytes and its contribution to positive and negative signaling pathways that are dysregulated in autoimmunity.

Keywords: Lyn, systemic lupus erythematosus, cell signaling, SFK, B cell, B-cell receptor, autoimmunity, lupus

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SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Systemic lupus erythematosus is a heterogeneous autoimmune disease with multiple clinical manifestations including auto-antibodies, dermatological rashes and ulcers, inflammatory markers, hematological deficiencies, arthritis, renal dysfunction, and neurological disorders (1). The incidence of SLE is approximately 1:1,500; however, the prevalence varies significantly with gender, ethnicity, and age (2). The progression of SLE symptoms is mediated by a dysregulation of innate (e.g., dendritic cells, mast cells, and macrophages) and adaptive immune cells (i.e., B and T lymphocytes) (3). Accumulating evidence emphasizes the contribution of B cells in mediating the development of autoimmunity, particularly through the breakdown in tolerance to self-antigens, the secretion of inflammatory cytokines, and the generation of auto-reactive antibodies that result in immune complex deposition in organs such as the kidneys (4–6). In keeping with this, numerous mutations affecting tyrosine kinases have been implicated in autoimmune disease progression (7), highlighting a critical requirement for the stringent regulation of intracellular signaling cascades in immune cells.

This review will focus on intracellular signaling within B cells, specifically on the role of Lyn in regulating these pathways and its contribution to the progression of autoimmune disease.

Lyn: SRC FAMILY TYROSINE KINASE

Lyn (Lck/yes-related novel tyrosine kinase) is a Src family, non-receptor tyrosine kinase found predominantly in myeloid cells and B lymphocytes (8), but it is also detectable in cell types outside of the hematopoietic compartment (9). *Lyn* is located on chromosome 4 A1 in mice and 8q12 in humans (9). In mice, an additional *lyn* gene encoding exons 1–10 is present within the genome but

is not transcribed (10). Alternate splicing of exon 2 results in the translation of two different isoforms of Lyn, annotated as Lyn A (56 kDa) and Lyn B (53 kDa), that differ by an insertion within the N-terminal variable domain (11, 12). Functional analysis of the individual isoforms indicates a similar capacity to phosphorylate substrate proteins (12–14); however, both isoforms are required for normal activation and regulation of internal signaling (13, 14).

Structural/Functional Regulation of Lyn

Lyn shares architectural and sequence homology with the other SFK members present in hematopoietic cells (e.g., Src, Fyn, Yes, Blk, and Hck) (15). The conserved domain organization of SFK members includes an N-terminal/unique domain, Src homology (SH) 3, SH2 and the catalytic/kinase domain. The N-terminal unique domain (SH4) contains sites for myristoylation and palmitoylation that promote localization and interaction with the cellular membrane and also integration into lipid rafts (16–18). Downstream of the N-terminus, SH3 and SH2 domains regulate the conformational and functional state of Lyn (19). Phosphorylation of the inhibitory tyrosine (Y529 in murine Src, Y508 in Lyn) on the C-terminus leads to a closed and inactive conformation via the binding and "latching" of the SH2 domain, which is further stabilized by the interaction with the SH3 domain (20, 21).

Dephosphorylation of the C-terminal tyrosine by phosphatases or competitive binding for the SH2 domain by an interacting protein relieves the inhibitory conformation imposed by SH3/2 domains, which then promotes phosphorylation of the activating tyrosine (Y416 in Src, Y397 in Lyn) within the kinase domain (19, 21, 22). Phosphorylation of Y397, either in *cis* or in *trans*, changes the conformation of the activation loop, permitting substrate binding and kinase activity (19, 22, 23). Kinase activity is mediated by interactions between the ATP binding loop (G loop) and the ATP hydrolyzing loop (catalytic loop) that facilitate optimal positioning and hydrolysis of the λ-phosphate group from ATP, respectively (20, 24, 25). The λ-phosphate group is then transferred to a protein substrate that is positioned adjacent to the catalytic loop (25). Dephosphorylation of the activating tyrosine or phosphorylation of the inhibitory tyrosine by Csk (c-Src kinase) decreases the kinase activity of Lyn and other SFKs (26).

Lyn REGULATES POSITIVE AND NEGATIVE PATHWAYS IN B CELLS

Activation of Lyn relies on membrane-bound receptor-type phosphatases such as CD45 and CD148 to dephosphorylate the C-terminal inhibitory tyrosine (27–29). After activation, Lyn binds and phosphorylates substrate proteins that possess tyrosine residues flanked predominantly by acidic residues (30–32). The affinity for particular substrates is further regulated by the phosphorylation of the SH2 domain (33). Key substrates of Lyn include proximal membrane-bound cellular surface receptors containing immunoreceptor tyrosine activating motifs (ITAMs) or immunoreceptor tyrosine inhibitory motifs (ITIMs) within their cytoplasmic tails (34, 35). Phosphorylation of these ITAMs and ITIMs leads to the recruitment and activation of other kinases, phosphatases, and adaptor proteins that enhance or inhibit downstream signaling.

Positive Signaling Cascade

The B cell receptor (BCR) comprises the membrane-bound immunoglobulin (Ig) and the heterodimeric signaling subunit Ig-α/Ig-β (CD79α/β) that contain ITAMs within their cytoplasmic tails (36). Antigen binding to the BCR induces a variety of signaling cascades that are initiated by the proximal kinase Lyn (**Figure 1**), which phosphorylates Ig-α/Ig-β ITAMs thereby creating docking sites for the recruitment and activation of Syk (37–39). Activated Syk leads to the phosphorylation and activation of downstream molecules such as the adaptor protein SH2 domain-containing leukocyte protein of 65 kDa (SLP-65) (also known as BLNK or BASH), Btk, and PLCγ2 (40). Upon phosphorylation, SLP-65 organizes a signalosome that promotes calcium (Ca^{2+}) flux and the differentiation of developing B cells (41). Phosphorylated SLP-65 also allows the recruitment of Bruton's tyrosine kinase (Btk), Vav Guanine Nucleotide Exchange Factor 1 (Vav1), and Growth Factor Receptor Bound Protein 2 (Grb2) (42).

Phosphorylation of the non-T-cell activation linker (NTAL) by Lyn leads to the recruitment of Grb2, murine son of sevenless homolog (mSOS), and GRB2-Associated Binding Protein 1 (Gab1) (43). Phosphorylation of Gab1 promotes complex formation between SH2 Domain-Containing Transforming Protein 1 (Shc), non-receptor SH2-containing tyrosine phosphatase 2 (SHP-2), and p85 subunit of phosphatidylinositol 3-kinase (PI3K) (44). Syk-mediated phosphorylation of Shc promotes the interaction between Grb2 and mSOS, which activates Ras-MAPK pathways (45), while interaction between Shc and Gab1 leads to the activation of PI3K pathway (46, 47).

Syk-mediated phosphorylation of CD19 enables the recruitment and activation of p85, which also promotes membrane localization of PI3K (48, 49). Syk also phosphorylates E3 ubiquitin-protein ligase Cbl, which permits the interaction with p85 and activation of PI3K (50). Similarly, Syk and Btk phosphorylate B cell phosphoinositide 3-kinase adapter protein 1 (BCAP), which localizes p85 to glycolipid-enriched microdomains (GEMs) after anti-IgM stimulation (51). PI3K catalyzes the generation of phosphatidyl inositol 3,4,5-trisphosphate (PIP3) from phosphatidyl inositol 4,5-trisphosphate (PI(4,5)P2), which in turn is used as a docking site to activate other effector proteins (52). PIP3 is essential for plasma membrane translocation of Akt, placing it in proximity to 3-phosphoinositide-dependent protein kinase 1 (PDK1) and rapamycin-insensitive companion of mammalian target of rapamycin (Rictor)/mechanistic target of rapamycin (mTOR2) allowing threonine and serine phosphorylation of Akt, respectively (53, 54). Akt phosphorylation and activation following BCR cross-linking leads to the inhibition of the pro-apoptotic protein Bad and activation of the pro-survival proteins Bcl-2 and Bcl-XL (55). Additionally, Akt phosphorylates the transcription factor Forkhead box protein O (FOXO) thereby inducing its exclusion from the nucleus and preventing apoptosis while promoting cell proliferation (56, 57). Akt also activates alpha and beta subunits of the IκB kinase (IKKα/β)

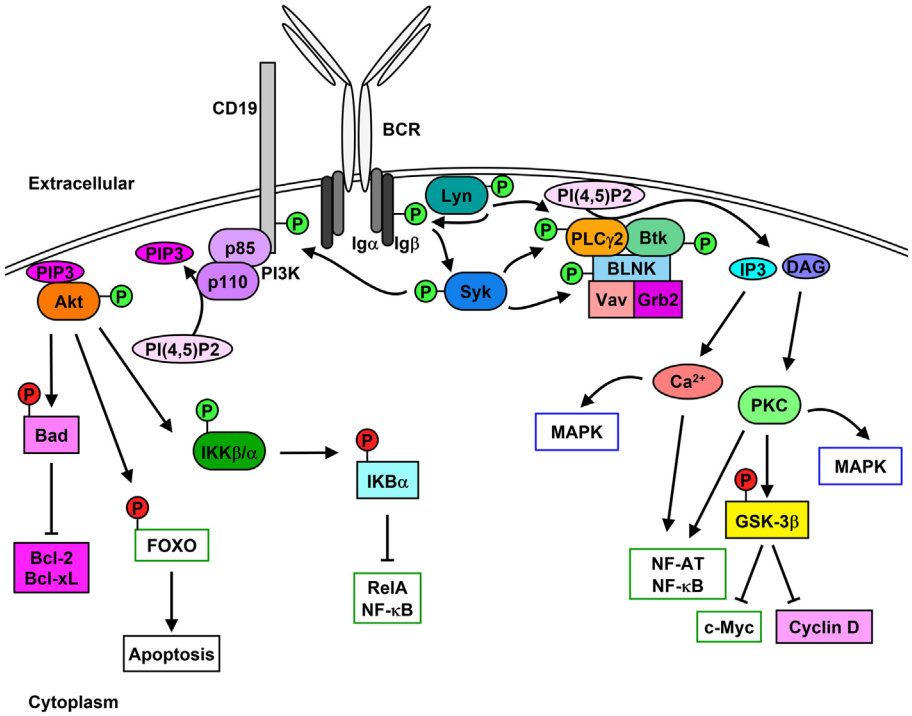

FIGURE 1 | Lyn initiates signaling cascades following B cell receptor (BCR) cross-linking. Stimulation of the BCR leads to the activation [green (P)] of Lyn and Syk which results in Igα/β phosphorylation. PLCγ2 activity leads to the hydrolysis of PI(4,5)P2 to IP3 and diacylglycerol (DAG), which stimulates Ca^{2+} mobilization as well as protein kinase C (PKC) and MAPK activation and cell-cycle progression. Phosphorylation of CD19 leads to the membrane recruitment of phosphatidylinositol 3-kinase (PI3K) and activation of Akt, which phosphorylates downstream proteins leading to their inhibition [red (P)] and up-regulation of pro-survival signaling.

which phosphorylates IκBα and the p65 nuclear factor kappaB (NF-κB) subunit/RelA (58). IκBα phosphorylation leads to its proteasome-mediated degradation and exposure of the nuclear-localization sequence of RelA, which leads to its translocation and up-regulation of pro-proliferation and pro-survival proteins such as c-Myc and Bcl-2 family proteins (59, 60).

Lyn, Syk, Btk, and Blk can also phosphorylate and enhance the activation of phospholipase C gamma 2 (PLCγ2), which hydrolyzes PI(4,5)P2 to create inositol 3,4,5-trisphosphate (IP3) and diacylglycerol (DAG), stimulating Ca^{2+} mobilization and protein kinase C (PKC), respectively (61–64). PLCγ2 phosphorylation also stimulates the activation of MAPK pathways and nuclear location of NF-κB and nuclear factor of activated T cells (NF-AT) (65–67). Additionally, PKC activation leads to the inhibition of glycogen synthase kinase 3 beta (GSK-3β), which promotes the accumulation of beta-catenin in the nucleus and thus up-regulates the expression of c-Myc and cyclin D (68).

B cell scaffold protein with ankyrin repeats 1 (BANK1) interacts with PLCγ2, which is mediated by B-lymphocyte kinase (Blk) (69). BANK1 promotes the Lyn-mediated phosphorylation of the inositol trisphosphate receptor (IP3R) located on the endoplasmic reticulum which mediates Ca^{2+} flux (70).

Negative Signaling Cascade

The role of Lyn in generating the positive signaling cascade in B cells is not essential and can be compensated for the other SFKs (71, 72). However, the role of Lyn in the initiation of negative feedback loops that not only regulate downstream signaling molecules but also limit the activity of SFKs is unique (35, 73). In addition to the phosphorylation of ITAMs after BCR stimulation, Lyn phosphorylates ITIMs contained within receptors including CD22, FcγRIIB, PIR-B, PD-1, CD66a (CEACAM1), CD5, and CD72 (35, 74–79), which act as docking sites for the binding and activation of non-receptor SH2-containing tyrosine phosphatases 1 and 2 (SHP-1 and SHP-2) and, in the case of FcγRIIB, phosphatidylinositol phosphatase 5 (SHIP-1) (76, 80–86). SHP-1 has been demonstrated to dephosphorylate and, therefore, down-regulate the activity of Btk, Syk, and Lyn, leading to the inhibition of signaling cascades (85, 87–89). Similarly, SHP-2 has been shown to dephosphorylate Ig-β, Syk, and PLCγ2 following BCR cross-linking (76). However, SHP-2 has also been implicated in enhancing various signaling pathways (90).

Upon BCR activation (**Figure 2**), SHIP-1 dephosphorylates PIP3 to P(3,4)P2, which decreases PI3K- and Akt-mediated signaling (91). The decrease of available PIP3 also leads to the down-regulation of Btk activity and subsequent reduction of PLCγ2-mediated Ca^{2+} mobilization (92). Activated SHIP-1 recruits downstream of tyrosine kinase 1 (Dok1/p62dok), which down-regulates MAPK signaling pathways (93). FcγRIIB phosphorylation leads to the Dok3-mediated recruitment of SHIP-1 into the Grb2/Shc/mSOS complex, which inhibits SFK-dependent activation of Syk and decreases NF-κB and MAPK signaling pathways (94–97). A more direct role of Lyn in negative signaling is the phosphorylation of the Csk-binding protein/phosphoprotein

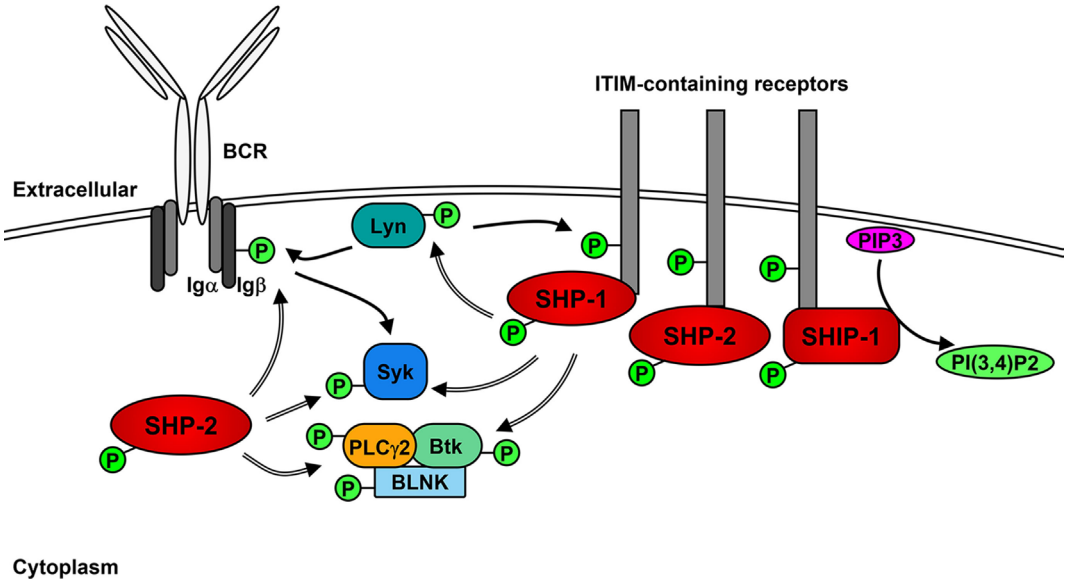

FIGURE 2 | Lyn activates negative receptors following B cell receptor (BCR) cross-linking. In addition to initiating positive signaling, Lyn phosphorylates receptors containing immunoreceptor tyrosine inhibitory motifs (ITIMs), leading to the recruitment and activation of phosphatases SHP-1, SHP-2, and SHIP-1. SHP-1 and -2 dephosphorylate (=> arrows) and inhibit the activity of Lyn, Syk PLCγ2 and Btk leading to the down-regulation of positive signaling events. SHIP-1 dephosphorylates PIP3 and prevents activation of Akt signaling pathways.

associated with glyco-sphingolipid microdomains (Cbp/PAG), which activates Csk to down-regulate the activity of Lyn and other active SFKs (98).

Lyn also mediates internalization of the BCR, which acts to dampen signaling by the BCR (99) and may also play a role in the dephosphorylation of BCAP, which down-regulates PI3K activity (51). Similar to the regulation of active Src (100), the kinase activity of Lyn also promotes its own ubiquitination and subsequent degradation in B cells, most likely via the activity of Csk and suppressor of cytokine signaling (SOCS) proteins (101–104).

IN VIVO MODELS OF Lyn ACTIVITY AND AUTOIMMUNITY

Consistent with the important role for negative signaling in the hematopoietic compartment, mice lacking Lyn ($Lyn^{-/-}$) progressively develop symptoms of autoimmunity that are comparable to SLE in humans (105). $Lyn^{-/-}$ mice produce high titers of anti-nuclear antibodies (ANA) and develop splenomegaly, systemic inflammation, antibody complex deposition in the kidneys and glomerulonephritis (105). B cells from $Lyn^{-/-}$ mice display hyper-phosphorylated Akt, MEK1/2, Erk1/2, and JNK compared to wild-type (WT) B cells after BCR stimulation, indicating enhanced positive signaling in the absence of negative feedback inhibition (23, 72, 106). In keeping with this, there is a reduced phosphorylation of FcγRIIB, CD22, SHP-1, and SHIP-1 (73, 74).

The activation of Lyn-dependent inhibitory signaling in mature B cells is essential for maintaining B cell tolerance (107), including B cell anergy (108). In Lyn-deficient mice, the absence of inhibitory signaling in mature B cells increases their sensitivity to antigen stimulation and this population of now auto-reactive cells is selectively depleted via clonal deletion (109). As such, $Lyn^{-/-}$ mice show a significant reduction in naive, mature B cells in the periphery compared to WT mice despite similar frequencies of newly formed, immature B cells (105). The naive B cells that do persist in the periphery of $Lyn^{-/-}$ mice are, however, hyper-responsive to anti-IgM stimulation, show delayed but increased Ca^{2+} mobilization and express markers of activation (107, 109–111). Additionally, antibody secreting B cells (plasma cells) persist in lymphoid tissues at 10-times normal frequency (105, 111). The myeloid compartment is also expanded, and T cells display markers of activation (101, 112). Increased serum levels of IL-6, IFN-γ, and BAFF, primarily produced by B cells, T cells, and myeloid cells respectively, promote the activation and proliferation of immune cells, further driving autoimmunity (112, 113).

Components of the autoimmune phenotype of $Lyn^{-/-}$ mice are B cell intrinsic. This has been demonstrated using a B-cell-specific deletion of Lyn ($Lyn^{fl/fl}$ mb-1Cre), which recapitulates the generation of hyper-responsive B cells, auto-antibodies and the development of glomerulonephritis (114). This phenotype may be dependent on signaling through toll-like receptor (TLR) adaptor protein myeloid differentiation primary response gene 88 (MyD88), as B-cell-specific deletion of MyD88 from $Lyn^{-/-}$ B cells ameliorated auto-antibody production, T cell activation, myeloid expansion, and the development of glomerulonephritis (114). Additionally, global deletion of MyD88 from $Lyn^{-/-}$ mice attenuated autoimmune disease development, which was considered to be due to reduced production of inflammatory

cytokines IL-6 and IL-12 by Lyn$^{-/-}$ dendritic cells (115, 116). This result was supported by Lyn being found to negatively regulate TLR-MyD88-IRF5-dependent expression of Type 1 IFNs in dendritic cells and by the double knock out of Lyn and IRF5 (Lyn$^{-/-}$ IRF5$^{-/-}$) in mice ameliorating SLE-like pathology (117). Perhaps surprisingly, the persistence of plasma cells was found to be independent of autoimmune disease and intrinsic to the Lyn$^{-/-}$ hematopoietic compartment (118). Similarly, mice double deficient in Lyn and IL-6 retain the plasma cell hyperplasia but show a dramatic reduction in kidney damage, splenomegaly, and the production of ANAs (112). As in B cells, Lyn controls intracellular signaling intensities within plasma cells in response to stimulation by cytokines thought to play a critical role in plasma cell survival (118).

The deletion of MyD88 in Lyn$^{-/-}$ mice also decreased spontaneous germinal center (GC) formation thus implicating GC reactions in the generation of pathogenic ANA in Lyn$^{-/-}$ mice (119). In line with this, removing T cells (TCRβ/δ$^{-/-}$) or deleting the adaptor protein SAP (SAP$^{-/-}$), thereby preventing T cell B cell interactions required for GC formation, leads to the reduction of IgG auto-antibodies in Lyn$^{-/-}$ mice (119). Similarly, deleting IL-21, a key regulator of GC responses and plasma cell formation (120), in Lyn$^{-/-}$ mice leads to reduced IgG ANAs but does not alleviate IgM ANA, plasmacytosis, or glomerulonephritis (121), suggesting that IgM and/or IgA can mediate the development of autoimmunity (122). In keeping with this, the autoimmune disease in the *sanroque* lupus mouse model also develops in the absence of germinal centers and depends on IgM (123).

While Lyn can act as a positive or negative regulator of signaling cascades, its role as a negative regulator is critical to the development of the phenotype seen in Lyn$^{-/-}$ mice. Indeed, the fact that dysregulation of other modulators of negative signaling, which are themselves targets of Lyn, also leads to autoimmunity (e.g., CD22, FcγRIIB, SHP-1, and SHIP-1) (124–128) helps define the pathways involved. This is also apparent in the compounding autoimmune disease phenotype in mice heterozygous in Lyn and SHP-1 (Lyn$^{+/-}$, Mev$^{+/-}$) (129). Finally, the importance of Lyn-regulated pathways in hematopoietic cells other than B cells is revealed by co-deletion of Btk, a key intermediate of several positive signaling pathways, from Lyn$^{-/-}$ mice. This alleviates symptoms of autoimmunity and the production of auto-antibodies, but B cells remain hyper-responsive to anti-IgM stimulation as measured by Ca^{2+} mobilization and the phosphorylation of Erk1/2 and Akt (130, 131). Consistent with this, mice deficient in Lyn and p110δ, a PI3K isoform, also show a reduction in inflammation, splenomegaly, T cell activation, ANA production, and glomerulonephritis, while hyper-phosphorylation of Akt and Erk1/2 compared to control mice after BCR cross-linking remained (132), indicating a unique requirement for Lyn in regulating these signaling responses, but one that is insufficient by itself to permit development of disease.

Lyn's enzymatic activity appears to be critical to its function, as mice expressing Lyn with no or impaired kinase activity still develop autoimmune disease, albeit with delayed onset and reduced severity (23, 24). B cells from Lyn^{Mld4} or kinase dead Lyn (Lyn^{KD}) mice, harboring a mutation within the activation loop, display similar signaling kinetics to Lyn$^{-/-}$ B cells, with hyper-Ca^{2+} mobilization, hyper-phosphorylated Erk1/2, Akt, and JNK and reduced phosphorylation of Syk, SHIP-1, and SHP-1 after BCR stimulation (23). Expansion of the myeloid compartment, splenomegaly, and the production of IgG anti-dsDNA antibodies were significantly reduced in Lyn^{KD} compared to Lyn$^{-/-}$ mice, but evidence of immune complex deposition in the kidneys remained (23). In contrast are Lyn^{WeeB} mice, with a mutation in the G-loop of Lyn that leaves partial kinase activity, conferring B cell signaling kinetics that are intermediate between WT and Lyn$^{-/-}$ (24). Stimulation of Lyn^{WeeB} B cells results in a partial decrease in the phosphorylation of Syk, Btk, PLCγ2, SHIP-1, and CD22 and a slight increase in the phosphorylation of Erk1/2, Akt, and JNK compared to WT B cells (24). However, in older Lyn^{WeeB} mice, splenomegaly, anti-dsDNA antibodies, and glomerulonephritis were comparable to those in Lyn$^{-/-}$ mice (24), indicating that the partial positive signaling permitted by Lyn^{WeeB} requires greater negative signaling to counterbalance its activity and prevent the development of autoimmunity. This is exemplified in mice expressing a mutant form of constitutively active Lyn (Lyn^{Y508F} or Lyn$^{up/up}$), as they develop an autoimmune disease with an increased rate of mortality (male-specific) compared to Lyn$^{-/-}$ mice (102). Lyn$^{up/up}$ B cells display constitutive phosphorylation of proteins involved in positive (Syk, PLCγ2) and negative (CD22, SHP-1, SHIP-1, FcγRIIB) signaling pathways, which is further increased after BCR stimulation (102). Despite the increased phosphorylation of ITIM-containing negative regulators, Lyn$^{up/up}$ B cells display enhanced Ca^{2+} mobilization compared even to Lyn$^{-/-}$ B cells, indicating that the increased positive signaling further outweighs the inhibitory signaling capacity within these cells (102). Thus, in the hematopoietic compartment, Lyn activity controls both negative and positive signaling and its dysregulation in mice is responsible for the breakdown of tolerance in B cells and progressive development of autoimmunity.

Lyn AND SLE

Systemic lupus erythematosus is a heterogeneous disease with varied clinical presentations and manifestations (1). It is therefore not surprising that its cause is multifactorial, with numerous genetic and environmental factors contributing to pathogenesis (1, 133). As such, a single gene defect in mice such as Lyn$^{-/-}$ is unlikely to replicate the complex phenotype seen within SLE in humans. Despite this, a reduction in Lyn expression, via increased turnover or reduced transcription, and altered sub-cellular localization are reported in patients with SLE (134–136). However, a significant susceptibility association of the *Lyn* locus with SLE has only been determined in a single case–control association study (137). Interestingly, proteins involved in the positive (Blk, BANK1) and negative (PTPN22/PEP, Csk, FcγRIIA, FcγRIIB, FcγRIIIA, FcγRIIIB, SOCS1) signaling pathways in B cells have all been linked to SLE via genome-wide association studies (GWAS) and this highlights the importance of regulating these signaling cascades to avoid disease onset or progression (138–143). Similarly, Blimp1 and Ets1, transcription factors involved in regulating plasma cell development, are also linked with SLE (141, 144). Mice deficient in *Ets1* show enhanced generation of

plasma cells and auto-antibodies, which are symptoms of autoimmunity (145). Recently, Lyn was linked with regulating *Ets1* expression, via the activation of CD22 and SHP-1 reducing the Btk-dependent down-regulation of *Ets1* (146, 147).

$Lyn^{-/-}$ mice are one of the numerous mouse models reported to develop an SLE-like illness, with none being a perfect recapitulation of the spectrum of human disease (148). Regardless, the $Lyn^{-/-}$ mouse model has been and remains a useful tool in dissecting the critical role that B cell signaling pathways play in the development of autoimmunity. Indeed, other models in conjunction with $Lyn^{-/-}$ mice have confirmed the importance of B cell dysregulation to the development of SLE-like autoimmunity. Deleting B cells in the MRL/lpr mouse model, for example, significantly decreases disease progression and mortality compared to mice that remain B cell replete (149). Furthermore, comparison of $Lyn^{-/-}$ with other mouse models has identified several potential novel therapies for patients with SLE such as inhibitors of BTK and HDAC (150, 151). Further determination of the molecular pathways responsible for B cell dysregulation in SLE-like autoimmunity will likely assist in the design of new treatments to ameliorate disease severity.

CONCLUDING REMARKS

The role of Lyn in B cells involves fine-tuning of BCR signaling, balancing positive and negative signals to maintain tolerance to self-antigens while permitting responsiveness to foreign antigens. The Lyn-mediated phosphorylation of ITIM-containing negative receptors and subsequent activation of the inhibitory phosphatases, SHP-1 and SHIP-1, that leads to the down-regulation of BCR-mediated signaling cascades and inhibition of SFK activity represents a critical component in B cell signaling that prevents the development of autoimmunity.

Although defects in $Lyn^{-/-}$ mice are not an identical model for human SLE, the investigation of Lyn and the pathways it modulates have highlighted the delicate balance inherent in B cell kinase signaling cascades and the devastating consequences that can occur when they are dysregulated. Numerous SLE susceptibility genes identified through GWAS are also linked with other autoimmune diseases indicating the involvement of shared pathways that ultimately lead to the loss of tolerance (152). Therefore, future experiments examining genomic regulation or global phospho-proteomics in models of SLE could be useful in identifying all the components of the intracellular pathways involved and through that, potential therapeutic targets.

AUTHOR CONTRIBUTIONS

EB, SI, and ML drafted the article. DT contributed to writing and provided critical review. All authors were involved in the revising process and approve the manuscript for submission.

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REVIEW
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TANK-Binding Kinase 1-Dependent Responses in Health and Autoimmunity

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The pathogenesis of autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is driven by genetic predisposition and environmental triggers that lead to dysregulated immune responses. These include the generation of pathogenic autoantibodies and aberrant production of inflammatory cytokines. Current therapies for RA and other autoimmune diseases reduce inflammation by targeting inflammatory mediators, most of which are innate response cytokines, resulting in generalized immunosuppression. Overall, this strategy has been very successful, but not all patients respond, responses can diminish over time and numerous side effects can occur. Therapies that target the germinal center (GC) reaction and/or antibody-secreting plasma cells (PC) potentially provide a novel approach. TANK-binding kinase 1 (TBK1) is an IKK-related serine/threonine kinase best characterized for its involvement in innate antiviral responses through the induction of type I interferons. TBK1 is also gaining attention for its roles in humoral immune responses. In this review, we discuss the role of TBK1 in immunological pathways involved in the development and maintenance of antibody responses, with particular emphasis on its potential relevance in the pathogenesis of humoral autoimmunity. First, we review the role of TBK1 in the induction of type I IFNs. Second, we highlight how TBK1 mediates inducible T cell co-stimulator signaling to the GC T follicular B helper population. Third, we discuss emerging evidence on the contribution of TBK1 to autophagic pathways and the potential implications for immune cell function. Finally, we discuss the therapeutic potential of TBK1 inhibition in autoimmunity.

Keywords: TANK-binding kinase 1, type 1 interferons, germinal center, autophagy, humoral immunity, autoimmunity

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INTRODUCTION

TANK-binding kinase 1 (TBK1) is an IKK-related serine/threonine kinase best known for the induction of antiviral type I interferons (IFN-Is) in innate immunity. However, a growing body of evidence highlights the relevance of TBK1 for other responses. In this review, we discuss our present understanding of the role of TBK1 in nucleic acid sensing pathways, antibody responses, and autophagy. We conclude by speculating how these diverse TBK1-regulated responses could potentially culminate in the induction, promotion, and maintenance of autoimmunity, as well as how

pharmacological modulation of TBK1 could represent an alternative treatment strategy, particularly in the context of humorally mediated autoimmunity.

TBK1 Overview

TANK-binding kinase 1 is an IKK-related serine/threonine kinase, best known for the induction of innate antiviral type I IFNs. However, TBK1 potentially has much broader functions, which we discuss in this review (**Figure 1**). TBK1 is ubiquitously expressed in both hematopoietic and non-hematopoietic compartments. Germline deletion of TBK1 is embryonically lethal in mice (1), highlighting its homeostatic functions during development. Through biochemical studies, TBK1 was shown to be activated by double stranded (ds)-RNA (*via* TLR3-TRIF), LPS (*via* TLR4-TRIF), viral RNA (*via* RIG-I-MAVS), and dsDNA (*via* cGAS-STING) in innate immune signaling pathways (2, 3). TRIF (TIR-domain-containing adapter-inducing IFN β), MAVS (mitochondrial antiviral-signaling), and STING (stimulator of IFN genes) are innate immune adaptor proteins that transduce signal downstream of their corresponding sensors to the activation of interferon regulatory factor 3 (IRF3). Mechanistically, TBK1 activation is thought to occur *via* trans-autoactivation, in response to adaptor proteins that shuttle TBK1 to specific signaling complexes and direct subcellular localizations, such as to the ER-Golgi compartments (4–7). Activated TBK1 then phosphorylates IRF3 and induces the production of type I IFN-Is (8–12). Other TBK1 substrates include AKT (13, 14) and PLK1, which are involved in TLR activation or oncogenicity of cancer cells (15). Closely related to TBK1, IKKε shares 60% homology and is initially thought to participate also in IFN-Is induction (8, 9). Subsequent studies show that IKKε is dispensable for IFN-I responses (16). IKKε is abundantly expressed in T cells and have been shown to regulate a number of T cell responses (17–19).

Interferons are a family of cytokines with potent antiviral priming effects, but are also associated with humorally mediated autoimmune diseases, most notably systemic lupus erythematosus (SLE) (20–22). Recently, TBK1 was also shown to associate with the inducible T cell co-stimulator (ICOS) in $CD4^+$ T follicular B helper (T_{FH}) cells that support efficient antibody responses (23). However, the downstream target(s) of TBK1 in ICOS signaling have not yet been identified. Finally, TBK1 is also implicated in promoting autophagy by phosphorylating autophagy receptor proteins, including optineurin (OPTN), SQSTM1/p62, and NDP52 (24, 25). TBK1-mediated regulation of autophagy is currently under evaluation because TBK1 haploinsufficiency is a major risk factor in neurodegenerative diseases (26, 27).

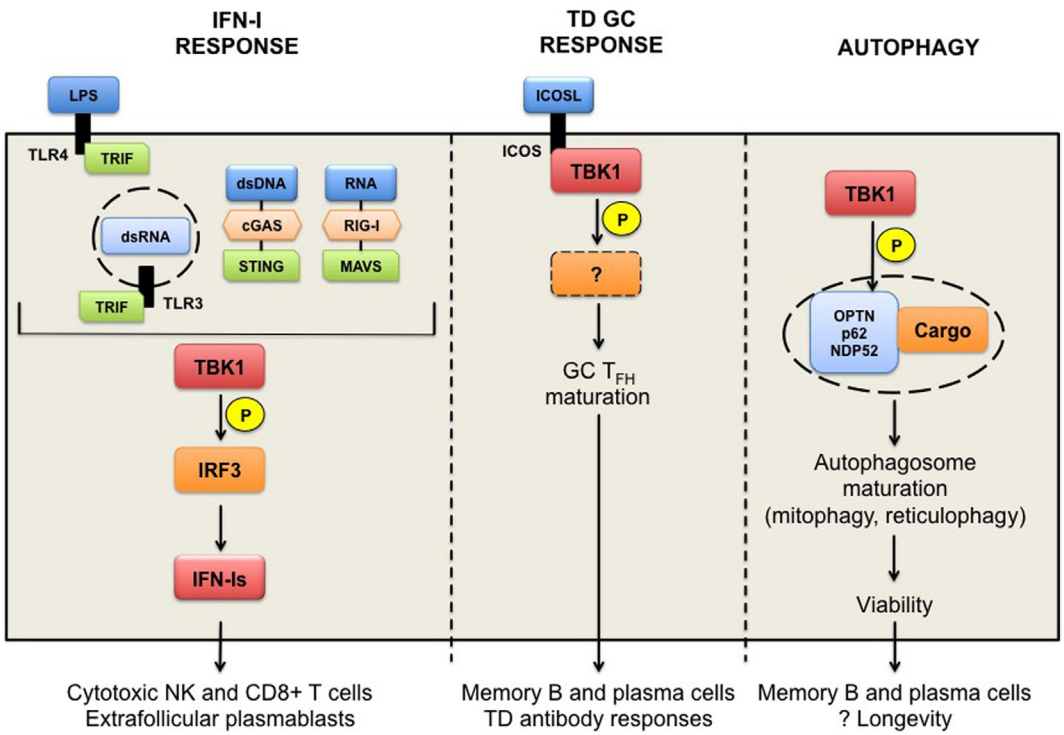

FIGURE 1 | TANK-binding kinase 1 (TBK1) in humoral responses. TBK1 functions downstream of TLR3/4-TRIF and DNA receptor cGAS-STING pathways leading to the activation of the transcription factor interferon regulatory factor 3 and the production of interferons (IFN-Is). Chronic IFN-Is prime cytotoxic functions promote the survival of NK and $CD8^+$ T cells, presumed to have pathogenic roles in autoimmunity, as well as the formation of extrafollicular plasmablasts. TBK1 is also implicated in the inducible T cell co-stimulator (ICOS) signaling pathway in T follicular B helper (T_{FH}) cells to thymus-dependent (TD) antigens. TBK1 is recruited to and activated upon ICOS engagement to ICOS ligand, and promotes the maturation of pre-T_{FH} to germinal center (GC) T_{FH} cells. TBK1 targets downstream of ICOS signaling remain to be determined. TBK1-driven ICOS signaling is necessary for the generation of GC-derived memory B and plasma cells, and TD antibody responses. Finally, TBK1 can promote autophagy through the phosphorylation of autophagy receptors proteins (optineurin, p62, or NDP52), which sequester ubiquitinated cargo (damaged or redundant organelles). Mitophagy in memory B cells and reticulophagy in plasma cells are required for their longevity *in vivo*. TBK1 involvement in these processes remains to be determined.

Autophagy is thought to protect senescent neuronal cells from the accumulation of defective or redundant organelles. TBK1 may likewise physiologically protect long-lived immune cells through autophagy (28). Although no particular TBK1 genetic variants have to date been directly linked to the development of autoimmune diseases, the diverse functions of TBK1 may contribute to one or more aspects of autoimmunity, which is the focus of this review.

TBK1 and Type I IFNs

TANK-binding kinase 1 has a well described role in activating the transcription factor IRF3 to induce type I IFNs production (8–12). IFN-Is are a family of cytokines with pleiotropic functions that have potent antiviral and antimicrobial effects against some intracellular bacteria, but are also implicated in pathogenesis of SLE (SLE, discussed below). TBK1 is ubiquitously expressed in both hematopoietic and non-hematopoietic compartments and it is activated by sensor-adaptor pairs, including TLR3/4-TRIF, RIG-I-MAVS, or cGAS-STING, in response to LPS, dsRNA, virus infection, and cytoplasmic DNA, respectively (2, 3). Consequently, TBK1$^{-/-}$ mouse embryonic fibroblasts (MEFs) have impaired production of IFN-Is (IFN-α and IFN-β) and IFN-inducible chemokines (CCL5 and CXCL10), among other genes, following activation with synthetic dsRNA (poly I:C) or viruses, or LPS (8, 10).

Molecular characterization of the sequence of events leading to TBK1 induction, IRF3 activation, and IFN-Is production have mainly been performed in cell lines and MEFs, in response to LPS stimulation or in the context of antiviral responses (2, 3, 8, 10). Elucidating roles for TBK1 in more complex biological settings *in vivo* has been challenging due to the embryonic lethality of germline TBK1-deficiency in mice. This is thought to be due to TNF-α-induced hepatocyte apoptosis and can be rescued by combined loss of TNF (i.e., TBK1$^{-/-}$ TNF$^{-/-}$ mice are viable) (1). Subsequently, TBK1 has been suggested to control cell survival through PAI-2/serpinB2 and transglutaminase 2 in the TNF-activated anti-apoptotic response (29).

High levels IFN-α or induction of IFN-stimulated genes (i.e., the "IFN signature") is a remarkably consistent feature of SLE and is associated with high titers of affinity-matured autoantibodies and worse disease outcome (20, 21, 22). A similar IFN signature and correlation with high levels of autoantibodies and disease activity is also found in some patients with RA and primary Sjogren's syndrome (30, 31) consistent with a pathogenic role for IFN-α in autoimmunity. Consequently, the possibility of targeting TBK1-dependent IFN-Is induction has received attention as a treatment strategy (32).

IFN-Is in Protective and Pathogenic Immune Responses

Among members of the IFN-I family in humans and mice, IFN-α and IFN-β are the best characterized and most broadly expressed. They signal through a shared, ubiquitously expressed heterodimeric receptor (IFNAR), and prime a rapid antiviral response that acts directly or indirectly on many cell types, including NK cells, T cells, B cells, DCs, and macrophages (33–35). IFNAR signaling mediates early attrition of existing memory CD8$^+$ T cells in response to viral infections, which is thought to permit a more vigorous, diverse, and efficient T cell response emanating from the naïve T cell pool (36). In later stages, IFNAR signaling in activated cytotoxic CD8$^+$ T cells (CTLs) (37) and NK cells (38) is important for long-term survival against perforin-mediated cytotoxicity, thereby preventing rapid elimination *in vivo* and sustaining antiviral immunity. IFNAR signaling is also required for optimal NK cell effector function through upregulation of granzyme B (38). IFN-Is is gaining attention in anti-cancer therapy, where it is generally considered pro-cytotoxic for CTLs and presumably NK cells. This is exemplified by the observations that IFNAR downregulation in CTLs endows colorectal cancers with an immune-privileged niche that promotes aggressive tumorigenesis, associated with poor prognosis, and lessens the response to immunotherapy. Conversely, IFNAR expression suppresses tumor growth and improves the efficacy of combined anti-cancer chimeric antigen receptor T cell transfer and PD-1 inhibition (39). Targeted intratumoral delivery of IFN-I-inducing (i.e., interferogenic) cyclic dinucleotide GMP-adenosine monophosphate (AMP), which activates the STING-TBK1 pathway and IFN-Is production in endothelial cells, has been shown to control tumor growth by boosting antitumor CD8$^+$ T responses in murine models of melanoma and colon cancer (40).

Emerging evidence also implicates dysregulated NK cells and CD8$^+$ T cells in SLE and potentially RA (41). Despite an overall reduction in circulating NK cell number in lupus patients and lupus mouse models, presumably owing to activation-induced death of these cells, NK cells with an activated phenotype infiltrate the kidneys of pre-disease lupus mice and may contribute to tissue injury by releasing cytotoxic granules (42). Another study showed that SLE patients have an expanded population of CTLs, which may contribute to tissue damage (43). Further studies are needed to determine whether IFN-Is contribute to the activation of these human effector cells.

Persistent IFN-Is exposure, particularly IFN-α, has long been implicated in immune dysfunction and autoimmune diseases, through a number of mechanisms. Some patients treated with IFN-α therapy develop autoimmunity, including RA and lupus-like autoimmune syndrome (44, 45). Chronic IFN-α overexpression *in vivo* induces rapid and lethal lupus, with immune complex glomerulonephritis in NZB/W lupus-prone mice (46). Such excess IFN-α can also induce sustained B cell proliferation *in vivo*, accompanied by uncontrolled production of proliferating, short-lived, autoantibody-secreting plasmablasts in secondary lymphoid organs of NZB/W mice (47). pDC-derived IFN-Is have been shown to increase the translocation of marginal zone B cells to the follicular region of the spleen, which disrupt the ability of marginal zone macrophages to clear apoptotic cells and promote the loss of immune tolerance to apoptotic cell-derived antigens in SLE (48). IFN-Is also promotes affinity maturation of antibodies by activating DCs to produce IL-6 (49). The severity of lupus-related pathology is attenuated with IFNAR-deficiency or IFNAR-blocking antibody in several murine lupus models (50–52).

While the involvement of IFN-Is-IFNAR signaling is a consistent feature of murine lupus models, there is less consensus in RA. In contrast to the association of IFN-α with humoral autoimmunity,

IFN-β has homeostatic and anti-inflammatory functions. In RA synovium, IFN-β reduced the secretion of RA-associated pro-inflammatory mediators, including IL-6, TNF-α, matrix metalloproteinases, and prostaglandin E2 (53). IFN-β also primes an anti-inflammatory phenotype of endothelial cells by upregulating the expression of CD73, an ecto-5′-nucleotidase that produces anti-inflammatory adenosine from AMP, at least in neuroinflammation (54). Other studies have shown that exogenous IFN-β can inhibit autoimmune collagen-induced arthritis (CIA) (55, 56). In contrast, IFN-β deficient mice develop prolonged CIA, with a higher incidence relative to control mice (57). IFN-β delivery has been used therapeutically in multiple sclerosis (58) and has been considered for RA (59). Monoclonal antibody therapies inhibiting IFN-Is signaling or depleting of IFN-overproducing plasmacytoid DCs (pDCs) are under evaluation for the treatment of SLE (60, 61). The opposing roles of IFN-α and IFN-β clearly require careful consideration in relation to these potential IFN-Is-targeted therapies in autoimmunity.

TBK1, IFN-Is, and Humoral Autoimmunity

The prevailing concept in SLE and murine lupus models is that immune complexes containing autoantibodies bound to self-DNA and RNA can act as interferogenic stimuli, following Fc receptor-mediated internalization and activation of endosomal TLR7 and TLR9 in pDCs (62). TLR7- or TLR9-mediated induction of IFN-Is, however, does not require TBK1. For instance, TBK1 is not required for IFN-I production in the TLR7-dependent pristane-induced lupus model (51). TLR9 ligand (CpG-B) induces IFN-Is production by B cells and DCs through IRF3, but independently of TBK1. Autocrine IFNAR signaling in B cells is required for enhanced IgM and IgG2a autoantibody production and these are dominant autoantibody isotypes in murine lupus (63).

Using viable TBK1$^{-/-}$ TNF$^{-/-}$ mice, Ishii and colleagues demonstrated functional distinctions between TBK1 signaling in hematopoietic and non-hematopoietic cells for the induction of Ag-specific responses in a plasmid-DNA immunization model (64). TBK1$^{-/-}$ TNF$^{-/-}$ mice had no difference in total serum IgG1 and IgG2a, suggesting normal B cell function. However, TBK1$^{-/-}$ TNF$^{-/-}$ mice had completely abrogated primary and secondary antigen-specific IgG responses upon vaccination with plasmid-DNA, relative to wild type, TNF$^{-/-}$ TBK1$^{+/+}$, Myd88$^{-/-}$, or TRIF$^{-/-}$ mice. Mechanistically, the DNA component of the plasmid-DNA vaccine was shown to activate DCs in a TBK1- and IFN-I-dependent manner, but this occurred independently of the CpG DNA sensor, TLR9 (64). Along the same lines, alum and hydroxypropyl-β-cyclodextrin adjuvants have been shown to induce cell death and DNA release as part of their immunogenic properties and TBK1$^{-/-}$ TNF$^{-/-}$ mice immunized with these adjuvants had reduced levels of antigen-specific IgG1 responses (65, 66). Ishikawa and colleagues subsequently demonstrated that intracellular DNA induced DC activation and IFN-Is production through the cGAS-STING-TBK1 pathway (67).

TBK1-Dependent IFN-Is Can Induce Lupus

As mentioned, TBK1-dependent IFN-Is responses are activated by cytoplasmic nucleic acids. In the autoimmune context, pathogenic TBK1-mediated IFN-Is responses can be caused by aberrant self-DNA that leads to chronic IRF3 activation, such as is the case in TREX1 deficiency (68). TREX1 is an endoplasmic reticulum (ER)-associated 3′–5′ exonuclease, which degrades cytoplasmic viral DNA before sensing occurs. TREX1 is also required to clear endogenous retroelements and genomic DNA. TREX1 deficiency in patients and murine models causes lupus-like autoimmune manifestations. TREX1-deficient mice develop aberrant interferogenic responses and features of lupus owing to the cytoplasmic accumulation of endogenous nucleic acids and chronic activation of the TBK1-dependent DNA-sensing pathway (68–70). Mutations in TREX1 are associated with human autoimmune disorders, including Aicardi–Goutières syndrome (71), familial chilblain lupus (72), and SLE (73). An inhibitor of TBK1 was effective in treating TREX1$^{-/-}$ mice (74).

In summary, TBK1 is an important signaling kinase for the induction of IFN-Is in response to a number of ligands that activate TLR3, TLR4, and the STING pathways. TBK1 may be less relevant in other IFN-Is induction pathways, including TLR7 and TLR9. Mutations leading to aberrant activation of TBK1 and IFN-Is overproduction can contribute to lupus. Limiting pathogenic IFN-Is production through TBK1 inhibition may alleviate lupus. However, TBK1-driven responses other than IFN-Is induction may also contribute to humoral autoimmunity and are discussed in the next section.

TBK1-Regulated Germinal Center (GC) Responses in Humoral Immunity

GC-Dependent Humoral Immune Responses

Antibody-mediated autoimmune diseases share underlying immune mechanism(s). High-affinity autoantibodies arise from a GC reaction occurring in the B cell follicles (75, 76). The GC is a specialized structure in secondary lymphoid tissues, where B cells undergo iterative rounds of somatic hypermutation in Ig variable (V) gene segments, class switching and affinity selection, as well as post-translational modifications. Normally, the GC reaction is transient (self-terminating) (77) and only B cells expressing affinity-matured, class-switched antibodies specific for the antigen exit GCs, and survive as long-lived memory B cells and/or antibody-secreting plasma cells (78–80). However, the GC reaction can persist and give rise to antibody-mediated autoimmunity (81).

Intrinsic B cell defects can directly contribute to the development of spontaneous GCs, breakdown of B cell tolerance and humoral autoimmunity, such as *Tlr7* gene duplication (80) or WAS (Wiskott–Aldrich syndrome) protein deficiency (82, 83) in lupus. However, CD4$^+$ T_{FH} cells provide another essential cellular component regulating GC B cells. T_{FH} cells are required for the generation of high-affinity antibodies by promoting the GC reaction, including B cell clonal proliferation, affinity selection and the development of high-affinity antibody-producing cells (75, 84, 85). T_{FH} cells are characterized by the expression of chemokine receptor CXCR5, which facilitates migration and proximity to follicular B cells. Here, they provide cognate help to B cells *via* stable interactions such as SAP (SLAM-associated protein), costimulatory molecules such as CD40L, and cytokines such as IL-21, IL-4, and IFN-γ (86–89). While T_{FH} cells are

critical for an optimal GC reaction and subsequent generation of protective antibodies following immunization, abnormal development and/or function of T_{FH} have also been implicated in loss of tolerance and the development of humoral autoimmunity.

An increased T_{FH} population in the GC as well as GC numbers may contribute to aberrant positive selection and autoantibody formation in SLE (90). This is exemplified in Sanroque mice, in which exaggerated T_{FH} generation occurs in a cell-intrinsic manner and leads to spontaneous GC formation, and lupus-like pathology (91, 92). Furthermore, adoptively transferred Roquin$^{san/san}$ T_{FH} cells are able to induce spontaneous GC B cell expansion and GC formation in naïve recipient mice (92). Similarly, an enlarged T_{FH} population accompanies increased GC size and more productive humoral responses in immunization models (77). Conversely, mice with conditional Bcl6 deficiency in T cells (Bcl6 is a transcriptional repressor that regulates both T_{FH} and GC B cell differentiation) have impairment of T_{FH} development, GC reactions, and antibody responses (93, 94). CD4 transgenic autoreactive T cells deficient in SAP (SLAM-associated protein, which mediates stable T-B interactions critical for GC formation), failed to mount GC reactions, develop IgG1 autoantibodies, and autoantibody-mediated arthritis (95, 96). Thus, the size of the T_{FH} population is directly coupled with GC function and ensuing humoral responses. Abnormal T_{FH} accumulation may also contribute to the production of pathogenic autoantibodies through enhanced positive selection of self-reactive B cells.

In clinical settings, the frequency of T_{FH}-like cells is increased in the peripheral blood of RA patients and correlates with higher elevated levels of anti-CCP (cyclic citrullinated peptide) autoantibodies, as well as disease activity (97, 98). Conversely, treatment responsive, new onset RA patients have a reduced frequency of circulating T_{FH}, which is accompanied by a decrease in anti-CCP antibody (98). SLE patients also demonstrate a similar expansion of T_{FH}-like cells, which correlates with disease activity, frequency of circulating plasmablasts, and anti-double-stranded DNA antibody positivity (99). T_{FH} expansion and its association with autoantibody responses have also been noted in other humoral autoimmune syndromes, including type 1 diabetes (100) and primary Sjogren's syndrome (101, 102). Given the robust correlation between T_{FH} numbers and high-affinity autoantibody levels, manipulation of the differentiation program and plasticity of T_{FH} cells may provide new therapeutic options in autoimmune diseases, such as SLE and RA.

ICOS in Humoral Immune Responses against TD Antigens and Humoral Autoimmunity

Among many determinants of optimal humoral immunity, ICOS has been consistently associated with GC reactions and the induction of GC-dependent thymus-dependent (TD) antibody responses. ICOS is a critical coreceptor, distinct from CD28, on activated or antigen-experienced T cells (103, 104) and is highly expressed on T_{FH} (105). Through interaction with ICOS ligand (ICOSL) on antigen-presenting cells (DCs and B cells), ICOS delivers robust costimulatory signals that promote T_{FH} positioning and thus supports GC function (106). ICOSL$^{-/-}$ mice mount comparable antigen-specific IgM and IgG3 responses, but have reduced IgG1 and IgG2a production upon immunization with thymus-independent antigens (107). ICOS$^{-/-}$ or ICOSL$^{-/-}$ mice have defective production of class-switched antibodies against TD antigens (particularly IgG1, IgG2a, and IgG2b isotypes, but not IgM), along with reduced number and size of GCs and a lack of B cell memory (107–110). Additionally, mice with a tyrosine-to-phenylalanine point mutation at residue 181 in the cytoplasmic tail of ICOS have abrogated T_{FH} generation, GC reactions, antibody class switching, and antibody affinity maturation (111). ICOS deficiency or antibody-mediated depletion of ICOS-expressing CD4$^+$ T cells in SLE1 lupus mice results in diminished pathogenic T_{FH} expansion, inhibited plasma cell generation, and a reduction in class-switched IgG autoantibodies (112). ICOSL$^{-/-}$ or B cell-specific ICOSL$^{-/-}$ mice have markedly inhibited development of proteoglycan-induced arthritis, with notable reductions in T_{FH} and GC B cells, IL-21 production, and proteoglycan-specific IgG antibody responses (113).

Blockade of the ICOSL pathway ameliorates autoimmune CIA, the K/BxN spontaneous arthritis model, and the SLE (NZB/NZW) F1 mouse model, with marked reductions in disease manifestations, numbers of T_{FH} and GC B cells, and pathogenic, class-switched, high-affinity autoantibodies (113–115). Interestingly, inhibition of CIA was observed even when ICOSL blocking antibody was given after the onset of disease (114). Sanroque mice have excessive ICOS activation due to genetic mutation of a RING-type ligase that represses ICOS (91). These mice develop spontaneous GC in the absence of foreign antigen, increased numbers of T_{FH} cells, spontaneous autoantibodies, including antinuclear antibodies, and lupus-like manifestations, such as glomerulonephritis. Intriguingly, although ICOS or ICOSL deficiency in Sanroque mice substantially reduced autoantibody production, it did not result in complete inhibition of autoantibody production (91). This observation suggests a contribution from residual GC-independent extra-follicular pathway (85, 113). Thus, ICOS/ICOSL signaling drives optimal GC-dependent TD antibody responses and inhibition of this pathway abrogates the GC reaction, autoantibody responses, and disease features in humorally mediated autoimmune disease models. Indeed, therapies targeting ICOS/ICOSL are under evaluation in early phase clinical trials of SLE (116).

TBK1 Mediates ICOS Signaling for T_{FH} Maturation and GC-Mediated Antibody Responses

Similar to other CD4$^+$ T cell subsets (Th1, Th2, Th17, and Treg cells), T_{FH} development is a multi-step process which involves initial priming of naïve CD4$^+$ T cells by dendritic cells in the T cell zone, followed by expansion and differentiation that are regulated through signaling pathways activated downstream of cytokines and cell surface molecules. Subsequent activation of lineage-defining transcription factors (T-bet for Th1, GATA3 for Th2, RORγt for Th17, FoxP3 for Treg cells, and Bcl6 for T_{FH}) promotes T cell differentiation (93, 94, 117).

T follicular B helper development can be separated into two stages—(i) naive to Bcl6$^+$ pre-T_{FH} and (ii) pre-T_{FH} to mature GC T_{FH}. Pre-T_{FH} development follows DC priming *in vivo*, through an ICOS costimulation signal and the phosphoinositide-3 kinase (PI3K) pathway. The ICOS-PI3K pathway instructs T_{FH} differentiation *via* induction of Bcl6 and the subsequent Bcl6-dependent

expression of CXCR5 on pre-T_{FH} (23, 105). The activation ICOS-PI3K signaling alone is, however, insufficient to drive full GC T_{FH} maturation and the GC reaction (23, 111, 118). For final differentiation of nascent T_{FH} into GC T_{FH}, pre-T_{FH} cells require a second costimulatory signal through ICOS (119, 120). This has been demonstrated by the inability of T cell-selective deletion of PI3K components to fully recapitulate the phenotype of $CD4^+$ T cells from $ICOS^{-/-}$ mice (111, 121).

A recent report identified TBK1 as a unique signaling kinase in the ICOS pathway (23). In this study, Pedros and colleagues identified a conserved TRAF-like motif in the cytoplasmic tail of ICOS (iProx motif), which mediated TBK1 recruitment and activation following a combination of strong TCR and ICOS signals. These authors showed that by deleting the iProx motif on ICOS specifically in $CD4^+$ T cells, TBK1 failed to associate with ICOS. T cells modified in this way displayed severely impaired differentiation into GC T_{FH} and TD antibody responses, despite generating pre-T_{FH} cells. A similar effect was obtained by $CD4^+$ T cell-specific TBK1 depletion. In a series of reconstitution experiments, transducing ICOS and TBK1 constructs into $ICOS^{-/-}$ TCR transgenic $CD4^+$ T cells, it was shown that intact ICOS is required for the generation of both nascent and final GC T_{FH} populations, while TBK1 controls progression from the pre-T_{FH} to mature GC T_{FH} phenotype (23).

Although the downstream mediators of ICOS-TBK1 signaling in T_{FH} have not been identified, FoxO1 is a potential candidate because ICOS signaling instructs the T_{FH} program *via* AKT-mediated FoxO1 phosphorylation (119). AKT has been shown to be a TBK1 substrate in some settings (13, 14, 122). FoxO1 is a transcription factor that, in its active unphosphorylated state, represses T_{FH} programming. FoxO1 phosphorylation results in its transient inactivation and cytoplasmic translocation from the nucleus (119). FoxO1 inactivation also reduces FoxO1-dependent KLF2 expression, together with expression of KLF2-dependent chemokine receptors, necessary for optimal repositioning of T_{FH} in the GC (120). FoxO1 inactivation, specifically in $CD4^+$ T cells (*Foxo1*$^{fl/fl}$:CD4-Cre mice) caused defective Tregs and systemic autoimmunity, characterized by accumulation of the T_{FH} population, with exaggerated Bcl6 induction and GC formation, and production of anti-DNA antibodies (123). While FoxO1-sufficient $CD4^+$ T cells give rise to effector T cells, pre-T_{FH}, and GC T_{FH} upon immunization, $FoxO1^{-/-}$ $CD4^+$ T cells generate pre-T_{FH} cells with higher expression of T_{FH}-defining markers (Bcl6, CXCR5, and PD-1) and lowered T cell zone chemokine receptors (CD62L, PSGL1) (119). ICOS-driven FoxO1 inactivation thus alters the chemokine receptor profile of pre-T_{FH}, facilitating migration from the T cell zone toward the B cell follicles (119). Conversely, ICOS/ICOSL blockade results in the relocation of fully developed T_{FH} back to the T cell zone. This relocation reverses their phenotype toward non-T_{FH} effector T cells, with a consequent reduction in antigen-specific GC B cells, as well as serum antigen-specific IgG1 and IgG2a responses, indicating collapse of the GC response (120). This study also concluded that ICOS is not required for T_{FH} survival or expression of T_{FH}-related transcription factors, but rather, it regulates the expression of T_{FH} homing markers. Changes in T_{FH} transcription factors are thus likely to be a secondary effect upon failure to maintain the positioning of pre-T_{FH} and impaired costimulatory signals from follicular B cells (120).

Although TBK1 has been identified as an ICOS-specific signaling kinase required for full maturation of GC T_{FH}, the role of TBK1-mediated FoxO1 regulation in this process has not been elucidated. One study using conditional $TBK1^{-/-}$ in $CD4^+$ T cells (*Tbk1*$^{fl/fl}$:CD4-Cre mice) and stimulation with TCR and CD28, suggested that basal TBK1 is required for constitutive AKT turnover to prevent hyperactivation of AKT upon T cell activation (124). These authors also reported a marked increase in IFN-γ production and activation markers in $CD4^+$ T cells derived from *Tbk1*$^{fl/fl}$:CD4-Cre mice, indicating the propensity of these cells to become a Th1-like population in the absence of basal TBK1 (124). Interestingly, $ICOS^{-/-}$ or $ICOSL^{-/-}$ mice also exhibit enhanced Th1 responses in secondary lymphoid tissues with marked elevation of IFN-γ in the context of infection (125–127).

Given the role of TBK1 in ICOS signaling in GC T_{FH} and downstream GC-driven antibody responses, TBK1 inhibition may curtail humoral autoimmunity through an ICOS-driven GC pathway. Importantly, targeting ICOS/ICOSL and/or TBK1 may not result in generalized immunosuppression, but rather reverse cell fate decisions in T_{FH}. Understanding how TBK1 signals in T_{FH}, how it affects cell fate decisions in T helper cell polarization, positioning and migratory pathways may provide new therapeutic strategies, especially for antibody-mediated autoimmunity.

TBK1-Regulated Autophagy in Immune Regulation

TBK1 Regulates Autophagy

The functional effects of TBK1 extend beyond innate immune signaling. Autophagy is a conserved homeostatic process in eukaryotic cells involving sequestration and lysosomal degradation of cytoplasmic contents, including damaged or surplus organelles (mitophagy for mitochondria, pexophagy for peroxisomes, ribophagy for ribosomes, reticulophagy for endoplasmic reticulum), cytotoxic macromolecular aggregates (aggrephagy), and intracellular microorganisms (xenophagy) (128–131). Autophagy will not be discussed in detail as it has been extensively reviewed elsewhere (128–131). Instead, we discuss reports which have implicated TBK1 in autophagic processes and how these translate to immune responses.

TBK1 in Antimicrobial Autophagy (Xenophagy)

TANK-binding kinase 1-mediated regulation of autophagy has been described in the context of antimicrobial defense (xenophagy), in which intracytoplasmic pathogens are sequestered into autophagosomes and targeted for lytic, lysosomal degradation. TBK1 and its homolog IKKε have been identified as binding partners of the autophagy receptor protein NDP52 that recognizes polyubiquitylated *Salmonella enterica* in human cells. However, only TBK1 is required for xenophagy of *S. enterica* (132, 133) and mycobacteria (134). Canonical IKKs initiate autophagy, while TBK1 knockdown suppresses the maturation of autophagosomes into autolysosomes. Mechanistically, TBK1 phosphorylates autophagy receptor proteins, including NDP52, OPTN on Ser177, and p62 (also known as SQSTM1) at Ser403 (located at the ubiquitin-associated/

UBA domain) (132–134). Phosphorylation increases the affinity of LC3-binding autophagy adaptors for K48- and K63-ubiquitinated cytoplasmic bacteria, as well as polyubiquitinated protein aggregates (135), and it promotes autophagic clearance (132–134). Knockdown or pharmacological inhibition of TBK1 impairs autophagic killing of *S. enterica* or *M. tuberculosis* (133, 134). Mice deficient in autophagic proteins (Atg3, Atg5, Atg7, Atg9, and Atg16L1) have embryonic lethality (129). Viable conditional autophagy knockout mice often have impaired pathogen clearance, reduced survival, and severe tissue injury due to enhanced inflammasome and cytokine responses, and in some cases, enhanced Th17 responses (136–138). In the same study, TBK1 was shown to be important for delivery of the lysosomal hydrolase cathepsin D to the autophagolysosomal compartment (134). TBK1, therefore, appears to play an essential role in late autophagic flux.

TBK1 in Mitophagy and Potential Implications in Neuronal Health

Autophagy is increasingly appreciated for its role in maintaining cell homeostasis through clearance or normal turnover of cytoplasmic contents or defective cellular organelles, including mitochondria (mitophagy). Mice deficient for Atg5 specifically in neural cells develop progressive decline in motor function in the absence of any disease-associated mutant proteins, accompanied by the accumulation of cytoplasmic inclusion bodies in neurons (139). Damaged mitochondria are detrimental to cellular homeostasis and efficient removal through autophagy is crucial for cell survival, particularly for senescent cells, such as neurons, which cannot dilute cytotoxic contents through cell division (140). Mitochondrial damage induces concomitant PINK1-PARKIN-mediated poly-ubiquitylation of damaged mitochondria and also activates TBK1. In turn, TBK1 can phosphorylate autophagy receptors (OPTN, SQSTM1/p62, and NDP52), thereby enhancing the ability of these receptors to associate with ubiquitinated cargo (e.g., ubiquitin-tagged, depolarized mitochondria) and autophagic membranes (24, 25, 28, 141). This post-translational modification creates a signal amplification loop that recruits and retains autophagy receptor/TBK1/ubiquitinated cargo complexes, thereby promoting mitophagy. Separate mutations that disrupt TBK1's association with OPTN, or of OPTN with ubiquitin, abolish the translocation and activation of TBK1, and, therefore, impair mitophagy (25).

Exome sequencing identifies TBK1 as a neurodegenerative disease gene in amyotropic lateral sclerosis (ALS) and frontotemporal dementia (26, 27). Further, it was shown that mutations of TBK1 at the C-terminal TBK1 coiled-coil domain, resulted in TBK1's dissociation from OPTN, while preserving its kinase activity (located at the N-terminal ubiquitin-like domain) (27). These studies provide a potential mechanistic basis for TBK's involvement in ALS. Mutations linking OPTN to impaired autophagy and neurodegenerative diseases have also been characterized (142). Thus, although it has not been directly demonstrated, TBK1-regulated autophagy appears to maintain cellular homeostasis of long-lived neuronal cells through mitophagy.

TBK1 and Autophagy in Immune Cell Lineage Development

TANK-binding kinase 1-regulated autophagy may also be important for regulation of immune cells. As mentioned above, mice deficient in autophagic proteins (Atg3, Atg5, Atg7, Atg9, and Atg16L1) have neonatal lethality, as do TBK1$^{-/-}$ mice (1, 129). In contrast, STING$^{-/-}$ mice are viable, but have impaired TBK1-dependent IFN-I responses to cytoplasmic DNA (67). Autophagy allows dynamic changes necessary for proper mammalian development through the recycling and provision of macromolecules and clearance of apoptotic bodies. Conditional *Atg7*$^{fl/fl}$:Vav-Cre mice (i.e., hematopoietic cell-specific deletion of Atg7) overcomes embryonic lethality, but these mice are anemic and lymphopenic, linking autophagy to erythropoiesis and lymphopoiesis (129). Similarly, T cell-specific deletion of Atg5 or Atg7 (*Atg5*$^{fl/fl}$:Lck-Cre or *Atg7*$^{fl/fl}$:Lck-Cre mice) or innate lymphoid cell (ILC)-specific deletion of Atg5 (*Atg5*$^{fl/fl}$:Nkp46-Cre) reduces peripheral T cells and ILC subpopulations, respectively (143, 144). Maturation of naïve T cells depends on autophagy to reduce mitochondrial and ER contents through mitophagy and reticulophagy, respectively (143, 145, 146). Defective autophagy in T cells results in accumulated mitochondrial biomass, disturbed Ca^{2+} homeostasis, higher levels of reactive oxygen species (superoxide), and enhanced susceptibility to apoptosis (143, 145, 146). In contrast to lymphopenia, the myeloid compartment in *Atg7*$^{fl/fl}$:Vav-Cre mice is expanded (147), implicating autophagy in the balance between lymphopoiesis and myelopoiesis. It was also recently shown that autophagy is required for full granulopoiesis (148). Autophagy regulates cellular differentiation and activation by accommodating metabolic adaptation, which can occur in parallel and independently of transcriptional regulators. Neutrophils from *Atg7*$^{fl/fl}$:Vav-Cre or *Atg7*$^{fl/fl}$:Cebpa-Cre (granulocyte-macrophage progenitor-specific deletion of Atg7) mice are numerically expanded, but are unable to complete maturation and, therefore, are functionally defective. In this case, autophagy-mediated lipolysis (lipophagy) provides free fatty acids to support a mitochondrial respiration pathway essential for neutrophil differentiation (148). Autophagy regulates cytosolic processing of antigen for presentation on MHCII in DCs. The absence of Atg5 in DCs results in failure to mount full Th1 cell immunity to viral infection (149). TBK1 has also been associated with metabolic adaptation of DC after TLR stimulation, whereby TBK1 phosphorylates AKT for the glycolysis which is necessary for DC activation. shRNA-mediated TBK1 knockdown in DCs results in a blunted glycolytic shift and reduced ability of these DCs to prime antigen-specific T cells *in vitro* (14).

In summary, separate lines of evidence have linked autophagy to cell metabolism, TBK1 to autophagy, and TBK1 to metabolism in immune cell development and activation. Further studies of TBK1's role in cellular autophagy and metabolism in various immune contexts could allow manipulation of immune function—either for protection against pathogens or rewiring toward tolerance in autoimmunity.

TBK1 and Autophagy Balance Age-Related Inflammation

In contrast to the maturation defect of neutrophils in the absence of autophagy, macrophages derived from *Atg5*$^{fl/fl}$:LysM-Cre mice

display a heightened proinflammatory phenotype. These mice develop greater hepatitis on a high fat diet and low dose LPS and also spontaneous uveitis (150, 151). Autophagy-deficient macrophages activate the NLRP3 inflammasome and develop IL-1β-mediated inflammation (151–153). Similar to macrophages, Atg16L1-deficient DCs have heightened activation in graft-versus-host disease (154). Loss of function polymorphisms of Atg16L1 have been associated with age-dependent development of inflammatory bowel disease (Crohn's disease) owing to impaired clearance of ileal pathogens or endogenous protein aggregates, and chronic elevation of inflammatory cytokine responses (155, 156). Interestingly, DC-specific deletion of TBK1 (*Tbk1*$^{fl/fl}$:CD11c-Cre mice) also display age-related cellular hyperactivation, with marked upregulation of costimulatory molecules on DCs, T cell activation, and autoimmune features (splenomegaly, lymphadenopathy, and tissue infiltration with lymphocytes) (157). These *Tbk1*$^{fl/fl}$:CD11c-Cre mice have an increased frequency of activated IFN-γ-producing CD4$^+$ and CD8$^+$ T cells, while Tregs remain comparable to TBK1-sufficient mice. Consequently, these mice are more sensitive to EAE and mount more robust antitumor immunity against poorly immunogenic B16F10 melanoma cells (157).

Enhanced macrophage or DC activation in the absence of TBK1 may be due to impaired autophagy, which normally limits age-related inflammasome activation. Of note, the age-dependent hyperinflammatory status of autophagy- or TBK1-deficient macrophages and DCs in Atg conditional knockout mouse models resembles aging macrophages. These cells shift from an anti-inflammatory to a proinflammatory phenotype, with an age-related reduction in autophagic activity and sensing of endogenous damage-associated molecular patterns (DAMPs) (158, 159). Thus, it is tempting to speculate that TBK1 maintains cellular autophagy and sustains immune cell longevity and homeostasis. Hallmarks of accelerated immune cell aging with chronic TBK1 deficiency are also notable in the *Tbk1*$^{fl/fl}$:CD19-Cre mice (B cell-specific ablation of TBK1) (160). These mice have normal B cell populations in the spleen and bone marrow, but develop age-related dysregulation of the non-canonical NF-κB pathway, uncontrolled production of IgA, increased levels of autoantibody antinuclear antigen and anti-dsDNA, with nephropathy-like disease (160). In this study, it was concluded that steady state TBK1 negatively regulates IgA class switching in B cells by attenuating noncanonical NF-κB signaling. This effect was thought to be due to TBK1-mediated phosphorylation and degradation of NF-κB-inducing kinase, downstream of BAFF or APRIL signaling (160). Intriguingly, it is possible that *Tbk1*$^{fl/fl}$:CD19-Cre mice phenocopy the aging B cell repertoire because the B1 population (responsible for IgA responses) and autoantibody production are enhanced by age (161). While chronic deficiency of autophagy or TBK1 results in amplified endogenous inflammation to DAMPs in myeloid cells and abnormalities in the B cell repertoire, inhibition of autophagy may be exploited to target long-lived autoimmune populations (discussed below) through acceleration of immune cell aging.

Autophagy Supports Long-Lived Memory Immune Cells—Implication of TBK1

As discussed above, autophagy is cytoprotective in senescent cells, such as neurons. This cytoprotective function also appears to apply in long-lived immune cells. Mice with B cell-specific deletion of Atg5 or Atg7 (*Atg5*$^{fl/fl}$:CD19-Cre or *Atg7*$^{fl/fl}$:CD19-Cre mice) have mostly normal B cell development, but are unable to maintain long-lived humoral antibody responses owing to the failure to maintain long-lived plasma cells and memory B cells, respectively (162, 163). In both studies, plasma cells and memory B cells arising from immunization and GC were shown to upregulate components of the autophagic machinery. In plasma cells, autophagy is thought to maintain longevity by reticulophagy (autophagic clearance of redundant endoplasmic reticulum) to limit excessive antibody synthesis and conserve energy balance (162). Further studies are needed to investigate whether TBK1 is involved in reticulophagy and physiological adaptation of plasma cells.

Autophagy also supports the lifespan of quiescent, antigen-experienced, long-lived, GC-derived memory B cells through mitophagy. *Atg7*$^{fl/fl}$:CD19-Cre mice mount normal primary antibody responses and have normal differentiation of post-GC memory B cells, but fail to generate secondary antibody responses to influenza virus due to spontaneous death of memory B cells (163). Memory B cells from *Atg7*$^{fl/fl}$:CD19-Cre mice are unable to efficiently remove damaged mitochondria, resulting in accumulation of reactive oxygen species, lipid peroxidation, and oxidative stress-induced death. Interestingly, these mice also develop enhanced Th17 responses to viral infection, possibly as a compensatory mechanism (163). Whether TBK1 also regulates mitophagy and, therefore, the survival of memory B cells requires further investigation. Mitophagy was shown to support the generation of LCMV-induced memory CD8$^+$ T cells (164, 165) and MCMV-induced memory NK cells (166). These studies highlight that autophagy is not apparently required for germline T cell development, nor for T cell activation and proliferation, but is important for established effector T cells to generate a pool of memory cells (165, 166). Autophagy is dynamically induced at various stages of immune cell development, activation, and differentiation, but plays a particular role in the formation and maintenance of long-lived immune populations, including memory B cells and senescent plasma cells. Because long-lived plasma cells or plasma cells deriving from memory B cells can drive persistent autoimmune disease (167), abrogation of autophagy through TBK1 inhibition might reduce resistance to autonomous cell aging and death, and diminish pathogenic autoantibody responses.

In summary, a number of studies demonstrate significant overlap between TBK1 and autophagy, most notably in antimicrobial xenophagy and maintenance of neuronal cell health. Similar to autophagy, TBK1 has a complex role in immune cells. It is known that autophagy is dynamically regulated to accommodate rapid metabolic adaptation and organelle turnover associated with cell development, differentiation, activation, and longevity. TBK1 regulates autophagy through post-translational modifications of autophagy adaptor/receptor proteins required for the maturation of autophagosomes. TBK1's involvement in other types of organelle autophagy and metabolic signaling pathways in immune lineage cells and immune responses is of great interest. Autophagy supports the extended lifespan of cells, such as neurons, immune memory populations, and long-lived plasma cells. Targeting autophagy through the inhibition of TBK1 may provide a novel approach for treating humoral autoimmune diseases.

Inhibition of autophagy or TBK1 may favor the generation of short-lived effector cells, rather than long-lived memory populations. Both autophagy and TBK1 have been shown to regulate the delicate balance between cellular adaptation for efficient immune response and aging-associated autoinflammation. Studies exploring how, when, and where TBK1 facilitates autophagy in distinct immune lineages will inform potential modulation of protective or pathogenic immune responses.

CONCLUSION

In this review, we discuss the remarkable functional diversity of TBK1 in the context of humoral autoimmunity. These pathways are summarized in the **Figure 1**. TBK1 is required for IFN-Is production in the context of sensing viral or aberrant cytoplasmic nucleic acids. Overactive TBK1 can precipitate IFN-Is and lupus, such as is the case for TREX1 deficiency. Recent literature reports that TBK1 is associated with humoral antibody responses *via* its recruitment to and activation of ICOS in the $CD4^+$ T_{FH} population in GCs of lymph nodes. ICOS is required for full maturation of the GC T_{FH} population, the GC reaction, GC-mediated generation of affinity-matured long-lived plasma cell and memory B cells, and productive GC-derived antibody responses. Thus, TBK1 inhibition may be useful in pathogenic autoantibody responses mediated by GC. Finally, we highlight the similarity of TBK1 deficiency to that of autophagy-deficiency. Therapeutic TBK1 inhibition may therefore lead to premature aging and/or death of pathogenic immune cells, such as long-lived plasma cells, and memory B cells in autoimmune diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the assembly and revision of this review manuscript.

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REVIEW
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Tailoring Immune Responses toward Autoimmunity: Transcriptional Regulators That Drive the Creation and Collusion of Autoreactive Lymphocytes

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T-dependent humoral immune responses to infection involve a collaboration between B and CD4 T cell activation, migration, and co-stimulation, thereby culminating in the formation of germinal centers (GCs) and eventual differentiation into memory cells and long-lived plasma cells (PCs). CD4 T cell-derived signals drive the formation of a tailored B cell response. Downstream of these signals are transcriptional regulators that are the critical enactors of immune cell programs. In particular, a core group of transcription factors regulate both B and T cell differentiation, identity, and function. The timing and expression levels of these transcription factors are tightly controlled, with dysregulated expression correlated to immune cell dysfunction in autoimmunity and lymphomagenesis. Recent studies have significantly advanced our understanding of both extrinsic and intrinsic regulators of autoreactive B cells and antibody-secreting PCs in systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune conditions. Yet, there are still gaps in our understanding of the causative role these regulators play, as well as the link between lymphoid responses and peripheral damage. This review will focus on the genesis of immunopathogenic CD4 helper and GC B cells. In particular, we will detail the transcriptional regulation of cytokine and chemokine receptor signaling during the pathogenesis of GC-derived autoimmune conditions in both murine models and human patients.

Keywords: autoreactive B cells, germinal centers, transcription factors, Bcl-6, T-bet, interferon-gamma

INTRODUCTION: ENTRY POINTS ON THE PATH TOWARD AUTOREACTIVE ANTIBODY PRODUCTION

Effective humoral immune responses depend on the ability to form and expand a population of B cells with high affinity for the foreign antigen. Both B cells and T helper cells employ a number of mechanisms to produce effector cells that help clear the antigen and form specialized immune memory cells. Yet, it is also the deployment of these mechanisms that put lymphocytes at risk of

Abbreviations: ABCs, age-associated B cells; cTfh, circulating T follicular helper cells; GC, germinal center; IFN, interferon; Ig, immunoglobulin; PC, plasma cell; SHM, somatic hypermutation; STAT, signal transducer and activator of transcription; Tfh, T follicular helper cells; Tfr, T follicular regulatory cells; YFP, yellow fluorescent protein.

creating and expanding autoreactive cells that attack the host rather than the foreign invader and can lead to lifelong chronic disease.

Production of autoreactive B cells can occur at multiple stages of B cell development or differentiation. B cells that develop in the bone marrow, as well as those that differentiate in the periphery, undergo a number of checkpoints to exclude autoreactive cells from the immune repertoire. In particular, cells that produce an autoreactive B cell receptor will undergo deletion, receptor editing, or will be made anergic, such that autoreactive cells cannot participate in an immune response [reviewed in detail by Ref. (1)]. After development in the bone marrow, B cells migrate to the secondary lymphoid organs and undergo the final stages of maturation. Together, these developmental checkpoints result in a mature naïve B cell repertoire that is ready to respond to virtually any foreign antigen without the risk of cells that are specific to self-antigens mounting an attack. It is when these checkpoints fail (2) or are subverted by excessive extrinsic signals (3) that autoreactivity ensues.

Another major risk for generating newly autoreactive cells arises during an immune response. During a T-dependent response, activated antigen-specific B cells can either form an early wave of plasmablasts, which are low affinity for the antigen and mainly IgM, or they can form transient sites of proliferating cells to fine-tune the affinity of their receptor to antigen. This major site for affinity maturation is the germinal center (GC). GCs are transient sites formed within secondary lymphoid tissue from which high-affinity memory B cells and plasma cells (PCs) emerge. GC B cells activate the enzyme activation-induced cytidine deaminase (AID) which permits somatic hypermutation (SHM), a process in which random mutations are introduced into the B cell receptor. This is followed by selection and survival of high-affinity clones mediated by follicular dendritic cells (FDCs) and T follicular helper cells (Tfh). As mutations are random, SHM may result in a number of different outcomes for the antigen receptor, which can range from helpful to detrimental. Ideally, SHM will increase the affinity of the receptor for antigen. However, resulting clones may also no longer be specific, or have lower affinity for the antigen. Finally, mutated receptors may detect self-antigen. If left unchecked, these cells may result in the production of autoreactive antibody-secreting cells.

Transcription factors are molecular regulators that can activate or repress programs of gene expressions. They have critical roles in regulating cellular behavior during immune responses, including proliferation, differentiation, and migration of cells in response to the microenvironment. Both B and T cells rely on cytokines, chemokines, and other extrinsic signals to dictate their behavior throughout a response. Following these environmental cues, it is the molecular regulators downstream of these signals that orchestrate changes in gene expression and make functional and fate decisions. In particular, transcription factors regulate formation of the immunologic repertoire, as well as the differentiation of antigen-specific cells into effector and memory subsets during an immune response. These same extrinsic and intrinsic mechanisms that promote effective antibody responses and formation of immunity can also lead to autoimmunity. This review will focus on the points during an immune response at which B and Tfh cells can become dysregulated, and the underpinning transcription factors that balance appropriate responses to foreign pathogens with autoreactive cell formation. As such, we will focus on GC-derived autoimmune conditions, principally systemic lupus erythematosus (referred to within as lupus).

TRANSCRIPTIONAL REGULATION OF CLASS BIAS IN T AND B CELLS

The production of an effective humoral response relies on the coordinate orchestration of B and T cell behavior in unique areas of secondary lymphoid organs throughout the response. Depending on the pathogen type, such as a virus or bacteria, antigen-activated CD4 T cells will be skewed toward a Th1 (driven by T-bet), Th2 (driven by Gata3), or Th17 (driven by BATF and Rorγt) phenotype early in the response (**Figure 1**) (4). This results in secretion of specialized cytokines by these subsets that modulate the microenvironment and, in turn, direct B cell behavior.

B cells will tailor their B cell receptor to utilize the heavy chain with the effector function most suited to clearing the infecting pathogen. This is termed immunoglobulin (Ig) isotype switching, and is a process which relies on CD4 T cells. Cytokines produced from CD4 T cells, such as IL-4, IFNγ, or TGFβ, are able to direct Ig isotype switching, thus modulating the effector function of the antibody (5). AID expression and the transcription factor BATF are required for switching to all isotypes downstream of IgM (6–9). In addition, diversity in antibody isotypes is regulated by a small group of transcription factors that play context-specific roles in switching to the appropriate isotype in response to different cytokines (**Figure 1**). The most well characterized of these is the T-box transcription factor T-bet, which mediates production of murine IgG2a/c (referred to within as IgG2a) in response to IFNγ (10) as well as type I interferons (IFNs). By contrast, Nfil3 is required for IL-4-mediated induction of IgE (11), and Rorα in TGFβ-mediated induction of IgA (12). Furthermore, the transcription factor Ikaros can block the induction of murine IgG2a and IgG2b (13). It has not yet been determined whether other IgG subclasses (14), such as IgG1 or IgG3, have specific transcription factors that regulate their production.

Specific isotypes can also mediate immune disorders. For instance, mouse models of lupus are linked with excessive production of autoreactive IgG2a, which triggers antibody-dependent cell-mediated cytotoxicity. T-bet-mediated switching in response to IFN signaling is important not only for anti-viral humoral responses in mice (15, 16) but can also be immunopathogenic in murine models of lupus. T-bet is regulated by the transcription factor c-Myb in B cells, which represses the expression of T-bet during Th2 cell-biased responses (17). In both T and B cells, T-bet induces a number of gene expression changes that can affect cellular function and migration. For example, T-bet regulates CXCR3 expression and thus migration to sites of inflammation (18) and into kidneys of mice with lupus nephritis (19). In lupus-prone mice, IgG2a immune deposition is found on kidneys, in a similar fashion to antibody deposition in human patients.

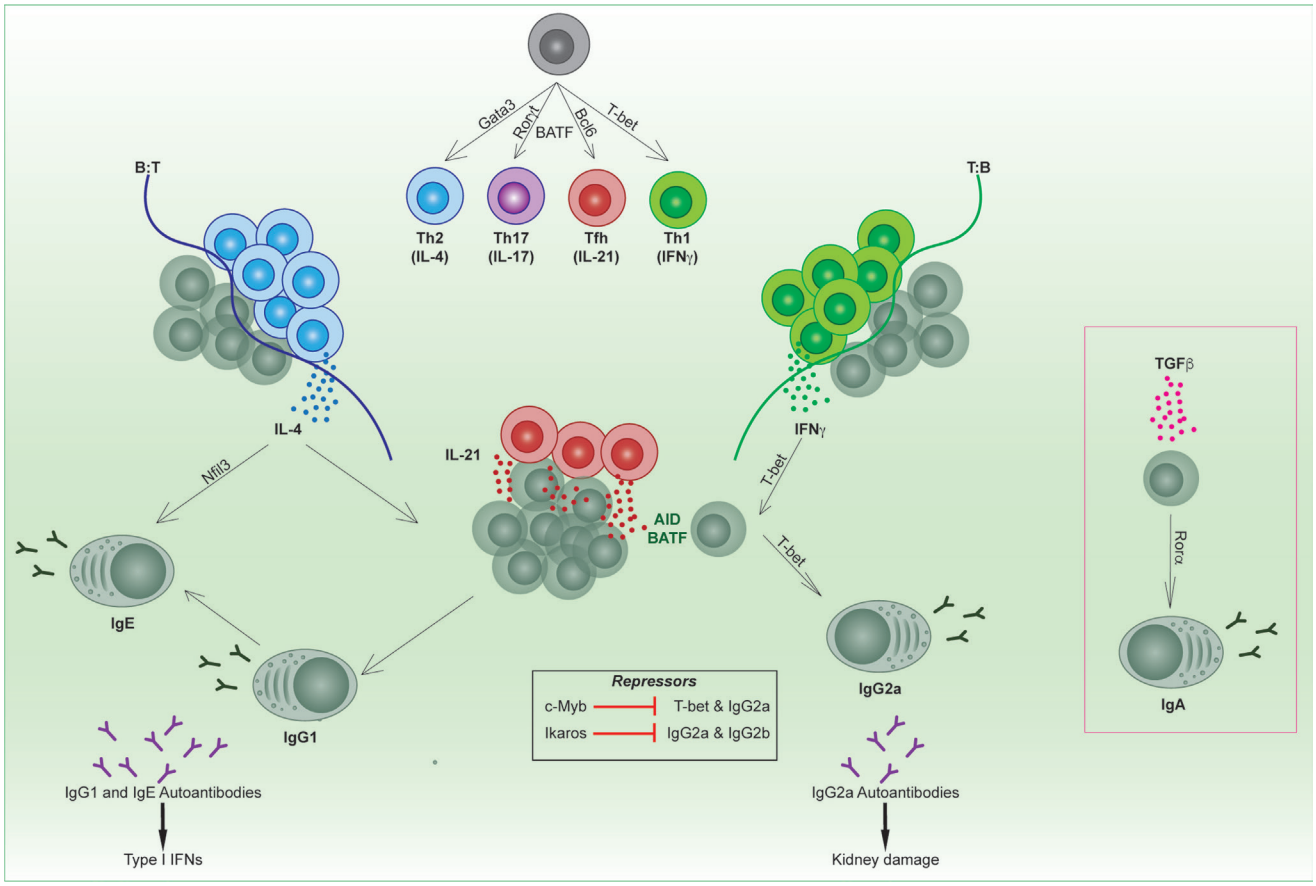

FIGURE 1 | Transcriptional regulation of B and T follicular helper cell subsets. Unique transcription factors regulate the differentiation of T helper subsets, which in turn regulate the tailoring of the B cell response. Cytokines produced from specialized T helper subsets switch on transcription factors in activated B cells (e.g., T-bet, Rorα, Nfil3) that direct isotype switching to downstream immunoglobulin isotypes. Specific isotypes can mediate autoimmune conditions such as lupus.

There are also other isotypes that have been linked to autoimmunity, such as IgE. For instance, inactivation of the apoptotic mediators FAS/FASL causes autoimmune lymphoproliferative syndrome (ALPS) in humans and is also the basis of some murine lupus models. It was recently revealed that FAS inactivation in mice results in the production of "rogue" GC B cells, which gave rise to a high frequency of IgE-secreting PCs (20). Correspondingly, a subset of ALPS patients exhibits hyper IgE (20). Furthermore, lupus patients can also exhibit anti-DNA IgE antibodies. Self-reactive IgE antibodies may synergize with IgG autoantibodies to trigger type I IFN responses, correlating with disease severity (21). While the transcription factor Nfil3 is associated with induction of IgE, it is not clear whether inhibition of Nfil3 may ameliorate disease. In sum, it is clear that pathogens influence the class bias and hence effector function of both B and T cells. This has critical downstream consequences for humoral responses both in infectious responses and autoimmunity. Understanding the molecular regulation that drives the context-specific production or repression of different isotypes could potentially lead to new clinical targets for modulation in disease.

THE INTERWOVEN PATHS OF B AND CD4 T CELLS DURING A HUMORAL RESPONSE

Positioning of B and CD4 T cells within different areas of secondary lymphoid organs regulates cellular interactions and exposure to signals within the microenvironment. The different migratory paths that B and T cells take during an immune response can dictate transcription factor expression, and determine the fate and function of these cells. In particular, a distinct T helper lineage, Tfh cells, is distinguished from other T helper cells subsets by its unique position within lymphoid organs, transcription factor expression (Bcl-6, c-Maf, BATF, IRF4, and Ascl2) (6, 7, 22–25), and cytokine production [predominantly IL-21, important for B cell proliferation, maintenance of GCs, and differentiation into antibody-secreting cells (26–29)]. The requirements for formation of these cells are imprinted *via* critical cellular interactions during the first few days of a humoral response (30–32), with DC–T cell interactions likely responsible for the initial upregulation of Bcl-6 within T cells (33). The expression of Bcl-6 regulates the gene encoding Ebi2 and is thus important for the convergence

of T and B cells (34, 35). Bcl-6 expression is also important for determination of Tfh from Th1 *via* expression of Bcl6 over T-bet [reviewed recently in Ref. (18)]. However, it is important to note that in contrast to previous reports, T-bet can be co-expressed with Bcl-6 (36–38) during anti-viral responses. Furthermore, the absence of Bcl-6 does not automatically commit T helper cells to Th1 or other lineages (30). The ability of T cells to co-express Bcl-6 and T-bet has implications for the induction of autoreactive GCs, as detailed later in the review.

In the initial phase of a T-dependent immune response, activated antigen-specific B cells and CD4 T cells migrate to the border between B cell follicles and T cell areas. At the B:T border, B and T cells cooperate to promote each other's differentiation into GC-precursor cells. This exchange of signals occurs both through direct cell surface ligand and receptor pairings, such as ICOSL–ICOS (32) and OX40L–OX40 (39, 40), as well as *via* SAP–SLAM signaling (41) and through T cell cytokine secretion. ICOS and OX40 have also been correlated to lupus pathogenesis in both humans and murine models (39, 40, 42). Tfh cells share this migratory path with other newly activated Th1 and Th2 effectors (43). Following Th1 cell-biased immunization, the ligands of CXCR3 are upregulated proximal to the B:T border and CXCR3-dependent migration into this area correlates with T cell-derived IFNγ production (44). Similarly, $CXCR5^+$ Th2 cells also align to the B:T border following nematode infection (45). Combined, this work suggests that these early encounters adjacent to the B cell follicle expose antigen-specific B cells to CD4 effector cytokines. This cytokine microenvironment regulates the transcription factor programs that determine B and T cell fate to balance continued Bcl-6 (30–32, 46) upregulation and thus progression into GCs, or Blimp-1-induced PC differentiation or effector T cell differentiation.

B cells and early Tfh cells have two main paths from the B-T border: forming an extrafollicular plasmablast response or migrating into the follicles to form GCs. Autoreactive cells may be generated and/or expanded in either the extrafollicular response or the GC response. For an initial burst of protective antibody and/or in responses to bacteria such as *Salmonella enterica*, B cells may move to the extrafollicular areas of secondary lymphoid organs and differentiate into plasmablasts driven by transcription factors such as Blimp-1 and IRF4. Bcl6-expressing T helper cells help program extrafollicular responses both in response to T-dependent and T-independent antigens (46), as well in an autoreactive model (47), all of which is dependent on IL-21 (46–48). In addition to IL-21, a number of signals that are derived from T helper subsets, or produced by other cells present in secondary lymphoid organs are influential in selection and subsequent expansion of autoreactive clones. These include type I and type II IFNs (49, 50), toll-like receptor (TLR) signaling together with the survival cytokine BAFF (51–53), and other cytokines such as IL-6 and IL-17 [reviewed in Ref. (54)]. Generally, they act within secondary lymphoid organs, but some (e.g., IL-17) also act in peripheral inflamed organs, and some of these cytokines are produced in ectopic lymphoid organs (see section below).

B and T cells that do not go down this path instead migrate up through the interfollicular areas and into the follicle (31, 44). Ascl2 mediates chemokine receptor expression such that Tfh downregulate CCR7 and PSGL1 and upregulate CXCR5, which is required to migrate into the B cell follicle (24). Bcl-6 is further upregulated in the interfollicular regions (30, 31), finalizing commitment to the Tfh lineage. Within the follicle, B cells and Tfh collaborate within GCs to produce high-affinity memory B cells, long-lived PCs and memory Tfh cells (36, 55).

GCs—A SITE FOR B AND T CELL COLLUSION

Germinal centers are specialized sites formed during immune responses that are responsible for the increase in affinity of B cells for the antigen (56–58). The three essential points of regulation of the GC response are: regulation of B cell behavior, regulation of Tfh, and resolution of the GC response itself (**Figure 2**). Dysregulation of any of these can lead to autoreactivity and/or the exacerbation of autoimmune disease.

Germinal centers are segregated into two zones—the dark and light zones—within which different functions occur. In the dark zone, B cells undergo proliferation and SHM of the B cell receptor. B cells will then migrate to the light zone, where they undergo selection *via* immune complexes on FDCs and compete for survival signals secreted by Tfh cells. Selected cells may then exit the GC and differentiate into memory B cells or long-lived PCs, or they will re-enter the dark zone to undergo another round of mutation and selection. T cell help of high-affinity GC B cells regulates cell cycle speed to mediate selection (56). This intricate process of cyclic migration between zones and interaction between different types of immune cells is important for appropriate regulation of affinity maturation. GC B cells have relaxed regulatory checkpoints within proliferating and mutating cells, and both clonal evolution (66) and the frequency of apoptotic cells (67) is similar between self-reactive clones and those specific to the immunizing antigen. Thus, once there is a break in tolerance to self-antigens, autoreactive clones can evade negative selection, undergo lymphoproliferation (68), with the consequential formation of B cell-mediated autoimmune conditions (69, 70). Dysregulation of T cell-intrinsic Bcl-6 (61) and overproduction of IL-21 by Tfh can further exacerbate disease (48, 54). The transcription factors Foxo1, BATF, and Myc mediate cycling between the light and dark zones, as well as selection of high-affinity cells (71–74). Whether dysregulation of these transcription factors within B cells enhance the conditions for selection and expansion of autoreactive cells remains largely uncharacterized.

REGULATORY FOLLICULAR T CELLS AND THE RESOLUTION OF THE GC

Foxp3-expressing follicular regulatory T cells (Tfr) are also important participants in GC responses (75). Their presence in the GC increases over time and they are thought to suppress Tfh numbers and function through molecules such as PD-1 (76). Chronic GCs increase the likelihood of generating autoreactive clones through epitope spreading (66, 68, 77); thus, the resolution of the GC response is essential to avoid standard immune

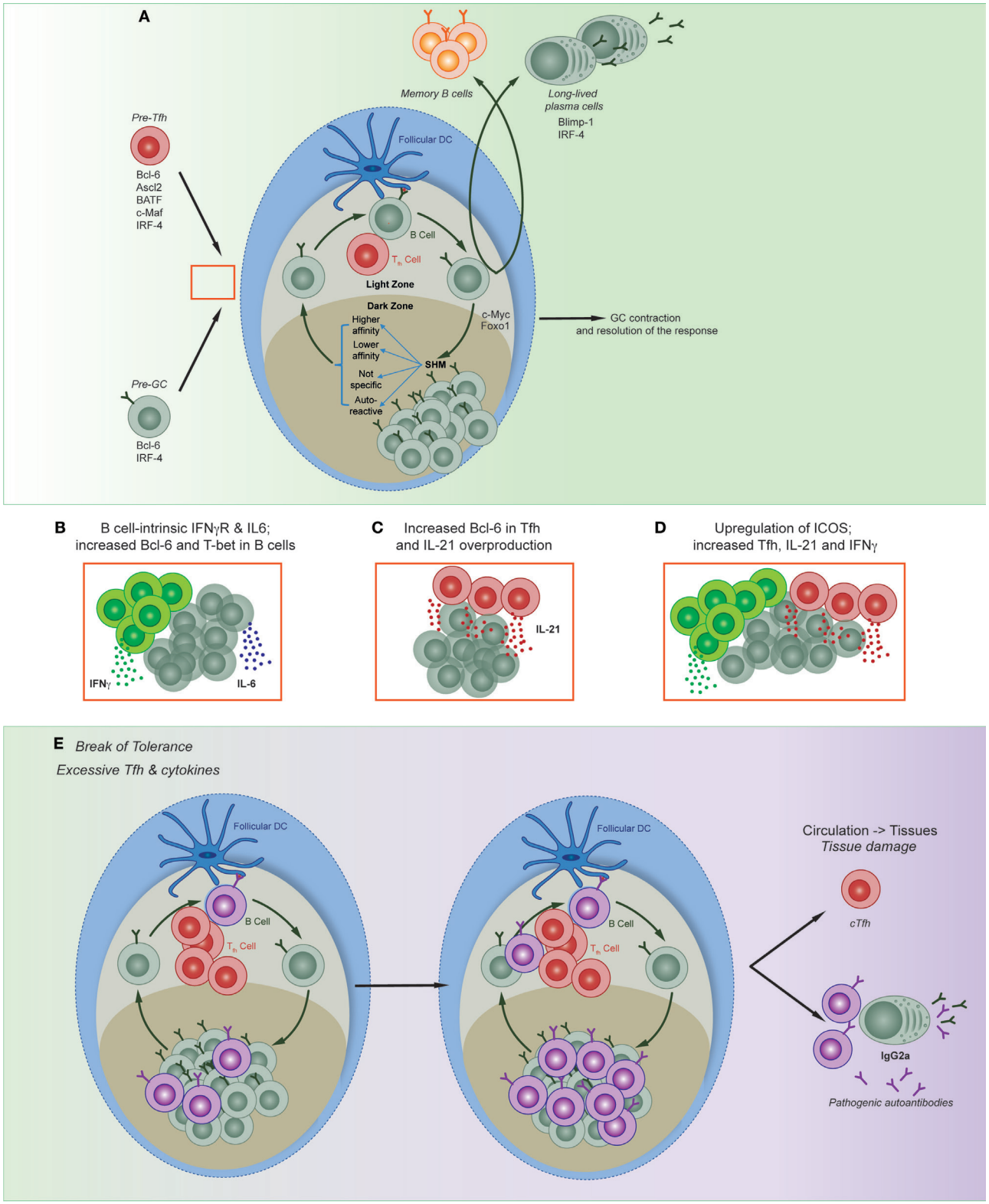

FIGURE 2 | Regulation of multiple phases of the germinal center (GC) reaction. **(A)** Appropriate regulation of Tfh formation and function, GC affinity maturation, and resolution of the GC are all required to form immune memory. Red box denotes possible point of collusion between pre-Tfh and pre-GC B cells in autoimmune disease. **(B)** Both B cell-intrinsic and T cell-intrinsic mechanisms have been shown to contribute to the production and expansion of autoreactive clones; shown are three models described in text: **(B)** (49, 59, 60); **(C)** (48, 61); **(D)** (42, 62–65). **(E)** Disruption of both B and T cell pathways result in the production and expansion of autoreactive clones, and can lead to the migration of circulating T follicular helper cells, autoreactive B cells, and plasma cells that may result in tissue damage.

responses against foreign pathogen from inducing autoimmunity. It is likely that Tfh, Tfr, and B cells all interact to shut down the GC; however, two Tfr-independent-related theories were recently put forward to explain the dissolution of the GC. The first was by McHeyzer-Williams and colleagues, who suggest that PCs directly interact with Tfh to downregulate Bcl-6 and IL-21 expression within Tfh (78). Supporting this, deletion of the PC transcription factor Blimp-1 specifically in B cells increased Tfh numbers (78). Furthermore, Toellner and colleagues demonstrated *in silico* that high-affinity antibodies feedback to mediate selection and to resolve the GC (79). Whether Tfr have a specific role in prohibiting autoreactive antibody formation is unclear, although there are two recent studies to suggest this possibility. Loss of Tfr through deletion of CD28 led to B cell-driven autoimmunity (80). Additionally, Ballesteros-Tato and colleagues recently demonstrated that IL-2-induced Blimp-1 suppression of Tfr resulted in an increase of anti-nuclear autoimmune antibodies after infection, by specifically promoting expansion of autoreactive antibody-secreting cells independent of GC and Tfh numbers (81). Furthermore, interactions between follicular T cell subsets and PCs through the inhibitory receptor PD-1 and its ligands may also suppress autoreactive GC cells (78, 82). Accordingly, PD-1 deficiency induces a lupus-like condition in mice (83) and PD-L1 deficiency can induce hyperactive Tfh responses in autoimmune arthritis (84).

THE ROLE OF IFNγ AND T-bet IN THE CREATION AND COLLUSION OF AUTOREACTIVE GCs

Both type I and type II IFNs play important roles during the development of lupus (85), yet until recently it was unknown whether both were able to drive the formation of autoreactive GC in a B cell-intrinsic manner. Two recent publications tested the requirement of IFN signaling and the downstream molecular mechanism in B cell autoimmune models (49, 59). Although B cell-intrinsic type I IFN-accelerated lupus development, it was not absolutely required (49). By contrast, B cell expression of IFNγR, as well as BCR signals and either TLR or CD40L signals, induced Bcl6 and hence spontaneous GC formation (49, 59). Furthermore, B cell-derived IL-6 synergized with IFNγ to mediate autoimmunity (60). Interestingly, this process was specific to autoimmune GC development, as the combination of IFNγR, BCR, and either TLR or CD40L was not essential for the formation of GCs in response to foreign antigen.

While Domeier and colleagues identified a role for T-bet in the formation of spontaneous GCs (59), another study in the same issue determined that the B cell-intrinsic deletion of T-bet did not impact on GC formation (49). This latter study utilized the Wiskott Aldrich syndrome chimera model of autoimmunity. Wiskott Aldrich is an X-linked immunodeficiency caused by mutations in the *WAS* gene. Patients are prone to develop systemic autoimmunity, and mice that lack WAS protein is B cells establish autoimmune disease (86). While the authors found a critical mechanistic role for IFNγR in the formation of autoreactive GCs, this was not through the induction of T-bet (49). Yet, there are other studies demonstrating B cell-intrinsic roles of T-bet; in particular for the formation of the T-bet-expressing age-associated B cell (ABC) subset. Multiple groups have identified and characterized ABCs in murine autoimmune models as well as in elderly and autoimmune patients (87–89). While T-bet expression had been used as a marker of these cells, it was initially unclear whether they were causative of autoreactivity. Rubtsova and colleagues addressed this by conditionally deleting T-bet in mature B cells in lupus-prone mice, resulting in the amelioration of autoimmune disease (90).

While these studies demonstrated a B cell-intrinsic role for IFNγR, the role of IFNγR was T cell-intrinsic in the Roquin model of lupus (a model in which a mutation in the *roquin* gene results in an aberrant number of Tfh) (62, 63). Specifically, a lack of ICOS repression resulted in excess INFγ and IL-21, concomitant with a substantial induction of Tfh and consequently GCs (42, 62, 64). Deleting one allele of Bcl-6 ameliorated the autoimmune symptoms, thus demonstrating the dependency on Tfh for generating disease in this model (65). Together, the commonality of these models is the prominent role of IFNγ in generating autoreactive responses, and the parallel pathways B and T cells can take (depending on the model) to generate autoimmunity. Studies into the molecular mechanisms that may tip an IFNγ-mediated anti-viral immune response to one that promotes autoreactivity are needed. Finally, regardless of the cell-intrinsic nature of T-bet-induced autoreactivity, it would be beneficial to determine regulators of this pathway [such as c-Myb (17)] to identify new clinical targets for autoimmune patients. This is particularly important in the B cell lineage, as cells with similar characteristics to ABCs have now been described in a number of different contexts (91, 92), particularly those that still require effective interventions such as in chronic infectious diseases (87, 93) or autoimmune conditions (94–96).

TRANSCRIPTIONAL REGULATION OF Tfh-DERIVED CYTOKINES

The transcriptional regulation of Tfh cytokine production is of central concern to lupus pathogenesis, given the number of mechanisms described above in which excessive cytokine production by Tfh promotes autoreactivity. In particular, the members of the signal transducer and activator of transcription (STAT) family of transcription factors are critical regulators of Tfh-derived cytokines. A recent study investigated the transcriptional regulation of Tfh-derived cytokines in viral infection (97). In this setting, STAT4-dependent upregulation T-bet, in line with previous studies showing STAT4 promotes both Th1 and Tfh downstream of IL-12 signaling (37). STAT4 was required for both IFNγ and IL-21, presumably acting as an upstream inducer of both T-bet and Bcl-6 (37, 97). STAT3 and STAT1 are also important regulators of Tfh differentiation and function. Functional STAT3 deficiency in humans compromises the generation of Tfh and production of IL-21 (98), while T cells from patients with lupus display increased levels of total and phosphorylated STAT3 (99). STAT3 regulates the production of IL-21 downstream of IL-6, and a positive feedback loop exists between STAT4 and STAT3 to further promote IL-21 production (37). STAT3 also works together with STAT1 to promote Tfh differentiation, again through

IL-6 induced Bcl-6 upregulation (100, 101). Interestingly, the reduction in Tfh differentiation observed in STAT3 deficiency is partially reversed with type I IFN blocking, which coincides with increased Bcl-6 expression (102). By contrast, STAT5 is induced in situations of high IL-2 to block Tfh differentiation in preference for Th1 *via* upregulation of Blimp-1 (103–105). Combined, the network of STAT transcription factors acts in concert with Bcl-6 and T-bet to specify key functional characteristics of Tfh which is relevant for lupus development.

MIGRATION TO SITES OF IMMUNOPATHOLOGY

While a lot of mechanistic insight has been gained by revealing the molecular factors underpinning B and T cell differentiation in secondary lymphoid organs, a lot less is known about whether these mechanisms also underpin local formation of autoreactive GCs in the tissues. Investigating human GC and Tfh dynamics and functionality is difficult as it relies on the attainment of tissues. As such, circulating Tfh (cTfh)—i.e., cells that possess some phenotypic and functional attributes of Tfh that are found in the blood—have been investigated as a proxy for what is occurring in the organs. It is currently unclear whether these cells are pre-Tfh or memory Tfh (106), as they do not express all markers of Tfh found in the tissues, notably Bcl-6. However, they have provided useful information about the clinical severity of diseases ranging from chronic infectious disease to autoimmune diseases. To that end, cTfh and associated serum cytokine levels such as IL-21 have been found to be elevated in patients with lupus and rheumatoid arthritis with active disease (107–112). Furthermore, a recent study also demonstrated that circulating Tfr were reduced in lupus patients, and that the ratio of Tfh/Tfr positively correlated with disease activity (113). As these cells do not express Bcl-6, other transcription factors may regulate their formation and/or migration. For instance, as previously noted, T-bet induces the expression of CXCR3, a chemokine receptor that is expressed by cTfh and is known to be important for directing cells to sites of inflammation. As cTfh do not express Bcl-6, it may be possible that cTfh are actually pre-Tfh that have been recruited into the blood before they fully differentiate in the follicle. Tfh-like cells in inflamed tissue from rheumatoid arthritis patients have been suggested to promote autoreactive plasmablast formation (114), but whether these cells originated from cTfh or were instead a non-Tfh subset produced locally (115) remains undetermined. Future work may resolve the following questions: (1) do cTfh cell phenotypes represent the cause or consequence of disease? (2) Have these cells been specifically recruited to sites of inflammation, or are they in the blood because they are dysfunctional and have simply been excluded from lymphoid organs?

CHEMOKINE RECEPTOR SIGNALING THAT REGULATES MIGRATION OF IMMUNE CELLS IN LUPUS

Lupus is a heterogeneous disease, in which the loss of varied tolerance checkpoints may result in similar disease phenotypes. Recently, transcriptional fingerprinting of patients has been highlighted as a means to deduce disease pathogenesis and stratify treatment protocols (116). This analysis has highlighted groups of patients with either primarily type I IFN or plasmablast signatures, suggesting a dichotomy of disease mechanisms. However, these patient phenotypes may be more intertwined. Several chemokines which may promote aberrant GC development are key IFN signature genes upregulated in lupus patients. Furthermore, the expression of the corresponding chemokine receptors has been correlated with B cells and/or PCs in autoimmune diseases, particularly lupus or rheumatoid arthritis. For example, elevated levels of serum CXCL10 (an interferon-inducible gene), CCL2, and CCL19 correlated with lupus activity (117–119). Furthermore, CCR6 has also been found to be upregulated on certain B cell subsets in lupus patients compared to healthy controls; however, the significance of this is currently unclear (120). The most well-characterized chemokine receptor in this context (regulation of cellular migration to sites of inflammation) is the T-bet-regulated CXCR3. CXCR3 mediates kidney disease in murine lupus nephritis (121, 122), and reduction of the transcription factor FLI1 results in amelioration of kidney disease in MRL/lpr mice with concomitant reduction in $CXCR3^+$ T cells and CXCL9/10 expression (123). Yet, the role of these chemokine families in directly facilitating the development of either self-reactive GC in secondary lymphoid organs or ectopic GC structures in inflamed tissues (124), or in mediating tissue damage by GC-independent mechanisms, remains to be determined.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It is clear that dysregulation of either B cells or Tfh cells can result in the production of autoreactive GCs and antibody-secreting cells. Unchecked proliferation of Tfh and excessive production of cytokines, such as IL-21 and IFNγ, can collude with B cell-intrinsic mechanisms to induce autoimmune responses. Furthermore, transcription factors common to both B and T cells, such as Bcl-6, T-bet, and Blimp-1, can be hijacked to assist in driving aberrant responses to infection that lead to the formation and migration of autoreactive clones. In particular, T-bet, and the T-bet-regulated gene CXCR3, appears to be key to controlling the relocation of cells from secondary lymphoid organs to other tissues where ectopic GCs and/or local autoreactive plasmablast formation can result. However, there are still a number of open questions relating to how these molecular networks may be dysregulated. Studies done in both B and T cells have demonstrated that the timing and level of expression of these transcription factors are important. For instance, the expression level of T-bet is critical in regulating T cell fate decisions, with high levels of T-bet favoring Th1 development over Tfh in the CD4 lineage, or effector CD8 over memory CD8 T cells. While a molecular mechanism underpinning graded expression has been put forth for CD8 T cells (125), whether there is a similar mechanism in other lineages that express T-bet is currently unknown. There is also evidence that gradient expression exists in B cells (17). Given that B cell-intrinsic T-bet has been postulated to be causative of autoimmune

disease in at least two murine models, it will be critical to understand whether the level of expression of T-bet can be causative of B cell dysregulation independent of extrinsic factors. Finally, while there has been a focus on IFN and T-bet in murine models in recent years, how this translates to pathogenesis in humans, and whether there are other transcription factor networks that drive the migration of cTfh, autoreactive B cells, and formation of ectopic lymphoid structures, requires further research. Thus, understanding the factors underlying the genesis of autoreactive GCs, *via* dysregulation of factors in the microenvironment and/or dysregulation of transcription factor networks, will be important in generating new targets for clinical intervention.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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Putting on the Brakes: Regulatory Kinases and Phosphatases Maintaining B Cell Anergy

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B cell antigen receptor (BCR) signaling is a tightly regulated process governed by both positive and negative mediators/regulators to ensure appropriate responses to exogenous and autologous antigens. Upon naïve B cell recognition of antigen CD79 [the immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling subunit of the BCR] is phosphorylated and recruits Src and Syk family kinases that then phosphorylate proximal intermediaries linked to downstream activating signaling circuitry. This plasma membrane localized signalosome activates PI3K leading to generation of PIP3 critical for membrane localization and activation of plecktrin homology domain-containing effectors. Conversely, in anergic B cells, chronic antigen stimulation drives biased monophosphorylation of CD79 ITAMs leading to recruitment of Lyn, but not Syk, which docks only to bi-phosphorylated ITAMS. In this context, Lyn appears to function primarily as a driver of inhibitory signaling pathways promoting the inhibition of the PI3K pathway by inositol phosphatases, SHIP-1 and PTEN, which hydrolyze PIP3 to PIP2. Lyn may also exert negative regulation of signaling through recruitment of SHP-1, a tyrosine phosphatase that dephosphorylates activating signaling molecules. Alleles of genes that encode or regulate expression of components of this axis, including SHIP-1, SHP-1, Csk/PTPn22, and Lyn, have been shown to confer risk of autoimmunity. This review will discuss functional interplay of components of this pathway and the impact of risk alleles on its function.

Keywords: B cells, anergy, SHIP-1, Pten, lyn, SHP-1, phosphatases, kinases

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INTRODUCTION

The stochastic nature of lymphocyte repertoire diversification leads to production of many B cells that are autoreactive. In fact, it is estimated up to 70% of newly generated B cells recognize self-antigens (1). These autoreactive cells must be silenced to prevent the production of pathogenic autoantibodies and presentation of autoantigen-derived peptides to potentially pathogenic T cells. Silencing of these cells occurs by three known mechanisms. If B cell antigen receptor (BCR) interactions with autoantigen are of high avidity and the cell is immature, antigen receptor signals activate receptor editing, wherein immunoglobulin light chain allele usage changes to an alternative allele (2, 3). If this process eliminates BCR autoreactivity, now harmless cell proceeds to the periphery where it can participate in protective immune responses. If alternate light chain usage does not remove sufficient autoreactivity, continued autoantigen-induced signaling results in apoptotic death, a process known as clonal deletion (4, 5). If remaining autoantigen avidity is significant, but too low to drive receptor editing or clonal deletion, the B cell proceeds to the

periphery where it exhibits reduced lifespan and is hyporesponsive to further antigen stimulation, a condition termed anergy (6). Anergic B cells fail to mobilize calcium (7), upregulate activation markers (8), and/or proliferate and differentiate in response to antigen (9).

It seems intuitive that among these silencing mechanisms, anergy is most fragile. Residence in the periphery increases the likelihood that anergic cells encounter inflammatory cytokines, stimulatory pathogen- and damage-associated molecular patterns, PAMPS, and DAMPS that may compromise their unresponsiveness. In addition, maintenance of the anergic state is dependent on continued occupancy of antigen receptors by antigen (10). Removal of autoantigen results in acquisition of responsiveness within minutes (10). This "reversibility" likely confers additional risk of participation in autoimmunity, but is also informative regarding the molecular mechanisms underlying anergy.

The rapid reversibility of anergy suggests that unresponsiveness is maintained by non-durable biochemical pathways and not by genetic reprogramming. Consistent with this possibility, analysis of differences in the transcriptomes of naïve and anergic B cells have failed to reveal mediators of anergic B cell unresponsiveness (11). This review is focused on the molecular regulatory mechanisms that are uniquely induced in anergic cells and are involved in maintenance of their unresponsiveness.

CHARACTERISTICS OF ANERGIC B CELLS

Naïve B lymphocyte recognition of antigen leads to transduction and propagation of signals that induce expression of activation markers and prepare the cell to interact productively with T cells. However, chronic binding of antigen in the absence of secondary signals provided by T cells, DAMPs, and/or PAMPs, leads to unresponsiveness (12). This anergy is maintained when as few as 20% of receptors are occupied (13), thus unresponsiveness is not caused by inability to bind self or cross-reactive exogenous antigen. Further, this state is not maintained by tonic regulators as it must be induced by antigen receptor stimulation (14–16). Therefore, the mechanisms that maintain the antigen unresponsiveness of anergic B cells can be expected to have the following properties: they require induction by chronic antigen receptor stimulation, they are non-durable, and they affect receptor-proximal signaling events.

Although the concept evolved from studies of normal mice (17), most of what is known about B cell anergy was learned by studying immunoglobulin transgenic mice in which all B cells share reactivity with an autoantigen. Perhaps most notable are MD4 immunoglobulin transgenic mice that express mIgM and mIgD BCR with high affinity for hen egg lysozyme (HEL). When these mice were crossed to ML5 mice expressing soluble HEL, B cells developed and occupied peripheral lymphoid organs, but the animals were unresponsive to immunization (9, 18). Transfer of naïve MD4 B cells or MD4xML5 B cells to B6 recipients with CD4 T cells that recognize sheep red blood cells (SRBCs), followed by immunization with HEL-SRBCs, led to a response by both populations, though the response of the latter cells was greatly reduced. MD4xML5 cells did not respond when transferred to ML5 recipients. The former must reflect gradual dissociation of autoantigen from BCR following transfer to the autoantigen free environment, with attendant loss of unresponsiveness, or "anergy" (19). The inability of anergic B cells to mount an equivalent immune response following transfer could be due to an inability of cells to cooperate with cognate T cells, due either to failure to process and present antigen or to respond to T cell derived signals. To determine whether the defect in these anergic cells lay in the ability to internalize, process, and present antigen to T cells, they were modified to constitutively express MHC class II with peptide, bypassing the need to process and present antigen, and allowing interaction with cognate T helper cells (7). If the defect lay only in antigen processing and presentation, adoptive transfer of these B cells into B6 or ML5 recipient mice, followed by immunization, should have led to an immune response. However, these MD4xML5 anergic B cells failed to respond by producing anti-HEL antibodies (7). Interestingly, naïve MD4 B cells adoptively transferred into ML5 recipients responded to antigen by proliferation and differentiation. This evidence suggests that the immune response defect in anergic B cells must reflect more than an inability to process and present antigen. To determine the ability of anergic B cells to respond to T cell help, naïve MD4 B cells and anergic MD4xML5 B cells were stimulated *in vitro* with IL-4 and anti-CD40 and responses assessed. Both naive MD4 B cells and anergic MD4xML5 B cells upregulated MHC class II and costimulatory molecules, i.e., CD86, in response to these stimuli that mimic T cell help (7, 8). These data demonstrated the reversibility of anergy, as well as suggest there is not an inherent defect in the ability of an anergic B cell to respond to T cell help. They left open the possibility that the defect could lie in an inability of the anergic cell to upregulate T cell costimulatory ligands such as CD86 in response to antigen.

Because the previous experiments indicated that the inability of anergic B cells to respond to antigen is not limited to an antigen processing and presentation defect, it seemed likely that there was defect(s) in antigen receptor signaling. To determine the ability of anergic B cells to respond to BCR ligation, *in vitro* responses of naïve MD4 B cells and anergic MD4xML5 B cells were compared. Unlike naïve cells, MD4xML5 failed to proliferate, increase RNA synthesis indicative of entry into cell cycle, or upregulate CD86 (7). These data suggest that there is an inherent defect in the ability of an anergic B cell to signal through their antigen receptors. Confirming this, anergic B cells failed to mobilize calcium in response to BCR stimulation. Antigen stimulation of anergic B cells did not lead to a significant increase in protein phosphorylation (7). Tolerant B cells show a decrease in cell surface IgM antigen receptors, possibly explaining the decrease in signaling. However, anergic B cells transferred into B6 recipients and "parked" for 36 h led to normalization of receptor levels and equivalent fluorescently labeled antigen binding, but the cells remained unresponsive to antigen based on calcium mobilization (7). It is important to note that while anergic B cells downregulate mIgM, they do not downregulate mIgD, which constitutes 90% of the antigen-binding capacity of most splenic B cells (20). This

alone would argue that hyporesponsiveness of anergic B cells is not attributable to reduced antigen-binding capacity.

Protein tyrosine phosphorylation is the earliest quantified event in BCR signaling. Loss of this event in anergic cells suggests that unresponsiveness may reflect a defect in initial transduction of signals across the plasma membrane (7, 21). Consistent with this possibility, it has been reported that antigen stimulation can lead to rapid destabilization of the interaction of mIgM with the CD79a/b (Igα/β) heterodimer (22). Reductionist studies using B cell lines ectopically expressing association-competent versus incompetent BCR demonstrated that incompetent BCRs can compromise competent receptor signaling within the same aggregate/complex. In fact, receptor complexes containing as few as 13% incompetent CD79-associated mIg showed defects in signaling (22). Thus, mechanisms that act to limit BCR signaling in anergic cells may somehow target the structural integrity of the antigen receptor itself.

The discussion above describes extant knowledge of biological and BCR signaling defects associated with B cell anergy in the MD4 anti-HEL model. The findings described were confirmed in another model, the Ars/A1 model, in which B cells are reactive with chromatin (13). Below, we drill more deeply into proximal BCR signaling pathways and negative regulatory mechanisms that limit the antigen responsiveness of anergic cells.

ANTIGEN RECEPTOR SIGNALING IN NAÏVE AND ANERGIC B CELLS

In naïve B cells, BCR stimulation leads most proximally to the tyrosine phosphorylation of two conserved tyrosine residues embedded in immunoreceptor tyrosine-based activation motifs (ITAMs) found in CD79a and CD79b, the heterodimeric signaling component of the BCR, as indicated in **Figure 1** (23–26). This phosphorylation appears to be governed by the balanced activity of phosphotyrosine phosphatases and SRC family kinases for which ITAMs are substrates (27–29). Phosphorylated ITAMs stimulate Lyn activation, presumably through association with the kinase SH2 binding and derepression of its enzymatic activity (30). ITAM bi-phosphorylation enables receptor binding of the Syk tyrosine kinase *via* its dual SH2 domains leading to its phosphorylation and activation (28, 31). BCR stimulation leads to concurrent Lyn-mediated tyrosine phosphorylation of CD19, a BCR accessory/co-receptor, enabling its association with Lyn and phosphoinositide 3-kinase (PI3-kinase) (32). CD19, functioning in conjunction with the adaptor BCAP, mediates activation of PI3-kinase and generation of PI(3,4,5)P3 (33). The head group of this inner leaflet phospholipid second messenger binds the pleckstrin homology (PH) domains of a number of critical downstream effectors, including PLCγ, AKT, PDK1, and BTK, localizing them

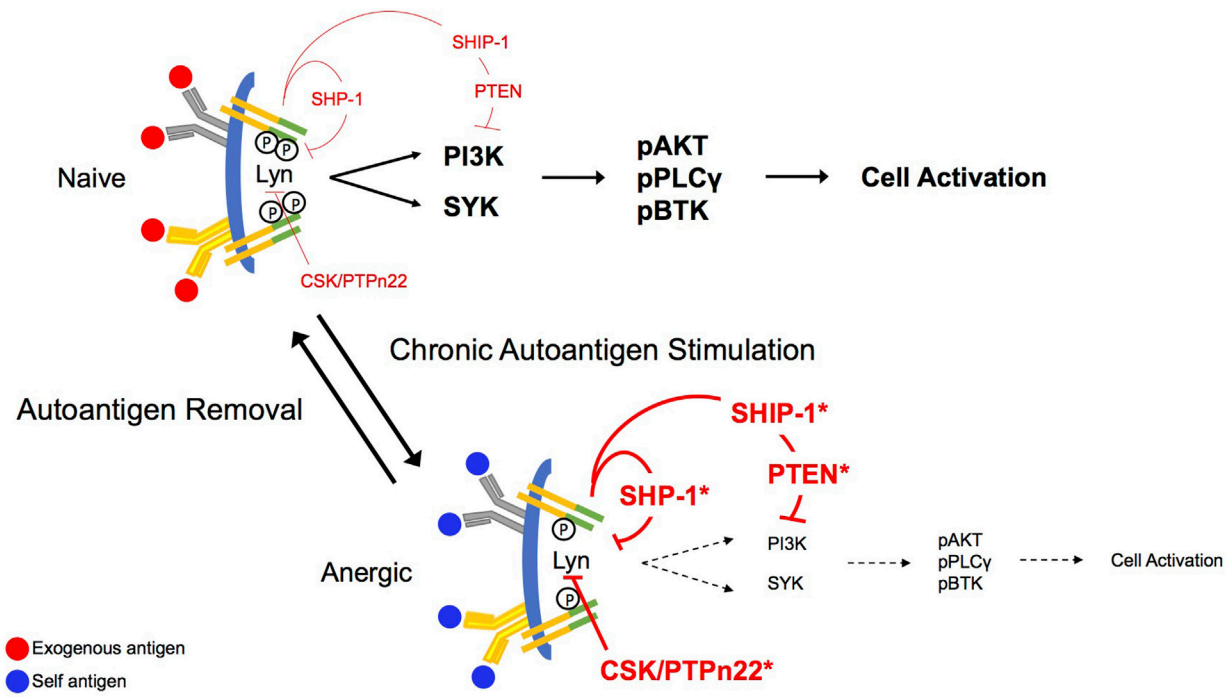

FIGURE 1 | Antigen receptor signaling in naïve and anergic B cells. B cell antigen receptor (BCR) aggregation in naïve cells leads proximally to phosphorylation of CD79a and CD79b immunoreceptor tyrosine-based activation motif (ITAM) tyrosines. This phosphorylation allows recruitment of the Src-family kinase Lyn to the receptor where it binds through its SH2 domain and its activity is derepressed. Lyn mediates activation of the phosphoinositide 3-kinase (PI3-kinase) pathway *via* phosphorylation of CD19, and the PI(3,4,5)P3 product of this pathway sequesters BLNK, PLCγ, and BTK for function in signaling. Phosphorylation of both ITAM tyrosines leads to binding of SYK *via* its dual SH2 domains and to subsequent activation. Under conditions of chronic antigen stimulation (anergy), the ITAMs on CD79a are preferentially monophosphorylated, recruiting Lyn to the complex. Lyn, in its negative regulatory role, recruits the function of the inositol phosphatases, SHIP-1 and PTEN, as well as the tyrosine phosphatase, SHP-1. These regulatory phosphatases will then exert their inhibitory function on downstream effector molecules, abrogating stimulatory BCR signaling. Bias toward stimulation of inhibitory circuitry is reversed upon removal of the antigen receptor stimulus.

to the receptor where they can be activated by phosphorylation (31). Multiple parallel pathways diverge from this activated receptosome, leading ultimately to cell activation.

Anergic B cells are chronically stimulated by autoantigen *in vivo*, but at least in MD4xML5 and Ars/A1 models, cell surface BCRs are not saturated. Indeed, on immediately *ex vivo* anergic cells most receptors are unoccupied (13). Additionally, chronic *in vivo* autoantigen stimulation results in monophosphorylation of receptor ITAMs (16). Findings from cell-free experiments in which the ability of specific kinases to phosphorylate CD79 ITAMs was assessed demonstrate that Lyn and Fyn, but not Syk efficiently phosphorylate these substrates. However, this phosphorylation occurs primarily on the more N-terminal ITAM tyrosines, and ITAM bi-phosphorylation is a minor event (~20%) (28, 30). This may indicate that the degree of ITAM tyrosine phosphorylation is highly regulated by factors in addition to degree of aggregation. In terms of downstream consequences, it should be noted that because activation of Syk requires binding of both SH2 domains to phosphorylated ITAM tyrosines, monophosphorylation is associated with Lyn, but not Syk activation (30). Consistent with this, induced phosphorylation of the Lyn substrate CD19 appears normal (Getahun and Cambier, unpublished) in anergic cells, but pathways downstream from Syk are silent. Phosphorylation of CD19 suggests that PI3-kinase is active in these cells. Anergic B cells exhibit increased basal and BCR-mediated tyrosine phosphorylation of the PI(3,4,5)P3 5-phosphatase SHIP-1 and its adaptor Dok-1 (34), previously shown to be associated with their activation (11, 35). They are also characterized by an increase in expression of PTEN, a PI(3,4,5)P3 3-phosphatase, that is subject to regulation by a number of microRNAs (36, 37).

REGULATION OF BCR SIGNALING IN NAÏVE AND ANERGIC B CELLS

Lyn

Interestingly, Lyn, the primary BCR-associated Src-family kinase, plays both positive and negative functional roles in antigen receptor signaling (38–40). Allelic differences in the LYN gene leading to reduced expression of the kinase confer increased risk of developing SLE, and patients with lupus have decreased Lyn expression in B cells (41, 42). In mice, Lyn deficiency increases negative selection in the bone marrow with fewer $Lyn^{-/-}$ B cells being found in the periphery of $Lyn^{-/-}$ MD4 mice (43). Peripheral $Lyn^{-/-}$ B cells fail to fully mature (43, 44). $Lyn^{-/-}$ B cells exhibit delayed but exaggerated and more sustained calcium response to antigen (39, 45), further suggesting both positive and negative roles in BCR signaling. In the absence of Lyn, other Src-family kinases expressed in B cells (Blk and Fyn) act to propagate BCR signaling, but down modulation of BCR signaling is abrogated, suggesting loss of anergy. The balance of Lyn and Fyn is further explained *in vivo* with Lyn deficiency exacerbating nephritis and arthritis, while loss of Fyn is protective from auto/inflammatory disease (46). Moreover, patients with SLE present in the clinic with a Fyn-activating signature, further suggesting a negative role for Lyn in BCR signaling.

$Lyn^{-/-}$ mice develop an SLE-like disease as indicated by autoantibody production and glomerulonephritis (47). B cells from these mice undergo enhanced proliferation in response to BCR crosslinking. Macrophages and dendritic cells also play a role in development of disease in $Lyn^{-/-}$ mice. However, B cell-specific conditional deletion of Lyn, achieved by crossing the $Lyn^{fl/fl}$ mouse to a mouse carrying Cre expressed under the CD79a promoter, leads to autoantibody production, IgG immune complex deposition ultimately resulting in glomerulonephritis (44).

Lyn may exert its negative regulatory function through phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on CD22, leading to recruitment of SHP-1 (48), a tyrosine phosphatase that dephosphorylates activating signaling molecules, such as CD79a/b ITAMs, Syk, and BLNK (49, 50). Lyn also drives inhibitory signaling by promoting the inhibition of the PI3-kinase pathway, by phosphorylating the inositol phosphatase, SHIP-1 and its Doc family adaptors, Doc-1 and Doc-3. SHIP-1 hydrolyzes PI(3,4,5)P3 yielding PI(3,4)P2, preventing recruitment, and activation of PH domain-containing effectors and consequent propagation of BCR signals. Alleles of genes that encode components of this regulatory axis, including SHP-1 (38, 51), Csk (52), PTPn22 (53–56), and Lyn (41), have been shown to confer risk of autoimmunity (57). Reduced PTEN and SHIP-1 levels presumably caused by increased in expression of microRNAs that regulate them are also seen in autoimmunity. Demonstrating their critical roles in maintenance of anergy, we have shown that acute deletion of these proteins from anergic B cells *in vivo* results in rapid cell proliferation and differentiation, and production of autoantibodies.

SHP-1

An allele of the regulatory SH2-containing tyrosine phosphatase SHP-1 has been associated with increased risk of developing SLE (38). SHP-1 and SHP-2 mediate the function of certain inhibitory ITIM containing receptors, such as CD22, PD1, and FcγRIIB, although there is evidence that SHP-1 is dispensable for the latter (58, 59). SHP-1 was first described as being crucial for FCγRIIB-mediated negative regulation of anti-BCR induced proliferation in motheaten mice (me) (60). The ultimate resolution of this inconsistency came from studies of Lasourne and colleagues who showed that the degree of packing of phosphorylated FcγRIIB ITIMs determined the relative involvement of SHIP-1 and SHP-1 in downstream inhibitory signaling. Higher level aggregation, as probably occurred in the D'Ambrosio studies, would be expected to evoke SHP-1 function.

Viable motheaten mice (me^v/me^v) have a mutation that interferes with a splice site in the gene that encodes SHP-1, *Ptpn6*, reducing the enzyme activity to 10–20% of wild type. These animals exhibit severe B cell immunodeficiency and autoantibody production. In me^v crossed to the MD4, SHP-1 low B cells undergo increased intracellular calcium flux responses to antigen. SHP-1 deficiency also leads to increased serum levels of IgM, IgG_1, and IgG_3 (61). Furthermore, B cell-specific loss of SHP-1 leads to an accumulation of B-1a cells and systemic autoimmunity (62, 63). Acute B cell targeted deletion of SHP-1 from anergic B cells *in vivo* leads to cell activation, proliferation, differentiation to plasmablasts and autoantibody production

(62). Genetic complementation studies indicate that SHIP-1 and SHP-1 act in distinct regulatory pathways both of which must be functional to maintain anergy.

Studies indicate that in a subset of SLE patients, B cells express reduced SHP-1 protein, suggesting these patients have a decreased ability to maintain B cell anergy (51). A decrease in SHP-1 (both protein and mRNA) in PBMCs isolated from multiple sclerosis patients is associated with an increase in inflammatory gene expression (64). This is particularly interesting given both me^{v} mice and the inducible mouse model of MS, experimental autoimmune encephalomyelitis, have more severe disease when SHP-1 deficiency is observed (65, 66).

SHIP-1 and PTEN: Cooperative Enforcement of Anergy

Phosphoinositide 3-kinases function in the promotion of numerous biological functions by the generation of lipid second messengers. Mice that lack the dominant PI3-kinase isoform found in B cells, p110δ, show a reduction in calcium mobilization, a decrease in serum immunoglobulin levels, as well as a decrease in germinal center formation in the spleen (67, 68). Tight regulation of this pathway by the inositol phosphatases, SHIP-1 and PTEN, is critical for maintaining tolerance to self-antigens.

Studies utilizing adoptive transfer of anergic B cells, followed by targeted deletion of SHIP-1, have shown that this inositol phosphatase is crucial for maintenance of anergic B cell unresponsiveness to self-antigen. Upon deletion of SHIP-1, B cells that were once anergic become activated, upregulating costimulatory molecules, and proliferate and differentiate into antibody secreting cells (62). Furthermore, B cell targeted deletion of SHIP-1 results in systemic autoimmunity (16, 69). In addition to its enzymatic activity, SHIP-1 functions as an adaptor protein, binding effectors such as rasGAP (34). To determine if SHIP-1's role in maintaining anergy is a function of its regulation of the PI3-kinase pathway as opposed to its adaptor functions, Getahun et al. used genetic models to conditionally delete PTEN or express a constitutive active PI3-kinase p110α in anergic B cells *in vivo* (62). Loss of PTEN expression or enforced over-production of PI(3,4,5)P3 led to a breach of anergy. Interestingly, while haploinsufficiency of either SHIP-1 or PTEN alone does not lead to loss of anergy, haploinsufficiency of both inositol phosphatases does, consistent with the fact that these phosphatases act in the same regulatory pathway.

SHIP-1 levels are decreased in Fas$^{MRL/lpr}$ mice due to an increase in the microRNA that regulates its expression, microRNA 155 (36). microRNA 155 is also elevated in SLE patients and has been correlated with disease activity (70). Additionally, descriptions of decreased PTEN expression, caused by an increase in expression of microRNA 7, in B cells from patients with SLE have been correlated with disease severity (37). These data confirm that induced inhibition of the PI3-kinase pathway is critical for maintaining tolerance and preventing anergic B cells from participating in an autoimmune response. They further implicate microRNA levels as indirect regulators of anergy.

Csk/PTPn22

PTPn22 variants are among the risk alleles most strongly linked to human autoimmunity, but the molecular mechanism of PTPn22 action and consequence of risk allele defect(s) remains unclear. The PTPn22 R620W allele is found at high frequency in patients with type 1 diabetes, RA, SLE, Grave's thyroiditis, and myasthenia gravis, but does not predispose to other diseases, such as multiple sclerosis, Crohn's disease, or psoriasis vulgaris. While there are additional PTPn22 risk alleles that are associated with increased risk of autoimmunity (71), we will focus on the more understood R620W allele.

PTPn22 is a nonreceptor tyrosine kinase that binds Csk, a known suppressor of antigen receptor signaling. PTPn22 has also been shown to negatively regulate signaling by dephosphorylating Src family kinases (72, 73). PTPn22$^{-/-}$ mice develop increased serum immunoglobulin levels and germinal centers although overt autoimmunity is absent. The R620 is located in a position critical for binding of PTPn22 to Csk, suggesting that R620W would decrease the interaction of the two molecules, possibly resulting in increased antigen receptor signaling. However, Liston et al. demonstrated R620W causes reduced TCR signaling, leading to reduced thymic selection and subsequent deletion, allowing for more autoreactive T cells to exit into the periphery to later become activated and participate in an autoimmune response (74). Furthermore, R620W human B cells have decreased BCR signaling and BCR-mediated responses, but have an increased autoreactive B cell compartment, coupled with less effective central and peripheral tolerance. Patients who carry the mutated form of PTPn22 have an increased autoreactive B cell compartment, coupled with less effective central and peripheral tolerance. This is also seen in mice that express the human equivalent mutation, R619W, in B cells only. These mice develop autoimmunity (75).

CONCLUDING REMARKS

This review summarizes a body of work that has defined B cell anergy and the molecular mechanisms that maintain antigen unresponsiveness of anergic B cells. It further describes the effects of allelic variations of regulatory signaling molecules that confer increased risk of autoimmunity. Role for these risk alleles in failed silencing of autoreactive B cells *per se*, underscore the potential of targeting B cells for therapeutic intervention in autoimmunity [reviewed in Ref. (76)]. In the era of precision medicine, therapy will be based on genetics in addition to symptomology. As with all therapies for autoimmunities, there is a balance to be struck between controlling the autoimmune response while still leaving patients competent to mount protective immune responses to pathogens.

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REVIEW
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Nuclear Factor-kappaB in Autoimmunity: Man and Mouse

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NF-κB (nuclear factor-kappa B) is a transcription complex crucial for host defense mediated by innate and adaptive immunity, where canonical NF-κB signaling, mediated by nuclear translocation of RelA, c-Rel, and p50, is important for immune cell activation, differentiation, and survival. Non-canonical signaling mediated by nuclear translocation of p52 and RelB contributes to lymphocyte maturation and survival and is also crucial for lymphoid organogenesis. We outline NF-κB signaling and regulation, then summarize important molecular contributions of NF-κB to mechanisms of self-tolerance. We relate these mechanisms to autoimmune phenotypes described in what is now a substantial catalog of immune defects conferred by mutations in NF-κB pathways in mouse models. Finally, we describe Mendelian autoimmune syndromes arising from human NF-κB mutations, and speculate on implications for understanding sporadic autoimmune disease.

Keywords: NF-κB, autoimmunity, self-tolerance, thymic development, mutation

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INTRODUCTION

NF-κB is family of proteins that mediate transcriptional regulation crucial to many biological functions (1). NF-κB was discovered and named for its action in regulating κ light chain expression in B cells. Remarkably, however, as elucidation of *Drosophila* immunity has demonstrated, NF-κB and its orthologs are highly conserved regulators of signaling and transcription crucial for cellular and humoral host defense even in organisms that lack adaptive immunity (2). In mammals, the actions of NF-κB are complex and extensive, and encompass activation, proliferation in cells of the innate and adaptive immune system, and organogenesis of lymphoid tissue and its microarchitecture (3–5).

Under normal circumstances, NF-κB proteins are latent in the cytoplasm, poised for rapid responses after their inhibition is temporarily removed. Uninhibited NF-κB molecules then shuttle between nucleus and cytoplasm as transcriptionally active homo- and heterodimers (**Figure 1**). In addition to this fundamental inhibitory constraint, many other negative regulatory loops exist to either prevent, dampen, or terminate NF-κB signaling, including sequestration in multi-molecular

Abbreviations: Aire, autoimmune regulator; BAFFR, regulation of B cell activating factor receptor; BIM, Bcl-2-interacting mediator; CBM, CARD/BCL-10/MALT1 complex; cTEC, cortical thymic epithelial cell; DC, dendritic cell; DUB, deubiquitinase; EAE, experimental autoimmune encephalomyelitis; ENU, *N*-ethyl-*N*-nitrosourea; Ep-CAM, epithelial cellular adhesion molecule; GC, germinal center; IκB, inhibitor of kappa B; IKK, inhibitor of kappa B kinase; LPS, lipopolysaccharide; mTEC, medullary thymic epithelial cell; MZ, marginal zone; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NIK, NF-κB-inducing kinase; nTreg, natural regulatory T cell; RANK, receptor activator of nuclear factor kappa-B; TAD, transactivation domain; Tconv, Conventional T cell; Treg, regulatory T cell; TSA, tissue specific antigens; UEA-1, *Ulex europaeus* agglutinin 1.

complexes, posttranscriptional regulation, and posttranslational modifications of proteins by phosphorylation and ubiquitination (of various forms). Furthermore, many components of NF-κB, including both positive and negative regulators, are under transcriptional regulation by NF-κB itself.

Despite this complex regulatory network, specific defects in individual molecules within the NF-κB pathway have been shown to disrupt cellular homeostasis, and immune pathology is an important consequence (1, 6). In this review, we will concentrate on how NF-κB contributes to immunological self-tolerance, and how defects in NF-κB contribute to autoimmune disease. Defects in NF-κB have also been shown to cause immune deficiency and autoinflammatory diseases, and somatic mutations are frequent drivers of lymphoid malignancy, for which authoritative reviews are available (7, 8). As will be discussed here, however, it is notable that in some cases, a single mutation confers both autoimmunity and immune deficiency, reflecting the complex regulatory actions of NF-κB.

OUTLINE OF NORMAL NF-κB SIGNALING

The NF-κB family of transcription factors form hetero- and homodimers that regulate transcription by binding to a palindromic DNA sequence, κB (1), located within promoters and enhancers of a large number of genes (9, 10). In vertebrates, there are five NF-κB family members, RelA, c-rel, RelB, NF-κB1, and NF-κB2. N-terminal Rel-homology domains (RHD, from v-Rel, reticuloendotheliosis viral oncogene homolog) are common to all and mediate κB binding and interactions with other proteins, including inhibitor of kappa B (IκB) (see below) (10–12).

NF-κB proteins are classified in two groups according to structure and function. p105 (NF-κB1) and p100 (NF-κB2) are precursor proteins that undergo partial proteolysis to remove their C-terminal ankyrin repeats, yielding p50 and p52, respectively. p50 and p52 lack transactivation domains (TAD) unless heterodimerized with Rel or coactivator non-Rel proteins (13). By contrast, RelA (p65), RelB, and c-Rel are active in the absence

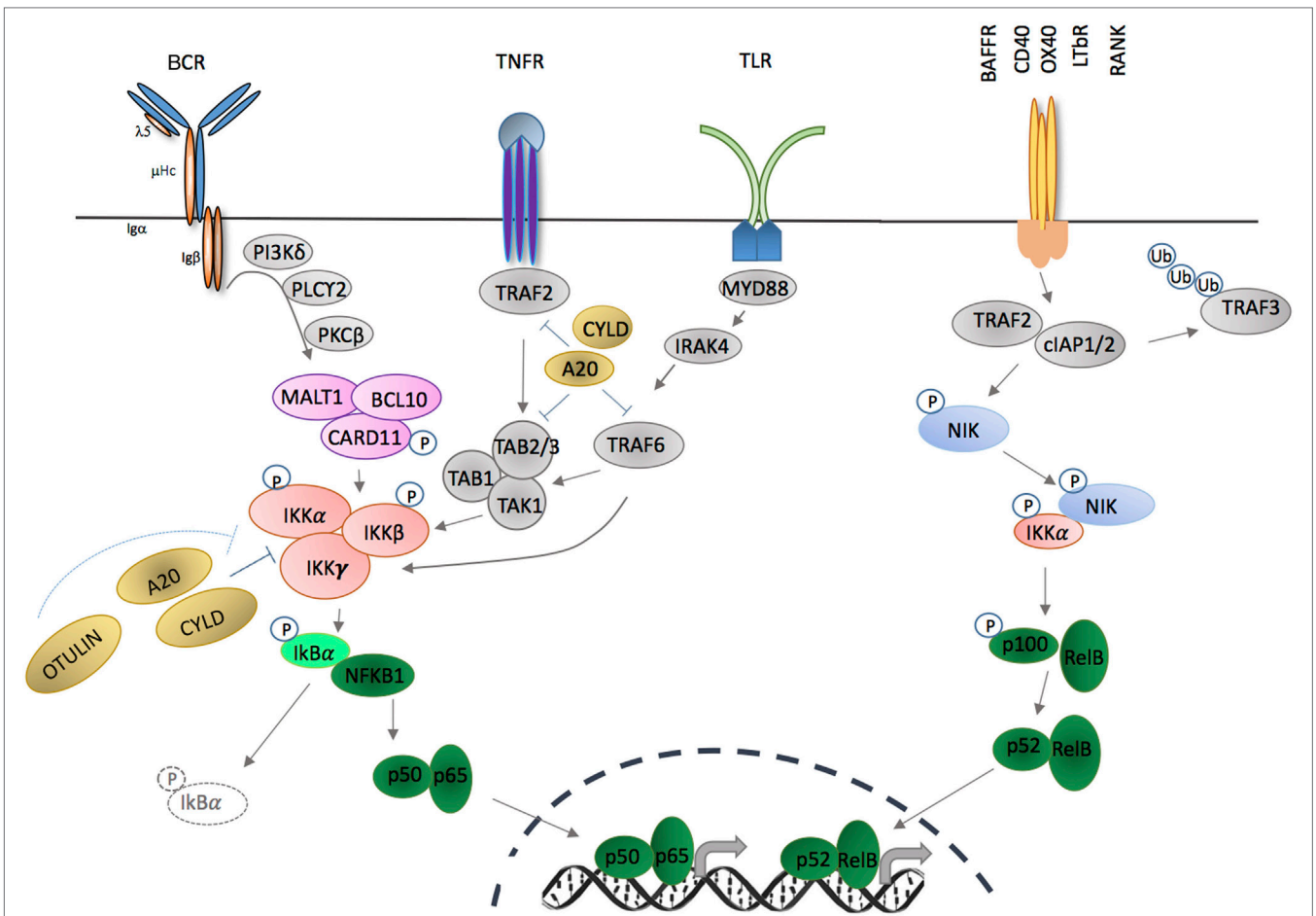

FIGURE 1 | Activation of canonical and non-canonical NF-κB signaling pathways through membrane-bound extracellular ligands. TNFR and toll-like receptor (TLR) family members, as well as antigen receptors activate the canonical pathway; and regulation of B cell activating factor receptor (BAFFR), CD40, OX40, LTβR, and receptor activator of nuclear factor kappa-B (RANK) activate the non-canonical pathway. Triggering of canonical pathway results in activation of p50/p65 (RelA), while the non-canonical pathway signaling leads to activation of p52/RelB complexes. Both pathways require phosphorylation and activation of inhibitor of kappa B kinase (IKK) subunit(s) in order to release NF-κB molecules that are sequestered by an inhibitor, e.g., IκBα or p100. Phosphorylation and ubiquitination of the inhibitors by IKKs release NF-κB that translocate into nucleus in the forms of homodimers or heterodimers complexes and bind to the κB site of their target genes.

of proteolysis because they contain TAD that positively regulate expression of target genes (14, 15).

Differences in transcriptional activity of NF-κB dimers helps explain the plasticity of responses to both quantitative and qualitative variation in cell stimulation (16). p50/65 heterodimers are near ubiquitous, and positively regulate NF-κB target genes (10). By contrast p50 homodimers repress TNF-α transcription in response to lipopolysaccharide (LPS) (17–19). Homodimers of p50 are abundant in resting T cells, but their expression is reduced after antigenic receptor ligation (20), when p50/p65 become abundant in cell nuclei, reversing the NF-κB-dependent suppression of the target genes, i.e., IL-2 or IL-6 and iNOS in response to LPS (21). RelB does not homodimerise, but confers transcriptional activity when complexed with p52 or p50 (22). RelB constitutively localizes to the nucleus, but binding may be inhibited by association with p100 (23–25). Under some circumstances, RelB represses NF-κB activity by forming RelA/RelB heterodimers that fail to bind DNA and sequesters RelA (9, 26, 27). Similarly to RelB, c-Rel is expressed in lymphoid tissues, and both c-Rel homodimers and c-Rel/p50 heterodimers are detected predominantly in hematopoietic cells. c-Rel homodimers and c-Rel/p65 are essential for B and T cells survival and effector cell function (28–30).

ACTIVATION OF NF-κB IN IMMUNITY

In the absence of specific signals, NF-κB is maintained in latent form bound to IκB in the cytoplasm. The IκB family includes IκBα, IκBβ, IκBε, BCL-3, IκBζ, IκBNS, as well as unprocessed p100 and p105, which are all characterized by multiple ankyrin repeats (31). In addition to their IκB function, so-called atypical IκBs (IκBζ, IκB-NS, and Bcl-3) have intrinsic nuclear localization propensity where they bind preferentially to p50 (Bcl-3, IκBζ) and p52 (Bcl-3), which under different conditions can either promote or terminate NF-κB binding to DNA (32, 33). This mechanism has been well characterized for Bcl-3, which has a TAD and can act as a coactivator when associated with p50 or p52 homodimers (34, 35), or by facilitating replacement of transcriptionally inactive p50 homodimers with transcriptionally active heterodimers (36, 37). On the other hand, Bcl-3 can also function as a repressor by stabilizing p50 homodimers on κB site of the target genes. For example, Bcl-3 has been reported to mediate LPS tolerance in macrophages by stabilizing p50 homodimer on the *TNF* promoter (38–40).

NF-κB members are liberated by the action of the IκB kinase complex [IKKα, IKKβ and NF-κB essential modulator (NEMO), encoded by *IKBKA*, *IKBKB* and *IKBKG*], which leads to temporary degradation of IκBs (**Figure 1**). IKKγ (NEMO) is a regulatory subunit that does not have intrinsic catalytic activity but is important for kinase activation of IKKα and IKKβ (41). IKKα and IKKβ are serine/threonine kinases that share an N-terminal kinase domain and are responsible for phosphorylating several members of the IκB family (42, 43). Serine phosphorylation of IKKα and IKKβ [serines 177 and 181 for IKKβ; serines 176 and 180 for IKKα (44, 45)] results in conformational change and kinase activation. Activated IKKβ operates within the inhibitor of kappa B kinase (IKK) complex to phosphorylate IκBα, leading to K48-linked ubiquitination and proteosomal degradation, which releases NF-κB factors for nuclear translocation (46, 47). Loss of IKKβ results in significant reduction of NF-κB activity in response to TNF-α and IL-1α, a defect that cannot be completely compensated for by IKKα. As result, *Ikbkb* deficiency confers a mouse phenotype similar to *Rela* deficiency (48, 49) (**Table 1**).

CANONICAL PATHWAY STIMULI

The canonical NF-κB pathway includes NFκB1 (p105), RelA (p65), and c-Rel and is activated by many ligands, including those that engage members of the tumor necrosis factor receptor superfamily, toll-like receptors (TLR), interleukin 1 receptor, and T and B cell antigen receptors (**Figure 1**). Different receptor-proximal signaling cascades connect these receptors to the IKK complex. TLR ligation activates a complex composed of TAK1, TAB 1, TAB 2, which phosphorylates IKKβ (106, 107). Activation of TNF receptor leads to interaction of series of adaptor proteins that contains TRAF-binding domains (108, 109). TRAF2 recruits the E3 ubiquitin ligases, cIAP1/2, which are necessary for IKK activation *via* recruitment of linear ubiquitination assembly complex (LUBAC) (directly) and TAK1 (indirectly) (110, 111). Ligation of B and T cell antigen receptors leads to phosphorylation of CARD11 by protein kinase C and assembly of the CARD/BCL-10/MALT1 (CBM) complex.

MODIFICATION OF CANONICAL SIGNALING

Posttranslational regulation by ubiquitination is crucial for NF-κB regulation. A series of ubiquitinating and deubiquitinating enzymes are known that both activate and modify NF-κB transcriptional regulation (112). The best characterized negative regulators of NF-κB are deubiquitinase (DUB) A20 (encoded by *TNFAIP3*), CYLD, and OTULIN (113–116).

TNFAIP3 is upregulated in response to TNF receptor and TLR ligation, and A20 negatively controls NF-κB-dependent gene expression (**Figure 1**) (117). A20 removes or modifies K63 polyubiquitin from several substrates within NF-κB signaling, including TRAF6, NEMO, MALT1, and TNFR1 (118–120). In addition to its deubiquitin domain in the N-terminus, A20 also contains seven zinc finger domains in the C-terminus with E3 ligase functions, enabling ubiquitin-editing. Thus, A20 replaces K63- with K48-polyubiquitin chains of RIPK1, flagging it for proteasomal degradation (99, 113). The importance of A20 in modifying NF-κB activity and immune responses was confirmed in A20-deficient mice that developed profound inflammation and cachexia, and died prematurely (117) (**Table 1**), and cell-specific deletion of A20 in myeloid cells, dendritic cells (DCs) or B cells, results in autoimmune phenotypes including polyarthritis and enteritis (**Table 1**) (121–124). Loss of catalytic activity might not account for this pro-inflammatory action, however, as specific introduction of a deubiquitination domain inactivating mutation resulted in a much less prominent inflammatory phenotype (125).

NF-κB is also modified by addition of methionine (M)1-linked linear ubiquitin chains by the LUBAC that consists of

TABLE 1 | Summary of autoimmune phenotypes in mice with genetic manipulation of NF-κB.

Gene	Protein	Mutation	B cell phenotype	Regulatory T cell (Treg) phenotype	Autoimmunity or inflammation	Reference
Map3k7	TAK1	Conditional deletion (T cell)		Treg deficient	Severe late colitis	(50)
		Conditional deletion (Tregs)		Peripheral Treg deficient	Mild autoimmunity, splenomegaly and lymphadenopathy, renal hemorrhage	(51)
		Deletion	Reduced B cells	Reduced T cells	Liver failure, ascites, jaundice	(52)
Card11	CARD11	L298Q [*N*-ethyl-*N*-nitrosourea (ENU)]	Absent B1 cells, abnormal B cell maturation, defective B cell responses	Treg deficient	Dermatitis with mast cell and eosinophil infiltrates. Concomitant defect in Tregs and conventional T cells	(53, 54)
		L525Q (ENU)	B1 cell deficiency, impaired B cell proliferation	Thymic Treg deficiency, reduced peripheral Tregs	Late onset dermatitis	(55)
		Deletion		Natural regulatory T cell deficiency, Treg precursor deficiency	No autoimmune due to concomitant defect in T cell function	(56)
Malt1	MALT1	C472A	Impaired B1 and marginal zone (MZ) B cells development	Defect in thymic Tregs with reduction in peripheral Tregs	Spontaneous autoimmune gastritis	(57)
		Deletion	Impaired follicular, B1, and MZ B cells development	Defect in thymic Tregs with reduced peripheral Tregs	Resistance to experimental autoimmune encephalomyelitis (EAE)	(58–60)
Bcl10	Bcl-10	Deletion	Defect in follicular, B1, and MZ B cell	Treg deficiency	Increased susceptibility to bacterial sepsis	(61–63)
Ikbka	IKKα	Conditional deletion (CD4)		Treg reduction	Increased susceptibility to colitis	(64)
		Deletion			Severe skin and skeletal abnormalities	(65, 66)
Ikbkb	IKKβ	Conditional deletion (T cell)	Defect in memory B cells and reduced germinal center (GC) B cells	Treg deficient	No autoimmunity or inflammation	(67)
Ikbkg	IKKϒ	Conditional deletion		Treg deficiency	Skin inflammation, epidermal granulocytic infiltration, liver apoptosis	(67, 68)
Map3k14	NF-κB-inducing kinase	G855R (*aly*)	Reduced B cells, defective GC formation	Treg deficiency	Spontaneous inflammation	(69–72)
		Deletion		Treg deficiency	Multi-organ inflammation	(73)
Nfkbia	IKBα	Conditional altered κB enhancer		Defect in T cell development and activation; low naive T cell, high memory T cells; Treg defect independent of IKBa-mediated feedback regulation of NF-κB	Sjogren's syndrome	(74)
		Deletion			Anemia; thymic atrophy; small spleen and liver	(75)
Nfkb1	p50	Deletion	Defect in terminal B cell differentiation, mature B cell apoptosis, reduced transitional and MZ B cells		Sepsis in response to selective pathogens; chronic inflammation; premature aging and premature death	(76–80)
		Deletion	Defect in T1 to T2 transition, and in MZ B cells; reduced GC B cells in young mice, absent GC structure		Lymphoproliferative disease and multi-organ autoimmunity	(81)
Nfkb2	p52	Deletion	Defect in follicular MZ and MZ B cells; impaired GC formation			(82, 83)

(*Continued*)

TABLE 1 | Continued

Gene	Protein	Mutation	B cell phenotype	Regulatory T cell (Treg) phenotype	Autoimmunity or inflammation	Reference
Rela	RelA	Conditional deletion (Treg)		Reduced Treg precursors, decreased Tregs with impaired function	Severe multi-organ inflammation, lymphoproliferation	(84, 85)
Relb	RelB	Deletion	Defect GC formation	Mild T cell depletion in spleen and lymph nodes	Multi-organ inflammation	(86, 87)
Rel	c-Rel	Conditional deletion (Treg)		Reduced Treg precursors, decreased Tregs with impaired function	Late onset mild inflammation	(85, 88)
		Deletion	Defect B cell proliferation	Defect T cell proliferation		(28)
Bcl-3	Bcl-3	Conditional deletion (T cell)		Impaired Th1 differentiation, fewer pathogenic Th17-like cells	Resistance to colitis and EAE	(89)
		Deletion	Increased MZ B cells, reduced follicular transitional B cells, defect GC formation		Susceptibility to *T. gondii*, multi-organ inflammation	(90, 91)
Nfkbid	IKBNS	Deletion		Treg deficiency	Resistance to Th17-dependent EAE	(92, 93)
Nfkbiz	IKBζ	Deletion		No effect on Treg, Impaired CD4 T cells and Th17 development	Resistance to TNFα- IL-17A- inducible psoriasis like skin inflammation, atopic dermatitis, resistance to EAE	(94, 95) (96)
Otulin	OTULIN	Conditional deletion (myeloid)	B cell hyperactivity		Autoimmunity, multi-organ inflammation	(97)
		Conditional deletion (immune cells)			Multi-organ inflammation	(97)
Tnfaip3	A20	Conditional deletion (B cells)	Increased B cell proliferation and activation, defect MZ B and B1 cells differentiation	Treg expansion	Autoimmunity	(98)
		Conditional deletion (dendrite cells)	Increased B cell activation and differentiation	Increased T cell activation and expansion	SLE-like phenotype; IBD-like phenotype	(99, 100)
Cyld	CYLD	Deletion	Increased MZ B cells in aged mice	Defect T cell development, hyper responsive to anti-CD3, -CD28	Spontaneous intestinal inflammation	(101–103)
		$CYLD^{x7/8}$ naturally overexpressed	Increased mature B cell, enhanced B cells survival	Enhanced Treg, defect Treg function and survival	Large spleen and lymph nodes	(104, 105)

HOIL-1L (*RBCK1*), HOIP (*RNF31*), and SHARPIN (112). LUBAC promotes canonical signaling in part through direct binding of the E3 ligase activity of the ring domains of HOIP to drive linear ubiquitination of NEMO and RIP1 (126), which promotes IKKβ phosphorylation and activation through TAK1 (107, 127, 128). CYLD modifies canonical signaling by removing K63 and M1-polyubiquitin chains from NF-κB signaling proteins, including TRAF2, TRADD, NEMO, and TAK1 (**Figure 1**). CYLD appears to be particularly important in promoting necroptosis *via* RIPK1. CYLD deficiency results in several non-immunological phenotypes, but also inflammatory bowel disease, defects in thymic medullary epithelial cells (see below), and increased B cell activity (**Table 1**) (105, 129). OTULIN specifically hydrolyzes M1-polyubiquitins. In mice, germline deficiency of *Otulin* is lethal, but conditional myeloid deficiency results in profound sterile inflammation (**Table 1**) (97). Interestingly, after caspase-mediated cleavage during apoptosis, the N-terminal fragment of HOIP also binds to the DUBs OTULIN and CYLD, which are down-regulators of LUBAC-mediated ubiquitination, providing a further regulatory feedback loop (130).

NON-CANONICAL SIGNALING

NFκB2 (p100) and RelB participate in the non-canonical NF-κB pathway (**Figure 1**). Unlike the canonical pathway, which is poised for rapid response, the non-canonical pathway depends on constant protein synthesis (131). This might explain the preferential action of the non-canonical pathway in cellular homeostasis and organogenesis, whereas the canonical pathway mediates acute inflammatory responses and immune activation.

Non-canonical NF-κB activation is stimulated by a relatively small number of ligands, including lymphotoxin, CD40, B cell activating factor (BAFF), and receptor activator of NF-κB (RANK). In resting cells, TRAF2 mediates ubiquitination of NF-κB-inducing kinase (NIK) in association with TRAF3 and cIAP1/2. NIK is maintained in limiting concentrations by rapid degradation after phosphorylation by TRAF6, but after receptor ligation, TRAF2-induced proteolysis and degradation of TRAF3 and TRAF6 leads to NIK accumulation, which phosphorylates IKKα (132, 133). pIKKα is crucial for phosphorylation of p100, leading to proteosomal processing to p52 (134–140).

Non-canonical signaling activates complex regulatory loops because p100 exerts IκB activity by associating with p52, p65 (RelA), c-Rel, and RelB. Interestingly, and by contrast with the IκB activity of the canonical counterpart p105, p52 is formed from recently synthesized p100. Elegant experiments have revealed that the balance between p100 activation and NIK degradation is maintained within a kappaBsome, which is a complex of NIK-IKKα-p100 and RelB. Almost all p100 is maintained within the kappaBsome, since free cytoplasmic p100 undergoes complete degradation, whereas processing from within the kappaBsome yields p52 (141). RelB promotes formation of the kappaBsome, and competes with IKKα–NIK complexes to inhibit p100 activation and processing. In addition to promoting p100 processing, IKKα serves to limit activity and abundance of NIK within the complex, and p100 inhibits NIK degradation. RelB dimerizes with both p52 and p100 in the complex to permit the proper processing of p100. In the absence of RelB, p100 undergoes complete degradation (141, 142).

TRANSCRIPTIONAL REGULATION BY NF-κB

NF-κB lacks the enzymatic activity to directly regulate transcriptional responses, and this is achieved through binding to transcriptional co-regulators including histone acetyltransferases (HATs) and histone deacetylases (HDACs). For instance, acetylation of p65 at different lysines by HATs (and the associated binding proteins) promotes its activity, while the co-repressor, HDACs, can deacetylate important sites on p65 and reverse the effect (143). NF-κB recruit these co-regulators to the enhancer region of the target genes, leading to conformational changes in chromatin to make genes accessible for transcriptional machinery (143–145).

Binding to HATs is also controlled partially by posttranscriptional modifications (PTMs). For example, kinases including PKA, MSK1, and MSK2 phosphorylate p65 at Serine 276, which is crucial for the interaction of p65 with HATs (21, 26, 146, 147). Interestingly, this amino acid substitution only affects expression of a subset of the genes, which suggests other PTMs might contribute to p65-dependent chromatin remodeling (148). One example is Akirin2, which binds to HATs and IκBζ and facilitates recruitment of other co-regulators to p50 (149).

NF-κB AND SELF-TOLERANCE

Canonical and non-canonical NF-κB pathways are crucial for T and B cell activation, regulation of inflammatory effector responses, antigen presentation and regulation of tissue-specific cellular targets of immunity. We will concentrate our attention on mechanisms that regulate lymphocyte self-tolerance, since these are most relevant to autoimmunity. These actions can be divided into those that act intrinsically within lymphocytes, and those that are mediated by lymphocyte extrinsic actions, particularly for thymic selection.

THYMIC DEVELOPMENT

The thymus is derived from the endoderm of the third pharyngeal pouch. Mature thymic epithelium is made up of cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC). Unlike other epithelia, thymic epithelium lacks apical polarity, and cells are dispersed in a three-dimensional reticular meshwork (150). The two best understood mechanisms of central T cell tolerance are negative selection of self-reactive conventional T cells (Tconv), and selection of regulatory T cells (Tregs). Both mechanisms depend on maturation of mTECs, and expression of autoimmune regulator (Aire), which in turn regulates expression of tissue specific antigens (TSA) (151–153).

Both mTECs and cTECs have been postulated to arise from $CD205^+$ $\beta5t^+$ and $CD45^-$ bipotential precursors (154). SSEA-1^+ Claudin$3/4^+$ expression identifies a self-renewing population of $CD80^{lo}$ $MHCII^{lo}$ mTEC precursors (155), which exhibit some epithelial stem cells characteristics. cTECs are characterized by

epithelial cellular adhesion molecule (Ep-CAM) β5t, CD205, and Ly51 expression (156). Mature mTECs are UAE-1^{+} Ly51^{-}, MHCII^{hi}, CD80^{hi}, CD40^{+}, and express Aire. mTECs are responsible for maintenance of T cell tolerance *via* negative selection of αβ T cells (157, 158), and positive selection of FOXP3^{+} Tregs, by virtue of promiscuous expression of tissue specific antigens (TSA) (154, 159, 160). Terminally differentiated mTECs (which form Hassall's corpuscles) are marked by loss of AIRE, MHCII, and CD80, acquisition of desmogleins 1 and 3, and of involucrin, a global marker of epithelial terminal differentiation (161).

NF-κB AND DEVELOPMENT OF THYMIC EPITHELIUM

Thymus development depends on an unusual hematopoietic–epithelial interplay between epithelial cell precursors and nascent lymphocytes (162). Interestingly, lymphocytes exert a similar influence on development of the specialized epithelium overlaying Peyer's patches (M cells) (163). In both thymus and Peyer's patches, epithelial development is at least partially dependent on NF-κB activation by RANKL and other ligands, delivered by lymphocytes (164–166). Selection of developing T cells depends on lymphocyte–epithelial interactions within the thymus, and interactions between immature T cells and thymic epithelium regulates T cell development by negative selection of self-reactive Tconv, and positive selection of regulatory Tregs (157, 161, 167).

Signaling *via* the non-canonical NF-κB pathway appears to regulate mTEC maturation and the size of the mTEC compartment, but not necessarily specification of the mTEC lineage. This action hinges on NF-κB activation by ligation of TNF receptor superfamily members RANK, CD40, and LTβR (86, 166, 168). Single positive thymocytes, γδ T cells, invariant NK cells, and lymphoid tissue inducer cells are all sources of RANKL and CD40L (165, 166). Fate-mapping studies have provided more information on the sequential actions of non-canonical stimuli for development and maintenance of mTECs. Thus, RANK operates after mTEC precursors have differentiated and coincides with onset of RelB expression (169). RelB appears to act cell-intrinsically to determine mTEC numbers (86, 168), while RelB expression mediates the non-canonical pathway, it is also modified by RelA and c-Rel, and therefore, mTEC development can be influenced by canonical NF-κB signaling (170). Late mTEC differentiation is also disrupted by altered CYLD function, suggesting that regulation of RANK signaling *via* TRAF6 deubiqutination might be important during late mTEC development (171), possibly by regulating RelB induction by the canonical NF-κB pathway.

As well as RelB deficiency, deficiencies of NIK, IKKα, LTβR, NF-κB2 (p100), and Bcl-3 result in mTEC deficiencies of varying severity (172–174). Interestingly, the thymic defect observed with *Nfkb2* deficiency is less marked than that observed with *Relb* deficiency (175). mTEC-specific deletion of LTβR results in disordered thymic architecture, but not altered negative selection, although negative selection is defective when LTβR is deleted from thymic DCs (159). Indeed, distinguishing the actions of defects in mTECs from those in DCs requires construction of chimeras in which the mutation is confined to either hematopoietic or stromal compartments, which has not yet been performed for all mutations.

Bcl-3 action appears to be redundant with NF-κB2 for mTEC development (176). Thus, mice doubly deficient for *Bcl3* and *Nfkb2* lack mTECs, thymic Aire expression, and some thymic DCs, and they develop severe organ-specific autoimmunity. NIK is crucial for non-canonical NF-κB activation, but the mechanism of Bcl-3 is less obvious. While Bcl-3 is an atypical IκB, it does not regulate RelA or c-Rel. Uncertainties regarding the mechanism of Bcl-3 notwithstanding, there is solid empirical evidence for a crucial cell-intrinsic action of non-canonical signaling for maintaining mTECs, and for central T cell tolerance. In addition, several findings suggest that these actions are modified by regulation of RelB expression and activation *via* the canonical pathway.

NF-κB DEFECTS CONFER SUSCEPTIBILITY TO TISSUE-SPECIFIC AUTOIMMUNITY

Deficiency of Aire leads to widespread tissue-specific inflammation in man and mouse as a result of impaired expression of TSA by mTECs (158, 177, 178). This discovery represented a landmark in autoimmunity research, providing empirical evidence that negative selection mediated by antigen expression in mTECs is a bulwark against autoimmune disease (160). NF-κB signaling is important for Aire expression independently of mTEC development and homeostasis. The conserved non-coding sequence 1 (CNS1) located 3′ to the Aire transcription start site is an Aire enhancer, and contains two NF-κB-binding motifs, one with a preference for RelA and c-Rel, and the other for RelB (179, 180). There is compelling evidence for the interaction of RelA with CNS1, implicating the canonical NF-κB pathway in Aire expression. CD40 and RANK ligation appears to be important for Aire induction by NF-κB, whereas Aire expression is independent of LTβR (181).

More recently, FEZF2 was also identified as a transcriptional regulator of TSA expression in mTECs. NF-κB has been implicated in *Fezf2* expression, although the relative importance of LTβR and RANKL remains controversial (159, 182). *Fezf2* deficiency results in a pattern of organ-specific autoimmunity targeted at lung, kidney, liver, and intestine, but not retina or pancreas, which is observed in Aire deficiency (182). PRDM1 is the most recent addition to transcriptional regulators of TSA in mTECs, and while the regulation of expression PRDM1 in mTECs is unclear, it is known to be induced by RANKL-mediated NF-κB signaling in other tissues (183, 184).

As might be predicted from its crucial action on mTECs, *Relb* deficiency results in multi-organ inflammation, indicating that non-canonical signaling acting to regulate mTEC development is crucial for maintaining self-tolerance and prevention of organ-specific autoimmunity (86, 168). The autoimmune phenotypes of *Relb* mutants and related strains are summarized in **Table 1**. The severity of the autoimmune phenotype conferred by *Relb* deficiency may reflect its importance not only for mTEC development but also on Aire expression. Mice deficient in NIK (*Map3k14*$^{-/-}$) also develop autoimmunity, providing additional

evidence that the non-canonical pathway regulates self-tolerance (70, 71). Similarly, autoimmune phenotypes have been induced by deletion of CD40L, RANKL, or LTβR, the ligands crucial for inducing non-canonical signaling for mTEC maturation and maintenance (166, 185, 186).

Mice rendered deficient in *Nfkb2* also display modest defects in central tolerance and an autoimmune phenotype of inflammatory infiltrates in lungs, liver, kidneys, and pancreas, together with autoantibodies, thus resembling the phenotype of Aire deficiency (175). In this case, reciprocal bone marrow chimeras confirmed the defect was in the radio-resistant stroma, and further analysis revealed a numerical defect in Ep-Cam^{+} (G8.8) and *Ulex europaeus* agglutinin 1^{+} mTECs, a cell-for-cell defect in LTβR-induction of Aire, and a reduction in expression of Aire-dependent TSA. No numerical or functional Treg defect was observed. Interestingly, and by contrast with complete NF-κB2 deficiency, specific deficiency of p52 conferred only a T-cell dependent antibody response with no overt autoimmune phenotype (83, 187, 188).

NF-κB AND Treg SELECTION

NF-κB appears to be important for maintaining Treg function *via* mTEC development, thymic dendritic cell development, and T-cell-intrinsic actions (**Table 1**). TCR ligation and co-stimulation, particularly *via* CD28, activates the canonical NF-κB pathway, which appears to be important for FoxP3 induction (189). Engagement of TCR and CD28 activates the CBM complex, *via* the upstream and mediators, TAK1 and PKCθ. Deficiency in each of these signaling components alone also results in Treg deficiency (54–56, 190, 191). Activation of the canonical pathway induces c-Rel, which binds the conserved non-coding sequence (CNS)-3 enhancer of FoxP3 to promote its expression (192, 193). Thus, c-Rel deficiency results in a similar Treg deficiency, while Tregs are preserved in the face of p50 deficiency (88). Furthermore, constitutive activation of IKKβ appears to be sufficient to drive Treg development even in the absence of TCR ligation (194).

NF-κB, Treg HOMEOSTASIS AND SUSCEPTIBILITY TO AUTOIMMUNITY

Once selected, ongoing NF-κB signals appear to be necessary to maintain Tregs and prevent end-organ inflammation. Comparison with Tconv reveals that there is greater accumulation of canonical NF-κB components in nuclei of Tregs (84). CD28 signals are important for Treg homeostasis in the periphery. Disruption of CD80/86 with CTLA4Ig results in a loss of Tregs (195, 196). T cell-intrinsic defects in the canonical pathway do not, however, always result in autoimmunity or organ-specific inflammation, as Tconv also exhibit a defect in activation.

Resting Tregs are predominantly located in secondary lymphoid organs due to specific chemokine receptor expression, while activated Tregs migrate into sites of inflammation and are characterized by a greater array of suppressive functions, including IL-10, Lag-3, TIGIT, CD73, and PD-1 expression (197). NF-κB also regulates Treg activation. Acquisition of the effector phenotype depends on TCR ligation (198). RelA appears to play a dominant role in maintaining effector Treg function in the periphery. *Rela* (p65) deletion is embryonic lethal, but recent evidence from conditional deficiency indicates that p65 is crucial for maintaining Treg lineage stability and activity (**Table 1**) (84, 85). Treg-specific p65 deficiency confers a multi-organ inflammatory phenotype. c-Rel deficiency confers a less prominent inflammatory phenotype (199). In addition to Treg homeostasis, canonical signaling appears to specify whether Tregs adopt either resting (CD62L^{hi} CD44^{lo}) or activated/effector (CD62L^{lo} CD44^{hi}) phenotypes (200).

NF-κB CONTRIBUTES TO B CELL HOMEOSTASIS AND SELF-TOLERANCE

In mouse models, B cell survival depends on signals *via* BCR and BAFF (201–203), implicating signaling though canonical and non-canonical NF-κB pathways. Several mechanisms normally operate to eliminate autoreactive B cells (204). In the bone marrow, high affinity ligation by antigen results in receptor internalization, attenuation of NF-κB-mediated induction of Bcl-2, increased Bcl-2-interacting mediator (BIM), and reduced expression of regulation of B cell activating factor receptor (BAFFR). V(D)J recombination continues by virtue of ongoing RAG1/2 expression resulting in receptor editing but if immature B cells remain autoreactive they undergo clonal deletion. Receptor editing is marked by enhanced canonical signaling (205).

Clonal anergy represents a third mechanism by which autoreactive B cells enter the periphery with diminished capacity for activation. B cell anergy is characterized by reduced BCR expression, which attenuates canonical NF-κB survival signals and BAFFR expression is reduced, while pro-apoptotic signals such as BIM continue (206). BAFFR signals predominantly through the non-canonical pathway, and enhances B cell survival *via* induction of Bcl-2, PIM2, and PKCδ (207, 208). These actions reduce the risk of activation and survival of autoreactive B cells. BAFF is normally available in limiting amounts, which regulates the size of the transitional B cell compartment, but also regulates survival of autoreactive anergic B cells. Experimental manipulations demonstrate that excessive BAFF production results in improved survival of anergic B cells (206, 209).

RARE GENETIC VARIANTS OF NF-κB AND HUMAN AUTOIMMUNE DISEASE

Rare single gene disorders provide important evidence to support mechanistic pathways of human disease. Mutations affecting NF-κB activation, and its proximal signaling pathways, have so far been implicated overwhelmingly in susceptibility to infection (i.e., primary immune deficiency diseases). For the most part, these are loss-of-function mutations arising from homozygous or biallelic mutations. An exception is gain-of-function (GoF) mutations in *NFKBIA* (IκBα), but since this is the fundamental negative regulator of canonical signaling, GoF reinforces lack of activation and, therefore, results in a clinical and cellular phenotype that resembles LoF mutations in IKKγ and IKKβ (210, 211).

Several Mendelian human diseases nevertheless provide evidence that NF-κB protects against autoimmunity (**Table 2**). In other cases, NF-κB defects have been shown to cause autoinflammatory diseases, which also result in tissue-specific inflammatory responses, but are distinguished by the absence of evidence for adaptive immune responses to autoantigens.

Syndromes arising from heterozygous mutations in *NFKB1* include autoimmune manifestations of arthritis, lung inflammation, gut inflammation, and immune-mediated thrombocytopenic purpura (ITP). There appears to be considerable clinical and cellular heterogeneity and postulated incomplete penetrance (212). The same mutation can also lead to antibody deficiency (213). Interestingly, as noted above, recent evidence from mouse studies showed that canonical signaling *via* RelA is important for maintaining Tregs in the periphery. Consistent with this, patients with *NFKB1* haploinsufficiency also show a defect in Tregs, with a diminution in effector Tregs (as judged by ICOS expression) (200).

NFKB2 mutations result in a syndrome of antibody deficiency with variable B cell deficiencies (214–216). Patients with *NFKB2* mutations exhibit other clinical features as well, including defects of the anterior pituitary and alopecia. In at least some cases, the alopecia is reversible, consistent with alopecia areata. The fundamental defect in self-tolerance conferred by *NFKB2* mutations is yet to be resolved. Mouse models (outlined above) point to the possibility of a defect in central tolerance, and human *NFKB2* defects have been shown to confer a reduction in circulating Tregs (214). Whether there is a causal association between this observation and end-organ pathology remains to be resolved.

RelB deficiency conferred by a rare homozygous nonsense mutation has been reported in a single patient with severe polyarthritis and inflammatory skin disease, as well as immune deficiency (217). Interestingly, this phenotype was associated with enhanced nuclear p65 translocation, but a defect in T cell activation and thymus biopsy was reported to show a defect in T cell production.

MALT1 and BCL-10 deficiencies both appear to result in an IPEX (immune dysregulation polyendocrinopathy, enteropathy, X-linked; OMIM 304790)-like syndrome, which was originally identified in young boys with *FOXP3* deficiency and subsequently also observed with heterozygous mutations in *CTLA4* and *IL2RA* (CD25) (222–225). Homozygous *MALT1* mutations result in a phenotype of primary antibody deficiency together with enteropathy and a reduction in Tregs (218). A similar phenotype of enteropathy and Treg deficiency has been observed with *BCL10* deficiency (219). Again, the precise mechanism of action remains to be determined, although as noted above, CBM defects in mice are typically associated with Treg deficiency.

Several patients have been reported with a Mendelian syndromes arising from haploinsufficiency of *TNFAIP3* (220, 221). This autoinflammatory syndrome is reminiscent of Behcet's disease, with manifestations, including aphthous ulceration, inflammatory bowel disease, and neutrophilic dermatoses. Biochemical analysis confirmed increased canonical pathway activation, prolonged nuclear translocation of p65, and enhanced transcription of NF-κB-dependent genes, including *IL6* and *TNF*. A similar autoinflammatory syndrome of panniculitis and dermatitis has been reported to arise from homozygous loss-of-function *OTULIN* mutations affecting the catalytic OTU domain. These mutations result in increased ubiquitination of several NF-κB proteins, including NEMO and RIPK1 (97).

POLYGENIC AUTOIMMUNITY

The mechanisms explaining sporadic autoimmune disease remain poorly understood. Rare monogenic cases provide clues to pathogenic pathways that become dysregulated in more common versions of the disease, even if in any given individual these cellular and biochemical defects arise from more than one gene defect. Human *AIRE* mutations provide evidence for the importance of promiscuous thymic TSA expression in protecting against organ-specific autoimmune disease (160, 177, 226). Interestingly, autosomal-dominant defects in AIRE have now been reported, raising the possibility of more prevalent single gene causes of autoimmunity (227).

Thymic hyperplasia and thymoma have been associated with some forms of organ-specific autoimmunity, most notably, myasthenia gravis (MG), an autoimmune disease of the muscle endplate. MG is characterized by muscle fatigability and weakness, is often associated with thymic hyperplasia or thymoma, together with defects in $CD4^+$ T cell selection and intrathymic cytokine production. Thymectomy is sometimes curative (228). Certain common *AIRE* polymorphisms segregate with MG, particularly in early-onset cases (229).

While plausible, there is no empirical evidence that genetic variants affecting non-canonical NF-κB signaling segregate with

TABLE 2 | Mendelian defects in NF-κB pathway genes that confer syndromes including autoimmune manifestations.

Gene	Protein	Inheritance	B cell and Ig phenotype	Regulatory T cell (Treg) phenotype	Autoimmunity or inflammation	Reference
NFKB1	p102/50	AD	Hypogammaglobulinemia	Reduced effector Tregs	Arthritis, pneumonitis, enteritis, ITP	(200, 212, 213)
NFKB2	p100/52	AD	Variable B cell deficiency; hypogammaglobulinemia	Reduced	Alopecia	(214–216)
RELB	RelB	AR	Memory B cell deficiency	ND	Arthritis, dermatitis	(217)
MALT1	MALT1	AR	Hypogammaglobulinemia	Reduced	Enteritis	(218)
BCL10	BCL-10	AR	Hypogammaglobulinemia	Reduced	Enteritis	(219)
TNFAIP3	A20	AD			Enteritis, dermatitis	(220, 221)
OTULIN	Otulin	AR			Enteritis, dermatitis	(97)

organ-specific autoimmune disease. The most consistent NF-κB genetic association with autoimmunity is *TNFAIP3*. Numerous coding and non-coding polymorphisms have been shown to segregate with autoimmune diseases, including lupus and organ-specific autoimmune diseases (230–232). Polymorphisms of *REL* (c-Rel) have been shown to segregate with both psoriatic arthritis and rheumatoid arthritis, and altered expression has been noted in other autoimmune diseases (233–236). Common *NFKB1* polymorphisms have been shown to segregate with several autoimmune and inflammatory diseases (237–239). More recently, a polymorphism within the *NFKB1* locus was shown to segregate with multiple sclerosis. Interestingly, MS patients were found to have increased levels of p65 (RelA) activation, and the disease associated polymorphism was found to be associated with increased expression of BCL-3, TNFAIP3, and CIAP1 (240). This represents important progress toward understanding how common variants in NF-κB might contribute to sporadic and more common autoimmune diseases, *via* pathways now well understood from investigations of animal models and patients with rare monogenic disease.

CONCLUSION

NF-κB is well established as a regulator of innate and adaptive mechanisms of host defense, and specific genetic defects that confer immune deficiency confirm the importance of these mechanisms. As described here, there is also abundant evidence that NF-κB is crucial for maintaining immunological tolerance, as a result of its actions during thymic selection, both for negative selection of autoreactive T cells, and selection and maintenance of Tregs. The non-canonical NF-κB pathway appears to be particularly important for normal mTec function; nevertheless, evidence has also emerged for a significant lymphocyte-intrinsic action of the canonical pathway for maintaining T cell tolerance. Further work is required to accomplish the challenging task of separating the actions of canonical and non-canonical components, cell-intrinsic versus cell extrinsic actions, actions on negative selection versus Treg defects. Finally, it is important to note that since NF-κB is crucial for Tconv function in the periphery, it is possible that defects in selection may be offset by defects in the Tconv compartment.

AUTHOR CONTRIBUTIONS

Both the authors contributed to conceptualizing and writing the manuscript.

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frontiers in Immunology

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Signal Transducer and Activator of Transcription 3 Hyperactivation Associates With Follicular Helper T Cell Differentiation and Disease Activity in Rheumatoid Arthritis

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Follicular helper T (Tfh) cells are the specialized CD4$^+$ T cell subset that supports B cells to produce high-affinity antibodies and generate humoral memory. Not only is the function of Tfh cells instrumental to mount protect antibodies but also to support autoantibody production and promote systemic inflammation in autoimmune diseases. However, it remains unclear how the activation of Tfh cells is driven in autoimmune diseases. Here, we report that in patients with rheumatoid arthritis (RA), excessive generation of CXCR5$^+$PD-1$^+$ memory Tfh cells was observed and the frequency of memory Tfh cells correlated with disease activity score calculator for RA (DAS28). The differentiation of Tfh cells is dependent on signal transducer and activator of transcription 3 (STAT3), the key transcription factor downstream of cytokine signal pathways. A drastic increase of phosphorylated STAT3 (pSTAT3) in CD4$^+$ T cells were detected in RA patients who also produced larger amounts of STAT3-stimulating cytokines, including IL-6, IL-21, IL-10, and leptin than those of healthy controls. Importantly, the phosphorylation status of STAT3 in CD4$^+$ T cells positively correlated with the plasma concentration of IL-6 and the frequency of memory Tfh cells. This study reveals an IL-6-pSTAT3-Tfh immunoregulatory axis in the pathogenesis of RA and reinforces its candidature as biomarkers and targets for diagnosis and therapy.

Keywords: rheumatoid arthritis, patient, follicular helper T cells, signal transducer and activator of transcription 3, phosphorylation, IL-6

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and immune-mediated arthritis that affects up to 1% of the population. It is characterized by synovial inflammation and hyperplasia as well as cartilage and bone destruction (1). Although the name "arthritis" speaks for itself that local tissue remodeling and damage represent the major pathology of RA, the systemic activation of the immune system is instrumental for the disease pathogenesis (1–3). Prior to the onset of clinical disease, increased generation of effector $CD4^+$ T cells, elevated production of pro-inflammatory cytokines, and the presence of anticitrullinated protein antibodies (ACPAs) can usually be detected in blood, therefore, being termed as the "pre-rheumatoid arthritis" phase (4).

Follicular helper T (Tfh) cells are a functional $CD4^+$ T cell subset that is specialized to support B cells to generate long-lived plasma cells and memory B cells and to produce high-affinity antibodies. By upregulating the chemokine receptor CXCR5, Tfh cells selectively migrate into B-cell follicles and help B cells through the expression of costimulatory receptors including CD40L and OX40 and the secretion of cytokines such as IL-21 (5). The critical role of excessive Tfh cells in the induction of systemic inflammation and the development of autoimmune diseases has been proven by many mouse models (5–8). For RA, there is also accumulated evidence to support a significant contribution of Tfh cells to the development of RA. First, the dependence of Tfh cells has been extensively studied in mouse models. In the commonly used collagen-induced arthritis model, upon the immunization of collagen, $CD4^+$ T cells differentiate into Tfh cells to initiate the pathogenic anti-collagen antibody responses (9, 10). In the K/BxN autoimmune arthritis model [KRN T cell receptor transgenic mice on the C57BL/6 (B6) background × non-obese diabetic mice], KRN T cells recognize self-antigen glucose-6-phosphate isomerase (GPI) and differentiate into Tfh cells to promote B cells to produce anti-GPI autoantibodies (11–14). Importantly, blocking of Tfh cell generation was able to prevent the development of diseases in these mouse models. Second, there is a strong involvement of B cells in human RA pathogenesis. The production of high-affinity IgG and IgA antibodies against citrullinated, carbamylated, and acetylated proteins is likely also dependent on Tfh cells (2). Third, several studies including ours have reported excessive Tfh differentiation and function in RA patients as compared to healthy controls (15–19). The association between increased circulating Tfh cells and the presence of high-titer ACPAs and disease activity again suggested a pathogenic role of Tfh cells in RA. The pressing question that remains to be unanswered is the causation of the aberrant Tfh activation in RA.

The development of Tfh cells is driven by signallings *via* T cell receptors through sustained antigen stimulation and co-receptors including CD28 and inducible T-cell costimulator (ICOS). The cytokine milieu also shapes the Tfh differentiation. IL-6 and IL-21 induce the activation of signal transducer and activator of transcription 3 (STAT3) and promote the differentiation of mouse Tfh cells (5). For human Tfh cells, additional cytokines including STAT4-stimulating IL-12 and SMAD2/3-stimulating TGFβ and Activin A were reported to also contribute (5, 20, 21). The activation of STAT3 is essential as the capability of Tfh differentiation was greatly impaired in STAT3-deficient mice (22) and patients carrying functional STAT3 deficiency (23). STAT3 has a strong implication in autoimmune diseases. Monogenic activating *STAT3* mutations were identified in individuals with a spectrum of early-onset autoimmune disease including juvenile-onset arthritis (24). The involvement of the activation of STAT3 and RA was also supported by studies showing enhanced expression of phosphorylated STAT3 (pSTAT3) and STAT3-inducible gene signature in RA patients (25–28). We, therefore, speculated that the hyperactivation of the STAT3 signaling may lead to abnormal Tfh differentiation in RA patients.

In this study, we examined and confirmed the excessive Tfh function in RA patients, shown by the increased frequency of circulating memory Tfh cells and its correlations with disease activity. We found a drastic enhancement of STAT3 phosphorylation in $CD4^+$ T cells in RA patients and the activation status of STAT3 positively correlated with the generation of Tfh cells. Major STAT3-stimulating cytokines including IL-6, IL-21, IL-10, and leptin were increased in RA patients and thus contributed to the STAT3 hyperactivation.

RESULTS

Characteristics of the Study Subjects

The demographic characteristics of healthy individual controls and patients with RA are shown in **Table 1**. Thirty-one RA patients including 25 females and 6 males participated the study. Their median age was 60 years. The disease activities

TABLE 1 | Demographics and clinical data of the study cohorts.

Characteristic	Value
Healthy controls	
Female/male, *n*	22/8
Age, median (range)	55 (18–73)
Patients with rheumatoid arthritis	
Female/male, *n*	25/6
Age, median (range)	60 (22–83)
Disease duration (year), median (range)	1 (0–10)
RF, *n* (%)	25 (81%)
Anti-CCP, *n* (%)	26 (84%)
DAS28, median (range)	4.92 (1.27–7.88)
Remission (<2.6), *n* (%)	4 (13%)
Low activity (2.6–3.2), *n* (%)	1 (3%)
Medium activity (3.2–5.1), *n* (%)	11 (35%)
High activity (>5.1), *n* (%)	15 (48%)
CRP (mg/L), median (range)	4.0 (<0.5–106.2)
ESR (mm/h), median (range)	29 (4–84)
WBC ($\times 10^9$/L)	6.5 (3.5–12.0)
Medication, *n* (%)	
Glucocorticoids	18 (58%)
DMARDs	24 (77%)

RF, rheumatoid factor; Anti-CCP, anti-cyclic citrullinated peptide; DAS28, disease activity score 28; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood count; DMARDs, disease-modifying anti-rheumatic drugs.

of the patient cohort ranged from remission (the disease activity score-28, DAS28 < 2.6) to high (DAS28 > 5.1), with the median value of 4.92. Four naïve patients had no treatment history. The rest patients were treated with low dose of glucocorticoids and/or disease-modifying anti-rheumatic drugs. Those with a treatment history with high-dose glucocorticoids (>10 mg/day) or biologics within the past 6 months were excluded from the study.

Increased Tfh Cell Differentiation in RA Patients Correlated Disease Activity

$CD4^+$ T cells play an important role in the pathogenesis of RA (1–3, 29) but very few efforts have investigated all major $CD4^+$ T cell subsets in a single study. Using multicolor flow cytometry, the frequencies of Treg ($CD25^{high}$) and conventional $CD4^+$ subsets including naïve ($CD25^-CD45RA^+CD62L^+$), Th1 ($CD25^-CD45RA^-CXCR3^+CCR6^-CCR4^-$), Th2 ($CD25^-CD45RA^-CXCR3^-CCR6^-CCR4^+$), Th17 ($CD25^-CD45RA^-CXCR3^-CCR6^+CCR4^+$), and Tfh ($CD25^-CD45RA^-CXCR5^+PD\text{-}1^+$) subsets in $CD4^+$ T cells were analyzed simultaneously (30) (**Figure 1A**; Figure S1 in Supplementary Material). The comparison between these subsets in healthy individuals and RA patients revealed a significantly higher frequency of Tfh cells in RA patients, increasing from an average of 1.6% in the controls to 2.6% in RA patients (**Figure 1B**). On the contrary, we did not observe any significant difference for other $CD4^+$ T cell subsets between healthy individuals and RA patients. We also examined the potential correlations between the frequencies of $CD4^+$ T cell subsets and disease activities as measured by DAS28. Again, only Tfh cells' frequency, but not others' showed a positive correlation with DAS28 values (p-value = 0.005) (**Figure 2**). The increase of the Tfh activation and the correlation between the aberrant Tfh differentiation and RA disease activity support the notion that Tfh cells participate in the pathogenesis of RA.

Hyperactivation of STAT3 in $CD4^+$ T Cells Correlated With Tfh Differentiation in RA Patients

The increase of Tfh cells in RA was reported in previous studies (15–19) and confirmed in our cohort. However, the reason that caused such immune dysregulation remained unknown. We hypothesized the constitutive activation of STAT3 in RA (25–28) could promote the Tfh differentiation since STAT3 is pivotal for the generation of Tfh cells (5). We took the advantage of Phosflow to quantify the phosphorylation of STAT3 in $CD4^+$ T cells. We could detect substantial expression of pSTAT3 in $CD4^+$ T cells but not in B cells (**Figure 3A**). Importantly, the expression of pSTAT3 in $CD4^+$ T cells from RA patients (mean value: 27.0%) was 2.4-fold higher than that of $CD4^+$ T cells from healthy controls (mean value: 11.4%) ($p = 0.002$) (**Figure 3B**). We did not detect any difference of pSTAT3 expression in B cells between the two groups. We further analyzed the expression on pSTAT3 in each $CD4^+$ T cell subset as shown in **Figure 1A**. To our surprise, all $CD4^+$ T cell subsets had significantly higher

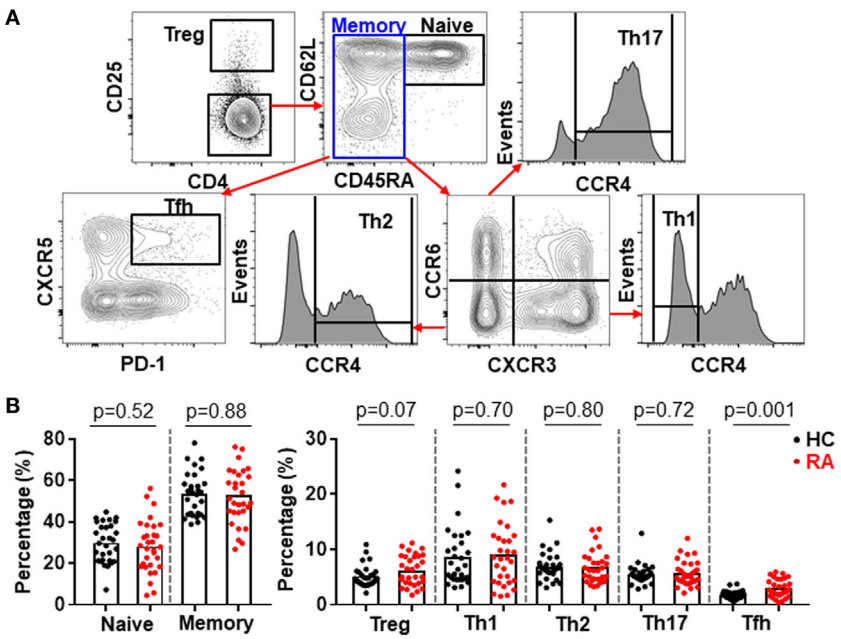

FIGURE 1 | Increased follicular helper T (Tfh) cell differentiation in patients with rheumatoid arthritis (RA). Peripheral blood mononuclear cells from RA patients and healthy control individuals (HC) were analyzed by flow cytometry. **(A)** FACS plots showing the gating of ZA^- $TCRab^+$ $CD4^+$ viable $CD4^+$ T cells for Treg ($CD25^{high}$) and conventional $CD4^+$ T cell subsets: naïve ($CD25^-CD45RA^+CD62L^+$), Th1 ($CD25^-CD45RA^-CXCR3^+CCR6^-CCR4^-$), Th2 ($CD25^-CD45RA^-CXCR3^-CCR6^-CCR4^+$), Th17 ($CD25^-CD45RA^-CXCR3^-CCR6^+CCR4^+$), and Tfh ($CD25^-CD45RA^-CXCR5^+PD\text{-}1^+$). **(B)** Statistics showing the percentages of $CD4^+$ T cell subsets in total $CD4^+$ T cells. Each dot represents the value of an individual subject with columns showing the mean values of each group. The p-values were obtained using Student's t-tests.

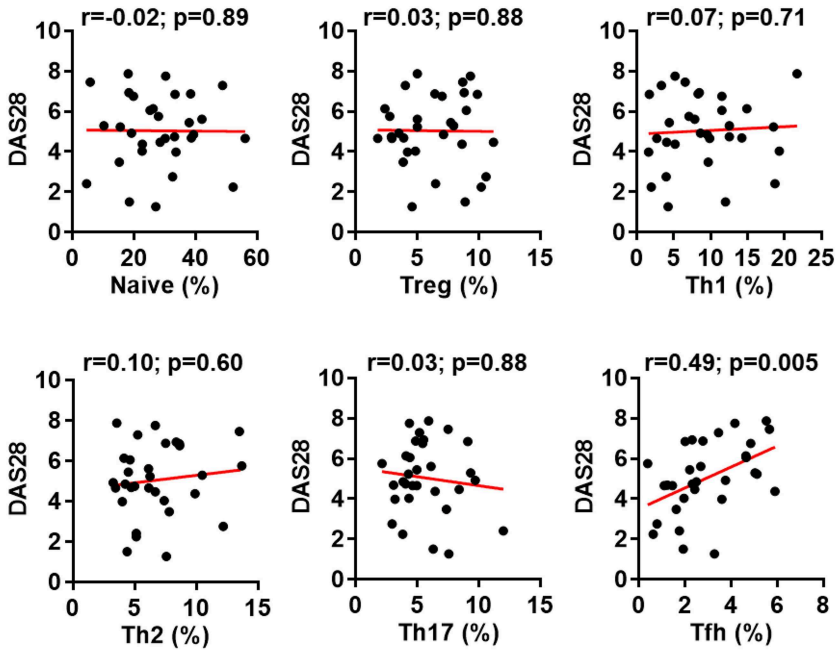

FIGURE 2 | Increased follicular helper T (Tfh) cell differentiation correlates with rheumatoid arthritis (RA) disease activity. The percentages of CD4+ T cell subsets in the peripheral blood mononuclear cells from patients with RA were analyzed as **Figure 1**. The correlation between the frequencies of these subsets and the disease activities measured by DAS28 were determined using Spearman's correlation coefficient.

level of pSTAT3 in RA patients than those in HC (**Figure 3C**). There was an approximate twofold increase of pSTAT3 in each of CD4+ T cell subsets in RA as compared to those in healthy controls (**Figure 3D**).

We then asked whether the phosphorylation of STAT3 associated with the generation of Tfh cells or other CD4+ T cell subsets in RA. By analyzing the relationship between the pSTAT3 expression in total CD4+ T cells and the frequencies of CD4+ T cell subsets, we could only detect a modest but significant correlation between the status of STAT3 phosphorylation in CD4+ T cells and the Tfh generation (p-value = 0.047) (**Figure 4A**). Intriguingly, we also detected a specific correlation of between STAT3 phosphorylation in CD4+ T cells and Tfh frequencies in HC (Figure S2 in Supplementary Material). It was possible that the differentiation of each CD4+ T cells might be more specifically affected by the phosphorylation of STAT3 in their own. To test this, we performed a similar study to examine the relationship between the pSTAT3 expression in individual CD4+ T cell subset and their frequencies. The STAT3 phosphorylation showed no correlation with the frequencies of naïve, Treg, Th1, Th2, or Th17 cells. The correlation with Tfh cells, nevertheless, became more obvious (p-value = 0.009) (**Figure 4B**). The results strongly suggest the hyperactivated STAT3 drove the Tfh differentiation in patients with RA.

In addition to dissecting a role of the STAT3 pathway in regulating CD4+ T cell differentiation, we also tested whether the status of STAT3 phosphorylation might affect the disease severity. We found a clear correlation between patients' disease activities and the pSTAT3 expression, not only in total CD4+ T cells (**Figure 5A**) but also in each CD4+ T cell subsets (**Figure 5B**). This indicates the contribution of STAT3 hyperactivation to RA is not limited to a specific CD4+ T cell population, but rather broad.

STAT3-Stimulating Cytokines Were Elevated in RA Patients

Cytokines IL-6, IL-10, IL-21, and leptin can activate STAT3 phosphorylation in CD4+ T cells (31, 32). Could these cytokines induce the enhanced STAT3 phosphorylation in RA patients? We first measured the production of these cytokines in RA patients' plasma. Results by ELISA demonstrated upregulated production of all four cytokines with the most prominent increase for IL-6: a 7.3-fold upregulation from 6.6 ± 1.1 pg/mL in healthy individuals to 48.06 ± 8.9 pg/mL in RA patients (**Figure 6A**). The increase for IL-10, IL-21, and leptin was about twofold (**Figures 6B–D**). To define the contribution of the elevated pro-inflammatory cytokines to the hyperactivation of STAT3 and the disease activities, we analyzed the relationship between cytokine levels and the disease activity DAS28 or the pSTAT3 expression. IL-6 levels significantly correlated with DAS28 (p-value = 0.006) and pSTAT3 (p-value < 0.001) (**Figure 7A**). Other cytokines also showed general trends for positive correlations but not significant (**Figures 7B–D**). These results spotlight a central role of IL-6 in inducing pSTAT3 in CD4+ T cells and for the development of the disease. We also test whether there was a relationship between the Tfh frequency and any of these STAT3-stimulating cytokines but could not identify any substantial link. We thus conclude that although the Tfh differentiation in RA patients is critically

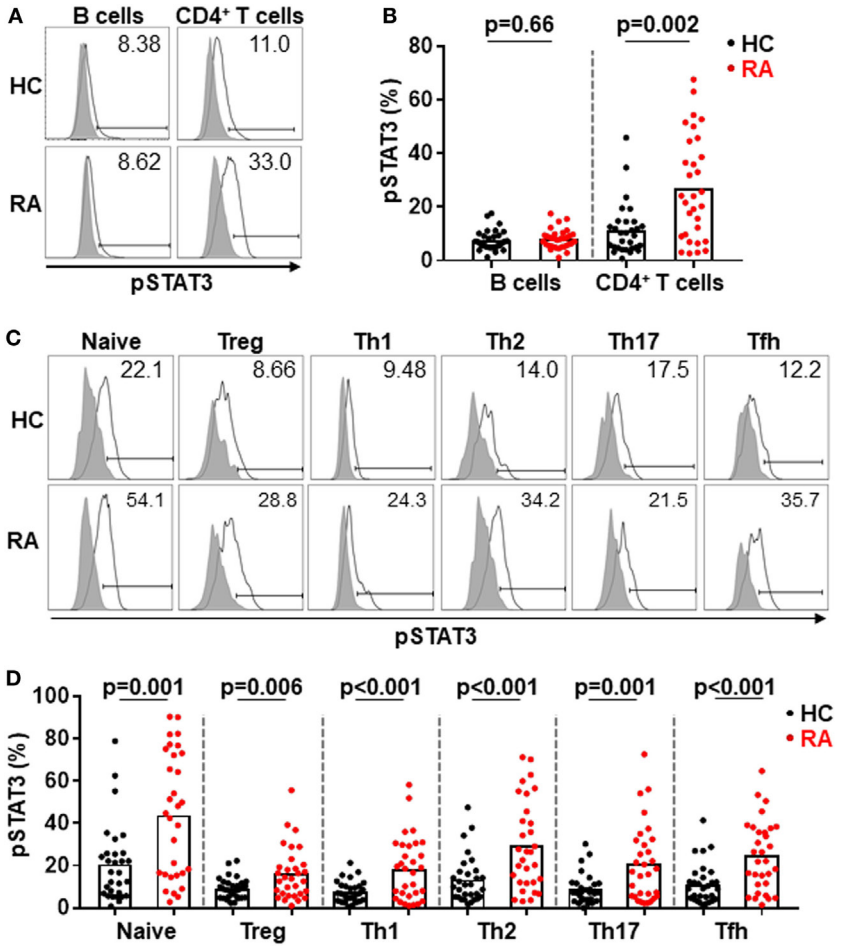

FIGURE 3 | Constitutive phosphorylation of signal transducer and activator of transcription 3 (STAT3) in CD4+ T cells from patients with rheumatoid arthritis (RA). The expression of intracellular phosphorylated STAT3 (pSTAT3) was analyzed using Phosflow assays in CD4+ T cells and B cells from RA patients. **(A)** FACS plots showing representative staining patterns for pSTAT3 (empty histograms) and an isotype control antibody (filled histograms) of indicated immune cell types; Numbers indicating the percentages of the pSTAT3 positive population. **(B)** Statistics showing the percentages of pSTAT3 positive population in total ZA−CD3−CD19+ viable B cells and ZA−TCRab+CD4+ viable CD4+ T cells from RA or HC groups. **(C)** FACS plots showing representative staining patterns for pSTAT3 and an isotype control antibody of indicated CD4+ T cell subsets; numbers indicating the percentages of the pSTAT3 positive population. **(D)** Statistics showing the percentages of pSTAT3 positive population in indicated CD4+ T cell subsets from RA or HC groups. Each dot represents the value of an individual subject with columns showing the mean value of each group. The *p*-values were obtained using Student's *t*-tests.

driven by the STAT3 phosphorylation, it may not depend on a single cytokine. A synergistic effect of multiple cytokines or other signals are candidate mechanisms.

DISCUSSION

CD4+ T cells have long been regarded as a key player in the pathogenesis of RA (29). Indeed, genetic variants in the major histocompatibility complex (MHC) region, especially MHC class II *HLA-DRB1* genes contribute to the overall 11–37% genetic risk of RA. Non-HLA genes encoding molecules directly involved in pathways of T-cell function including *PTPN22*, *IL23R*, *CTLA4*, *STAT4*, and *CD40* are also ranked high in the identified RA-risk loci (33). Although early studies discovered synovial effector CD4+ T cells predominantly produce Th1 cytokines IFNγ and TNFα (34), more recent research has been focusing on later discovered CD4+ T cell subsets: Th17 and Tfh cells.

In the cohort of RA patients we examined, there was a significant increase of CXCR5+PD-1+ memory Tfh cells in blood, as compared to that of healthy controls. The frequency of Tfh cells positively correlated with the disease activities, as measured by DAS28. This observation was largely in agreement with several published reports showing the aberrant function of Tfh cells in RA patients (15–19). The circulating memory Tfh cells can be analyzed in two different ways (35, 36). Studies using CXCR5+PD-1+ or CXCR5+ICOS+ to mark Tfh cells indicated an active Tfh differentiation in RA (15, 16, 18) while those applying CCR6 and CCR3 to stratify Tfh cells demonstrated a biased Tfh polarization into CXCR5+CCR6+CXCR3− (Th17-type Tfh) and/or CXCR5+CCR6−CXCR3− (Th2-type Tfh)

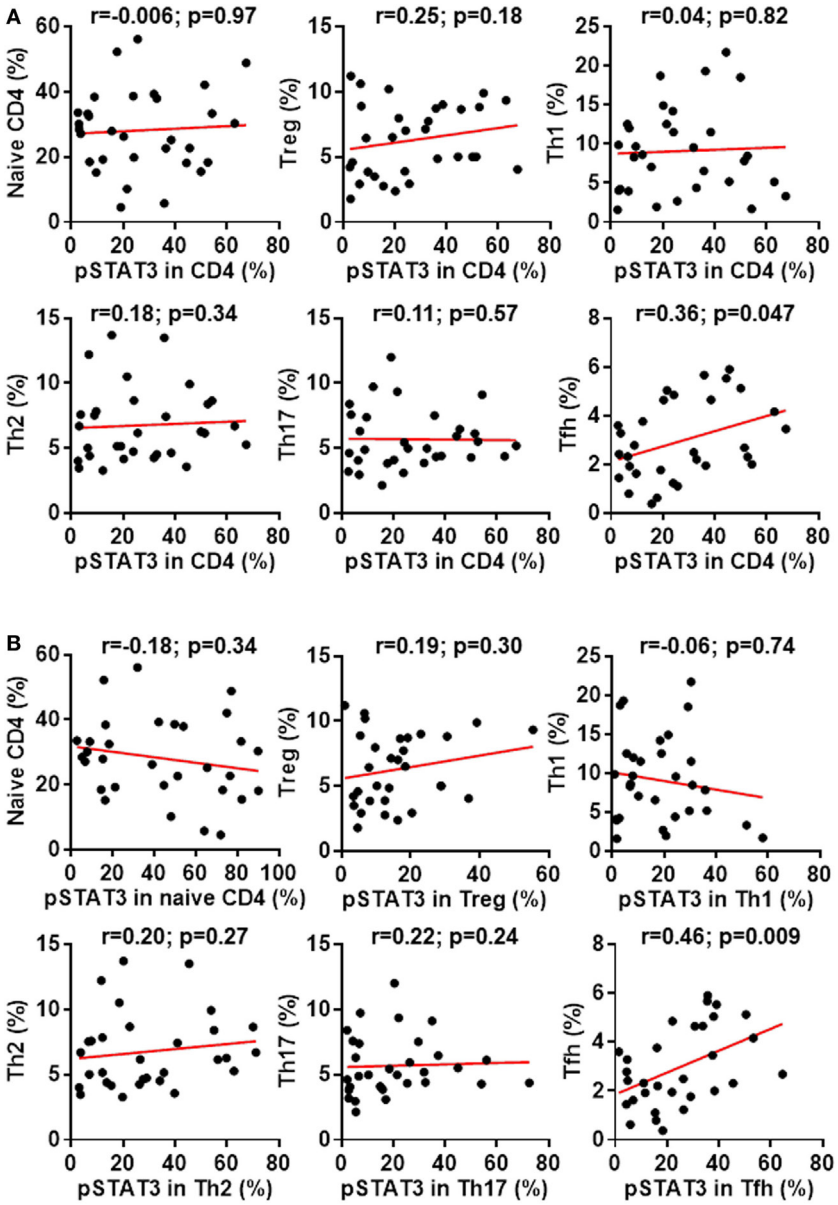

FIGURE 4 | Signal transducer and activator of transcription 3 (STAT3) hyperactivation correlates with aberrant follicular helper T (Tfh) differentiation in patients with rheumatoid arthritis (RA). Statistics showing the relationship between the frequencies of indicated CD4$^+$ T cell subsets with the phosphorylated STAT3 (pSTAT3) expression in total CD4$^+$ T cells **(A)** or in each individual subsets **(B)** in the peripheral blood mononuclear cells from patients with RA. The correlation was determined using Spearman's correlation coefficient.

subsets in RA patients (18, 19). Despite different analytic methodologies, such evidence strongly supports a significant contribution of Tfh cells to systemic inflammation and autoimmunity that drive the development of RA. To be noted, the phenotype of circulating Tfh cells is different from B-helper T cells infiltrated in inflammatory synovial tissues in RA. The latter CD4$^+$ T cell population, though expressing conventional Tfh markers including PD-1, ICOS, CXCL13, and IL-21, do not upregulate CXCR5 (37). It remains unclear whether these B-helper T cells in joints also participate in systemic immune activation in RA.

There is a major question left unanswered by the studies that characterized Tfh cells in RA patients—what induces the aberrant Tfh differentiation? We set to test the hypothesis that the constitutive activation of STAT3 in RA led to enhanced Tfh differentiation. By measuring the phosphorylation of STAT3, we found a drastic increase of pSTAT3 in CD4$^+$ T cells but not in B cells. The increase of pSTAT3 was detected in all CD4$^+$ T cell subsets with highest phosphorylation status in naïve CD4$^+$ T cells. In human CD4$^+$ T cells, naïve CD4$^+$ T cells were shown to express a lower level of suppressor of cytokine signaling 3 (SOCS3), which interacts with cytokine receptors and

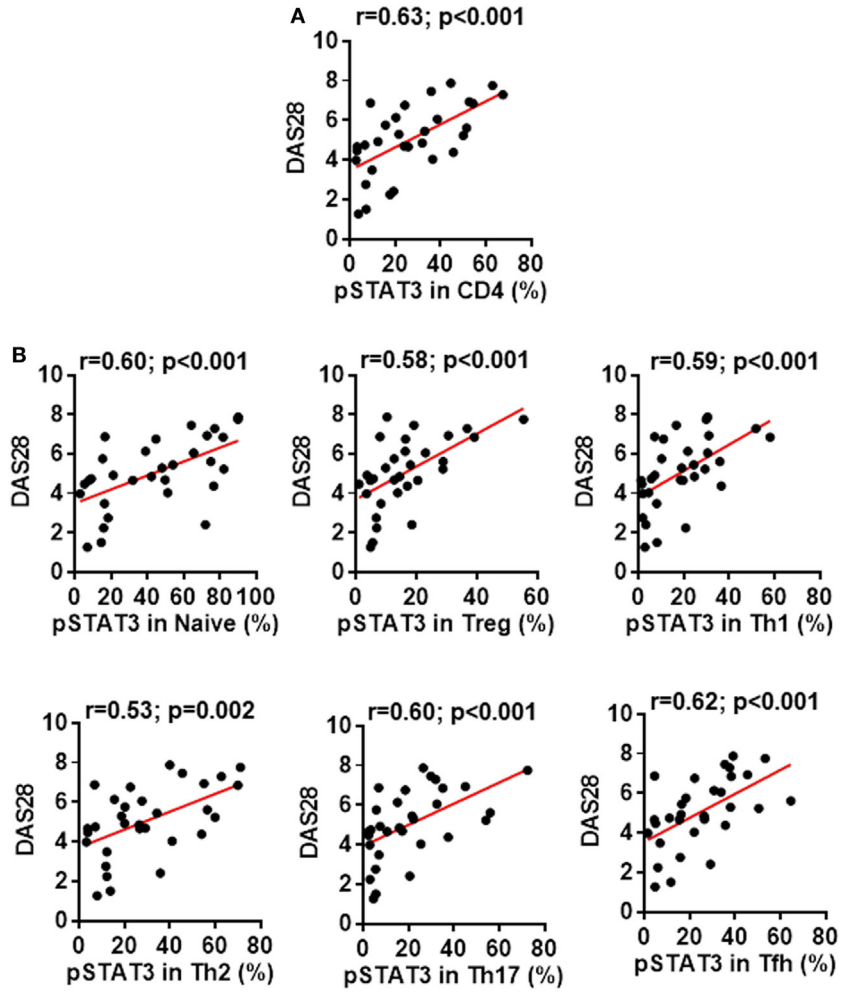

FIGURE 5 | Signal transducer and activator of transcription 3 (STAT3) hyperactivation correlates with rheumatoid arthritis (RA) disease activity. Statistics showing the relationship between the disease activities measured by DAS28 with the phosphorylated STAT3 (pSTAT3) expression in total CD4+ T cells **(A)** or in each individual subsets **(B)** in the peripheral blood mononuclear cells from patients with RA. The correlation was determined using Spearman's correlation coefficient.

inhibits receptor-mediated signal transduction (38). As the induction of SOCS3 represents the key mechanism to block the activation of STAT3 (39), the low expression of SOCS3 may lead to a high pSTAT3 in naïve CD4+ T cells. For effector CD4+ T cell subsets, pSTAT3 was detected lowest in Th1 cells, reflecting the fact that STAT3 functions to promote Th2, Th17, and Tfh differentiation but inhibit Th1 differentiation (22, 23, 39–43). Despite the high levels of pSTAT3 in all three subsets, the intensity of pSTAT3 in CD4+ T cells only correlated with the frequency of Tfh cells but not Th2 or Th17 cells, suggesting redundant factors other than STAT3 potently regulate Th2 and Th17 differentiation. The key role of pSTAT3 in promoting the Tfh generation was further recognized by a strong correlation between the frequency of Tfh cells and the pSTAT3 expression in Tfh cells.

Since the STAT3 hyperactivation was observed across all CD4+ T cells, we believe it was caused a systemic change of the immune system that is not specific for certain subsets. We measured the serum concentrations of cytokines IL-6, IL-10, IL-21, and leptin, all of which have been shown to induce the phosphorylation of STAT3 in T cells (31, 32). All of these cytokines were upregulated in RA patients as compared to healthy controls, with a prominent increase of IL-6 for more than sevenfold. By analyzing the correlation between the plasma cytokine concentrations and the pSTAT3 status in CD4+ T cells, IL-6 appears to play a central role in determining the activation of STAT3 in CD4+ T cells. Other cytokines may synergistically enhance pSTAT3 as there were general trends for positive correlations. It has been previously shown that serum IL-21 concentrations modestly correlated with the frequencies of Tfh cells in RA patients (44). In our cohort, we also observed a similar trend of positive correlation between IL-21 concentration and Tfh activation, but it was not statistically significant (p-value = 0.06). There were no significant correlation between the frequency of Tfh cells and the concentration of any of tested STAT3-activating cytokines. It could be well explained by the fact that the Tfh differentiation is

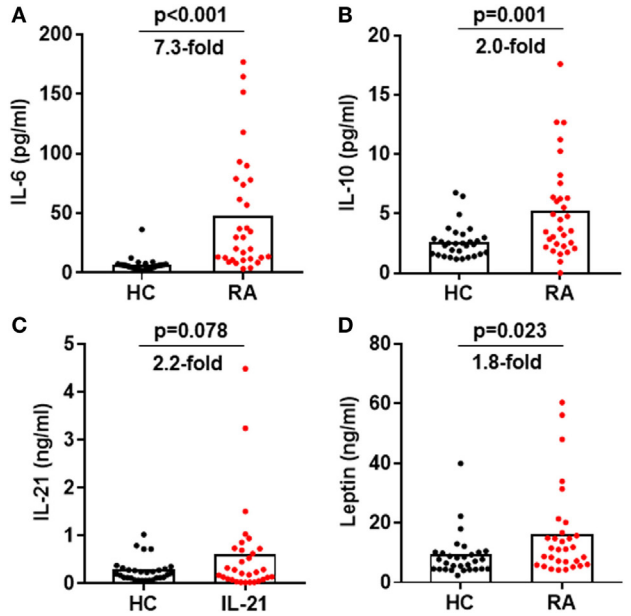

FIGURE 6 | Increased signal transducer and activator of transcription 3-stimulating cytokines in the plasma of rheumatoid arthritis (RA) patients. Plasma was isolated from blood from RA patients and healthy controls. The amount of IL-6 **(A)**, IL-10 **(B)**, IL-21 **(C)**, and leptin **(D)** was measured by ELISA. Each dot represents the value of an individual subject with columns showing the mean values of each group. The *p*-values were obtained using Student' *t*-tests.

induced by the combination of multiple cytokines (20). It is also possible that cytokines may preferably execute in supporting Tfh differentiation in local lymphoid tissue spleen and lymph nodes so the plasma concentrations were not always representative of its tissue concentrations.

Our study revealed an IL-6-pSTAT3-Tfh axis in regulating immune responses in RA patients. As the status of each of three components individually correlated with the disease activity indicator DAS 28, this immunoregulatory axis is likely to reside proximal to the center of RA pathogenesis. Therapeutics have been developed to target this axis including monoclonal antibodies to block IL-6 and small molecules to inhibit Janus kinases (JAKs) that act upstream of the STAT3 pathway. IL-6 blockers such as tocilizumab and JAK inhibitors such as tofacitinib have demonstrated profound efficacies and been approved to treat RA (45). Intriguingly, tocilizumab therapy was shown not only reducing pSTAT3 in $CD4^+$ T cells (46) but also inhibiting the Tfh differentiation (47). The evidence gained here and those from previous studies justify targeting the IL-6-pSTAT3-Tfh axis for biomarker discovery and drug development for RA diagnosis and therapy.

MATERIALS AND METHODS

Study Subjects

Thirty-one patients diagnosed as RA according to the 2010 RA classification criteria by American College of Rheumatology/European League Against Rheumatism (48) were recruited. The cohort included both newly diagnosed patients ($n = 4$) and patients with long-standing disease ($n = 27$). No patients had a history of treatment with biologic agents or high-dose corticosteroids (>10 mg/day) 6 months preceding the study. Thirty healthy individuals with comparable demographics were recruited as controls. All participants consented in writing to donate blood for the study. Ethics was approved by human ethics committees of Renji Hospital and Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China) and Affiliated Hospital of Hubei University for Nationalities (Hubei, China).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Plasma

Blood from healthy individuals and patients with RA were collected in BD Vacutainer® Blood Collection Tubes (BD). After the centrifugation (300 *g*, 20°C, 5 min), plasma was collected and stored in −80°C for further analysis. Cells were diluted with PBS (1:1), and gently loaded to Ficoll-Paque Plus (GE) layer at the ratio of 1:1 (PBS + blood cells:Ficoll), followed by density gradient centrifugation (450 *g*, 20°C, 20 min, no brake). Mononuclear cell layer was transferred to a new tube and washed with cold PBS. Cells were suspended in cold FACS buffer (1% BSA, 0.05% NaN3) for further analysis.

Flow Cytometric Analysis

Freshly isolated PBMCs were incubated with following fluorochrome-conjugated monoclonal antibodies in FACS buffer for surface staining. Antibodies (from BD Biosciences and BioLegend) were TCRαβ (clone IP26), CXCR3 (clone G025H7), CD45RA (clone HI100), CD3 (clone UCHT1), PD-1 (clone EH12.2H7), CD19 (clone SJ25C1), CCR6 (clone 11A9), CD8a (clone SK1), CCR4 (clone L291H4), CD62L (clone DREG-56), CD25 (clone BC96), CXCR5 (clone RF8B2), CD4 (clone RPA-T4), and dead cells stained with Zombie Aqua were excluded from analysis. After the surface staining, cells were washed with FACS buffer and then fixed with pre-warmed Phosflow™ Fix Buffer I (BD) in 37°C for 15 min. Cells were washed and resuspended in pre-chilled Phosflow Perm Buffer III (BD) in 4°C for 25 min. After wash, cells were stained with anti-pSTAT3 (BD, clone 4/pSTAT3) at 37°C for 30 min to detect pSTAT3 (pY705). The same procedure was performed to stain with an isotype control antibody (BD, clone G155-178). The expression of surface markers and intracellular pSTAT3 were analyzed by a FACS analyser (LSRFortessa X-20, BD). The results were analyzed with FlowJo software (TreeStar).

ELISA

Cytokines in plasma samples were measured by ELISA kits (Biolegend: IL-6 and IL-21; Invitrogen: IL-10; R&D systems: leptin) following the manufacturers' protocol. Plasma was diluted at 1:5 for IL-6, IL-10, and IL-21 and 1:100 for leptin, using ELISA diluent (1% BSA in PBS-T). Standards and diluted-plasma samples were incubated at room temperature for 2 h, followed by incubations with detecting antibodies

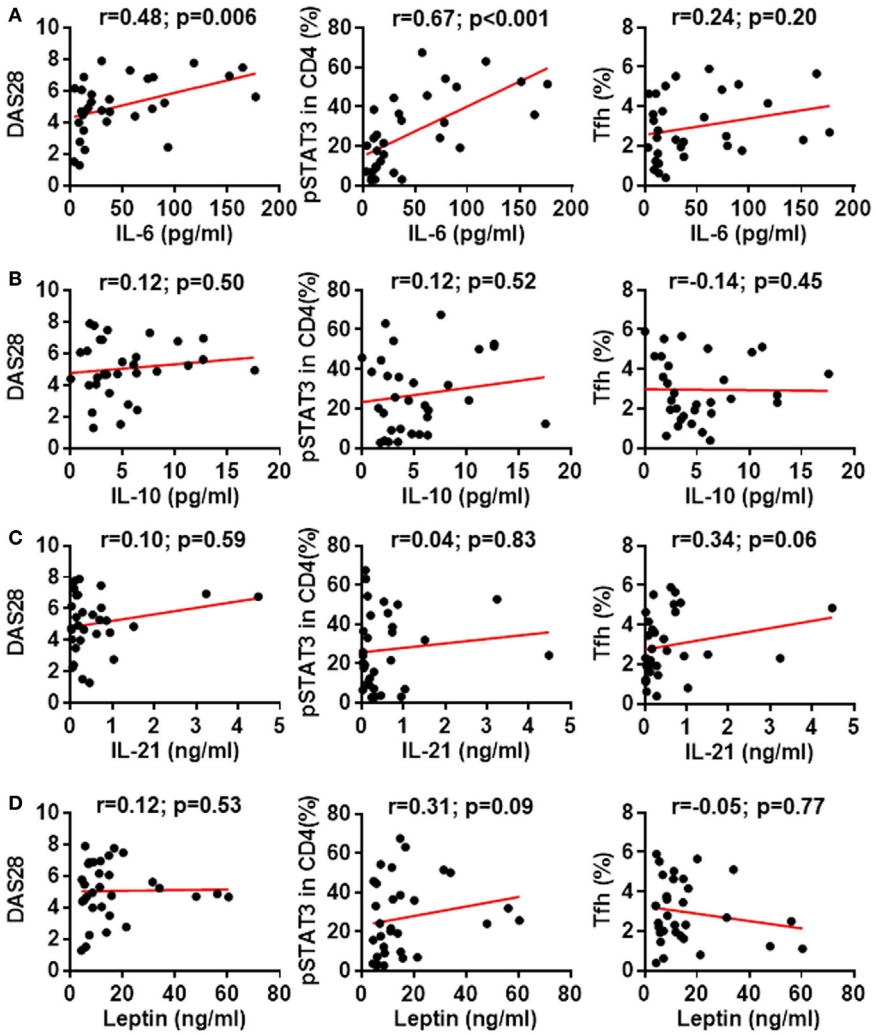

FIGURE 7 | Association between signal transducer and activator of transcription 3 (STAT3)-stimulating cytokines with STAT3 phosphorylation, disease activity or follicular helper T (Tfh) differentiation. Statistics showing the relationship between the expression of phosphorylated STAT3 (pSTAT3) in total CD4+ T cells, the disease activities measured by DAS28 or the frequency of Tfh cells with the amount of plasma IL-6 **(A)**, IL-10 **(B)**, IL-21 **(C)**, or leptin **(D)**. The correlation was determined using Spearman's correlation coefficient.

and streptavidin-horseradish peroxidase. The substrate 3,3′,5,5′-Tetramethylbenzidine was added before the values of optical densities were obtained by a microplate reader (SpectraMax 190, Molecular Devices).

Quantitative PCR

Naïve ($CD25^-CD45RA^+CD62L^+$), Th1 ($CD25^-CD45RA^-CXCR3^+CCR6^-CCR4^-$), Th2 ($CD25^-CD45RA^-CXCR3^-CCR6^-CCR4^+$), and Th17 ($CD25^-CD45RA^-CXCR3^-CCR6^+CCR4^+$) cells were sorted with a FACS cell sorter (BD Aria III). mRNA samples were extracted with Trizol reagents, and cDNA were synthesized with cDNA Synthesis Kit (Takara). Following, *Tbx21* forward 5′-ATTGCCG TGACTGCCTACCAGA-3′ and reverse 5′-GGAATTGACAGT TGGGTCCAGG-3′; *Gata3* forward 5′-ACCACAACCACACTC TGGAGGA-3′ and reverse 5′-TCGGTTTCTGGTCTGGATG CCT-3′; *Rorc* forward 5′-GAGGAAGTGACTGGCTACCAGA-3′ and reverse 5′-GCACAATCTGGTCATTCTGGCAG-3′; *Ifng* forward 5′-GAGTGTGGAGACCATCAAGGAAG-3′ and reverse 5′-TGCTTTGCGTTGGACATTCAAGTC-3′; *Il4* forward 5′-CC GTAACAGACATCTTTGCTGCC-3′ and reverse 5′-GAGTGTC CTTCTCATGGTGGCT-3′; *Il17a* forward 5′-CGGACTGTGA TGGTCAACCTGA-3′ and reverse 5′-GCACTTTGCCTCCCA GATCACA-3′; *Gapdh* forward 5′-GTCTCCTCTGACTTCAAC AGCG-3′ and reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3′, were used to measure the transcript levels using SYBR (Takara) with a Real-Time PCR Systems (QuantStudio 7 Flex, ABI). Relative fold change of gene expression was calculated by $2^{-(\mathrm{DCT\ experiment} - \mathrm{DCT\ control})}$. $\mathrm{DCT} = \mathrm{CT}^{\mathrm{gene\ of\ interest}} - \mathrm{CT}^{\mathrm{GAPDH}}$.

Statistical Analysis

Data were analyzed with GraphPad Prism (version 7.0, GraphPad Software). The correlations of indicated parameters were

determined by Spearman's correlation coefficient. Two-tailed *t*-tests were used to compare parameters between healthy individuals and RA patients. Results were considered statistically significant when p-values < 0.05.

ETHICS STATEMENT

Ethics was approved by human ethics committees of Renji Hospital and Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China) and Affiliated Hospital of Hubei University for Nationalities (Hubei, China).

AUTHOR CONTRIBUTIONS

DY, JD, XC, and LLu conceived the study. DY, JD, and XG designed the experiments. XC, LLu, CF, RG, JF, YX, SX, SG, LY, WeiS, WenyanS, LLin, TJ, and DH helped to recruit patients and collect samples. JD, XG, QZ, DG, ZC, YC, and YW performed experiments and collected data. JD, XG, CF, LLu, XC, and DY interpreted the data and wrote the manuscript. All authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01226/full#supplementary-material.

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Potential Pathways Associated With Exaggerated T Follicular Helper Response in Human Autoimmune Diseases

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Convincing lines of evidence in both mice and humans show that exaggerated T follicular helper (Tfh) responses is pathogenic in autoimmune diseases. However, the cause of exaggerated Tfh response in humans is still much less clear than in mouse models where genetic factors can be manipulated for *in vivo* testing. Nonetheless, recent advances in our understanding on the mechanisms of human Tfh differentiation and identification of multiple risk loci in genome-wide association studies have revealed several pathways potentially associated with exaggerated Tfh response in human autoimmune diseases. In this review, we will first briefly summarize the differentiation mechanisms of Tfh cells in humans. We describe the features of "Tfh-like" cells recently identified in inflamed tissues of human autoimmune diseases. Then we will discuss how risk loci identified in GWAS are potentially involved in exaggerated Tfh response in human autoimmune diseases.

Keywords: T follicular helper, autoimmune diseases, IRF5, *TNFSF4*, STAT4, ustekinumab, IL-23, IL-12

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INTRODUCTION: T FOLLICULAR HELPER (Tfh) CELLS

T follicular helper cells represent a $CD4^+$ T cell subset that plays fundamental roles for antibody responses against protein antigens [see also previous reviews for Ref. (1–4)]. Tfh cells are critical for the generation of antibody responses as well as for the establishment of long-lived and high-affinity B cell clones. The most mature Tfh cells reside in germinal centers (GC) in secondary lymphoid organs and provide help specifically to high-affinity B cells that have undergone somatic hypermutations within the GCs. The selected B cells undergo clonal expansion and eventually differentiate into long-lived plasma cells and memory B cells. Less mature Tfh cells also interact with antigen-presenting B cells outside GCs and induce B cell proliferation and differentiation into antibody producing cells. Among them, extrafollicular helper cells, another Tfh-lineage $CD4^+$ T cell subset, induce the differentiation of extrafollicular plasma cells. This extrafollicular mechanism mainly contributes to the early generation of specific antibodies after antigen challenge. Extrafollicular helper cells share the phenotype, gene profiles, and the functions with GC Tfh cells.

T follicular helper cells and their precursors are endowed with features to perform these tasks (1–4). After interacting with antigen-presenting dendritic cells (DCs) in T cell-rich area of secondary lymphoid organs, Tfh precursors increase the expression of the chemokine receptor CXCR5 and the G protein-coupled receptor S1PR2, while decreasing the expression of CCR7 and the selectin ligand PSGL1. These changes in the cell surface molecular profile are required for their migration toward and retention within B cell follicles enriched with the chemokine CXCL13. Tfh cells produce

large amounts of IL-21, which potently promotes the growth, differentiation, and class-switching of B cells (5, 6). Tfh cells in GCs express high levels of inducible co-stimulator (ICOS), a co-stimulatory molecule crucial for their interactions with B cells. CD40 ligand (CD40L) expressed by Tfh cells provides signals to B cells through CD40 for their differentiation and class-switching. The surface markers commonly used to define human GC Tfh cells and their precursors in lymphoid organs are CXCR5, ICOS, and PD-1. GC Tfh cells express these molecules at high levels, whereas their precursors express at low levels (**Table 1**). CD127 and CCR7 are expressed by Tfh precursors, but not by GC Tfh cells (7). The combination of CXCR5 and PD-1 is often used to define GC Tfh cells and their precursors also in mice, but ICOS can be broadly expressed by activated CD4$^+$ T cells in lymphoid organs (8) and is rarely used to define mouse GC Tfh cells.

Prolonged Tfh response results in enhanced generation of mature Tfh cells and/or extrafollicular helper cells, and eventually leads to autoimmunity (3, 4). This is convincingly demonstrated in many autoimmune-prone mouse models including Roquin$^{san/san}$ mice, in which CD4$^+$ T cells overexpress ICOS and excessive signals through ICOS cause exaggerated Tfh and GC responses (9). Molecular mechanisms for Tfh cell differentiation and the causal link of genetic mutations to exaggerated Tfh responses are relatively well defined in mice (3, 4). However, less is known for human autoimmune diseases. In this review, we will first summarize the differentiation mechanisms of Tfh cells in humans briefly. We will also summarize the features of pathogenic "Tfh-like" cells recently characterized in inflamed tissues of human autoimmune diseases. Last, we will discuss how risk loci identified in GWAS overlap with Tfh-differentiation pathway and how they are potentially associated with exaggerated Tfh response in human autoimmune diseases.

TABLE 1 | Cell surface makers commonly used to define T follicular helper (Tfh) subsets in lymphoid organs.

	Humans		Mice	
	Germinal centers (GCs) Tfh	**Pre Tfh**	**GC Tfh**	**Pre Tfh**
CXCR5	++	+	++	+
PD-1	++	+	++	+
Inducible co-stimulator	++	+	+	+
CD127	−	+	−	+
CCR7	−	− ~ ±	−	− ~ ±

DIFFERENTIATION OF Tfh CELLS IN HUMANS

When interacting with antigen-presenting DCs, naïve CD4$^+$ T cells receive signals *via* three major receptor families, which ultimately determine the fate of T cell differentiation: the T cell receptor (TCR, signal 1), receptors for co-stimulatory molecules (signal 2), and receptors for cytokines (signal 3). Recent studies show that each signal provides parameters that negatively and positively affect Tfh differentiation in humans and mice (**Figure 1**).

Signal 1: TCR

Studies in mice demonstrated that strong TCR signals are required for the differentiation of fully mature Tfh cells (8, 10). Consistent with this, stimulation with stronger TCR signals

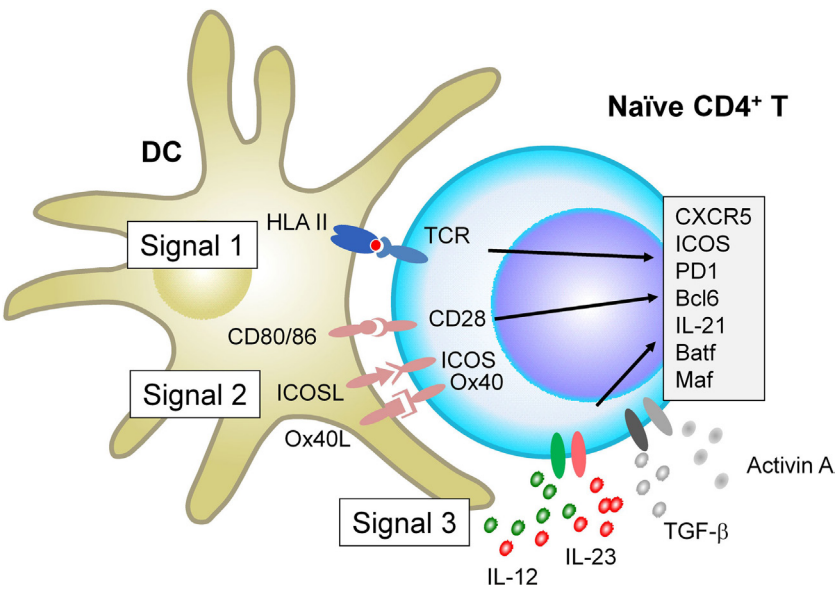

FIGURE 1 | Factors that positively regulate human T follicular helper (Tfh) cell differentiation. When interacting with antigen-presenting dendritic cells (DCs), naïve CD4$^+$ T cells receive signals *via* three major receptor families: the T cell receptor (TCR, signal 1), receptors for co-stimulatory molecules (signal 2), and receptors for cytokines (signal 3). For signal 1, evidence in both mice and humans shows that strong TCR signals promote Tfh cell differentiation. For signal 2, in addition to CD28 signals which is essential for optimal T cell activation, signals *via* inducible co-stimulator and Ox40 promote human naïve CD4$^+$ T cells to express multiple Tfh molecules. For Signal 3, among inflammatory cytokines that activated DCs produce, IL-12 and IL-23 play dominant roles for human naïve CD4$^+$ T cells to express Tfh molecules. The effect of IL-12 and IL-23 is further enhanced by the co-presence of TGF-β family molecules, TGF-β and Activin A. Given that TGF-β and Activin A are often highly expressed in human inflamed tissues, the source of these cytokines might be both from interacting DCs and from microenvironment.

in vitro induces human naïve CD4+ T cells to express higher levels of multiple Tfh molecules, including CXCR5, Bcl6, IL-21, and Ox40 (11). As demonstrated in experimental mouse models (8, 10), it is possible that human Tfh cell clones display relatively higher TCR affinity than non-Tfh cell clones, yet this remains to be tested.

Signal 2: Co-Stimulatory Molecules

Inducible co-stimulator is critically involved in Tfh cell biology at multiple levels, including the differentiation program at early stages (12, 13), their migration into B cell follicles (14), and the functions when interacting with B cells (15, 16). Patients with ICOS deficiency display severely impaired Tfh response accompanied by severely impaired memory B cell formation, indicating the essential role of ICOS in humans (17). Ox40 is another important co-stimulatory molecule promoting human Tfh cell differentiation. Ox40 signals together with TCR and CD28 signals promote human naïve and memory CD4+ T cells to express multiple Tfh molecules, including CXCR5, ICOS, PD-1, and Bcl6 (11). The direct contribution of Ox40 signals to Tfh cell differentiation was also recently demonstrated in mice with vaccinia viral infection (18). Unlike ICOS deficiency, however, loss-of-function (*LOF*) mutations in human Ox40 do not affect Tfh and antibody responses (19), suggesting that deficiency of Ox40 signals can be compensated by other mechanisms for Tfh differentiation. Instead, excessive Ox40 signals drive human CD4+ T cell differentiation toward Tfh cells and contribute to autoimmunity by increasing Tfh responses (11).

Signal 3: Cytokines

In humans, IL-12 produced by DCs is the major DC-derived cytokine driving human naïve CD4+ T cells to become Tfh-like cells (20–24). This is in contrast to mouse CD4+ T cells which acquire multiple features of Tfh cells in response to IL-6 and IL-21 (25, 26). Children who lack the expression of functional IL-12 receptor β1 chain, a common receptor for IL-12 and IL-23, display less Tfh cells and memory B cells in blood circulation and impaired GC formation in lymph nodes, providing *in vivo* evidence of the significance of this pathway for intact Tfh response in humans (21). Another important set of cytokines for human Tfh cell differentiation is TGF-β family cytokines TGF-β and Activin A, which activate the Smad signaling pathways including Smad2 and Smad3. Although only marginally effective by themselves, TGF-β and Activin A co-operate with IL-12 and IL-23 to promote human naïve CD4+ T cell differentiation toward the Tfh lineage (23, 27). TGF-β signals render STAT4 and STAT3 (activated by IL-12 and IL-23) to promote human naïve CD4+ T cells to acquire Tfh gene signature, while suppressing Th2 and regulatory T cell gene signatures (23). Furthermore, both TGF-β and Activin A also induce human CD4+ T cells to produce CXCL13 (27, 28), the major chemokine that human mature Tfh cells produce (7). TGF-β and Activin A are often strongly expressed in inflammatory sites, such as synovial fluid in rheumatoid arthritis (RA) (29, 30). Of note, neither TGF-β nor Activin A, even in the presence of Tfh-promoting cytokines, such as IL-6 and IL-21, induces Tfh molecules in mouse CD4+ T cells, and, therefore, this pathway is not shared in mice (23, 27).

Several cytokines are known to inhibit human Tfh cell differentiation. Type I (IFN-α, β, and ω) and type III (IFN-λ1 and λ2) interferons are potent inhibitors of Tfh cell differentiation in humans, and strongly diminish the expression of Tfh markers and gene signature by human naïve CD4+ T cells (23). This suggests that exaggerated Tfh cell responses in human autoimmune diseases with dominant IFN signature, such as systemic lupus erythematosus (SLE), is not mediated by the direct effect of type I IFNs on T cells, but by an indirect effect on APCs. Type I IFNs promote human DCs to produce Tfh-promoting cytokines, such as IL-12, IL-23, and IL-6 (31). Similarly, mouse studies demonstrated that type I IFN signals act as an adjuvant for antibody responses by promoting DCs to produce IL-6 (32, 33).

In mice, the IL-2–STAT5–Blimp1 axis inhibits Tfh cell differentiation at multiple differentiation stages (34, 35), and deprivation of IL-2 signals in the microenvironment is important for Tfh cell differentiation (36). There is evidence that IL-2 suppresses also human Tfh cell differentiation. IL-2 inhibition by a neutralizing Ab increased CXCR5 expression by human naïve CD4+ T cells cultured in Tfh-promoting culture conditions *in vitro* (27). Furthermore, treatment with low dose IL-2 decreased $CXCR5^{+}PD\text{-}1^{+}CCR7^{lo}$ cTfh cells in SLE accompanied with marked decrease of disease activity (37). It is of note, however, that it is unclear whether decrease of disease activity was dependent on decreased Tfh activity and whether decreased Tfh activity was the primary effect of IL-2 on Tfh cells. Current evidence highly suggests that the major target of low dose IL-2 treatment was Tregs. Low dose IL-2 treatment selectively induced STAT5 activation in Tregs but not conventional CD4+ T cells *in vitro* (38). In chronic GVHD patients, low dose IL-2 expanded circulating Treg frequency and their suppressive functions (38, 39). Therefore, it is possible that decrease of Tfh activity was secondary to increased activity of Tregs.

"Tfh-LIKE" CELLS IN INFLAMED TISSUES OF HUMAN AUTOIMMUNE DISEASES

Recent studies have identified "Tfh-like" cells involved in autoantibody production in inflamed tissues of human autoimmune diseases. These T cells share properties with Tfh cells only partly. Characterization of CD4+ T cells in inflamed synovial tissues in RA patients revealed a massive expansion of $CXCR5^{-}PD\text{-}1^{+}$ CD4+ T cells. While these cells expressed many human Tfh markers, including IL-21, CXCL13, ICOS, CD84, TIGIT, and c-Maf, and were able to induce memory B cells to produce IgG *in vitro*, they lacked the expression of CXCR5 and Bcl6. $CXCR5^{-}PD\text{-}1^{+}$ cells instead expressed Blimp-1 and the chemokine receptors CCR2, CCR5, and CX3CR1 (40). Analysis of inflamed salivary glands in Sjogren's syndrome showed an increase of $CXCR5^{-}$ $ICOS^{+}$ $CCR9^{+}$ CD4+ T cells. $CCR9^{+}$ CD4+ T cells isolated from blood samples of patients produced IL-21 upon activation and were capable of inducing memory B cells to produce IgG (41). These studies

suggest that the precise nature of B helper T cells associated with autoantibody production in inflamed tissues might differ among diseases, likely reflecting the differences in microenvironment. The chemokine receptors and integrins expressed by these "Tfh-like" cells might be critically involved for their migration to and retention in inflammatory tissues. It will be important to clarify how the developmental mechanisms and the functional regulations of "Tfh-like" cells are different from Tfh cells in lymphoid organs.

TRANSCRIPTION FACTOR NETWORK IN Tfh CELL DIFFERENTIATION

$CD4^+$ T cell differentiation is controlled by transcription factors which govern the expression of a set of target genes. Recent studies in mice identified a number of transcription factors and pathways that affect Tfh cell differentiation. Transcription factors positively regulate Tfh cell differentiation include Bcl6, Ascl2, Batf, cMaf, IRF4, STAT3, STAT1, STAT4, NOTCH1, NOTCH2, Tcf1, and Lef1 (42–48). Among these, Bcl6 is specifically required for the differentiation of Tfh cells, but not other $CD4^+$ T cell subsets (7, 44, 49–51). The transcription factor Blimp-1, an antagonist of Bcl6, inhibits Tfh cell differentiation (51). Genome-wide analysis of Bcl6-binding sites by using ChIP-Seq demonstrated that Bcl6 binds to genes associated with the differentiation of other $CD4^+$ T cell lineages, including Th1, Th2, Th17, and Tregs, and genes that negatively affect $CD4^+$ T cell localization in GCs (52). Thus, Bcl6 primarily acts as a repressor to inhibit the differentiation toward other $CD4^+$ T cell lineages and to diminish the molecules that exclude $CD4^+$ T cells from GCs (49–52). Interestingly, many genes repressed by Bcl6 contained AP1 and STAT motifs but lacked Bcl6 motif, suggesting that AP1 and STAT recruit Bcl6 to the target sites and repress the target genes (52).

RISK LOCI IDENTIFIED IN GWAS OF AUTOIMMUNE DISEASES AND THEIR POTENTIAL ASSOCIATION WITH EXAGGERATED Tfh RESPONSE

The genome-wide association studies (53) are an experimental design used to detect associations between genetic variants and traits including diseases in samples from populations (54). GWAS in autoimmune diseases have successfully identified loci associated with an increase or a decrease of risk (53–55). A fraction of the identified risk loci is located within or in the proximity of genes encoding transcription factors and cell surface molecules expressed by T cells and/or antigen-presenting cells. How risk haplotypes are associated with their dysregulated function and linked to the immunological consequences largely remains to be established. Nonetheless, several key risk loci are located in the proximity of genes that regulate Tfh differentiation in humans, and thus might contribute to the exaggerated Tfh response in human autoimmune diseases. Here we focus on three genes, *STAT4*, *IRF5*, and *TNFSF4*, which are identified and validated as risk loci in GWAS in multiple autoantibody mediated autoimmune diseases with a strong disease association at the genome-wide p value of at least 5×10^{-8} (**Figure 2**).

STAT4

STAT4 is among the top risk loci (other than HLA genes) identified in GWAS on SLE, RA, and Sjogren's syndrome (56). STAT4 is dominantly activated by IL-12 and to a lesser extent by IL-23 and type I IFNs (57). As discussed above, the IL-12–STAT4 pathway strongly promotes Tfh cell differentiation in humans. Human naïve $CD4^+$ T cells primed in the presence of IL-12 highly upregulate expression of multiple Tfh molecules (21, 58), which are inhibited by decreasing STAT4 expression by specific siRNA transfection (22, 23). STAT4 in $CD4^+$ T cells in the proximity

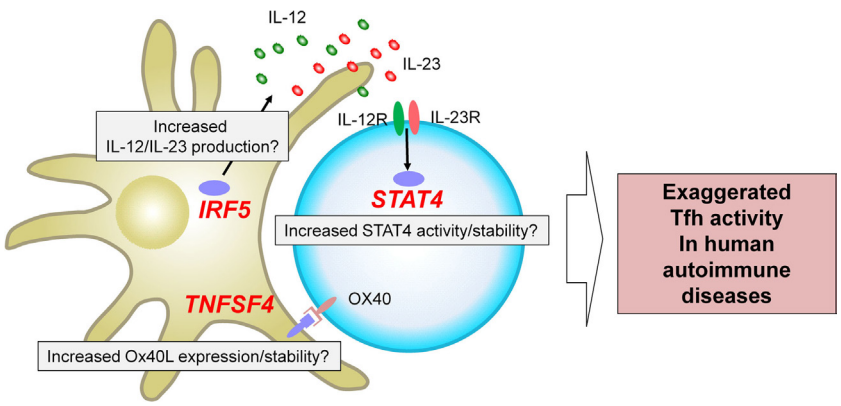

FIGURE 2 | Three risk loci potentially associated with exaggerated T follicular helper (Tfh) response in human autoantibody-mediated autoimmune diseases. GWAS in autoimmune diseases have identified multiple loci associated with disease risk. *STAT4*, *IRF5*, and *TNFSF4* have been identified and validated in multiple studies on autoantibody mediated autoimmune diseases as risk loci with a strong disease association at the genome-wide p value of at least 5×10^{-8}. These risk loci might contribute to the exaggerated Tfh response in human autoimmune diseases. For example, *IRF5* risk loci might be associated with an increased production of Tfh-promoting cytokines, including IL-12, IL-23, and IL-6. *TNFSF4* risk loci might contribute to increased Ox40 signals that promote the expression of Tfh molecules by human $CD4^+$ T cells. *STAT4* risk loci might affect the activity and/or stability of STAT4 and might enhance the signals of IL-12 and IL-23.

of GCs in inflamed human tonsils is strongly phosphorylated, indicating that the IL-12–STAT4 pathway is active *in situ* (23). Previous studies showed that DCs and macrophages in inflamed lymphoid organs and tissues of autoimmune diseases express IL-12 and IL-23 (31). It is plausible that STAT4 allele variants [such as rs11889341-T in SLE (59)] might be directly involved in the exaggeration of Tfh response, for example, by increasing their signaling efficacy and/or stability of STAT4 in $CD4^+$ T cells stimulated by IL-12 and/or IL-23. Of note, the IL-12–STAT4 pathway contributes to the Tfh differentiation also in mice, and STAT4 directly binds to multiple Tfh genes in IL-12-stimulated mouse $CD4^+$ T cells, including *Il21*, *Cxcr5*, *Pdcd1* (encoding PD-1), and *Icos* (60, 61).

A recent report showed that ustekinumab, a human recombinant antibody against IL-12p40 (a component of IL-12 and IL-23), was effective for active SLE in a phase II trial (presented in ACR 2017 meeting). Although this encouraging outcome remains to be confirmed, it is of great interest to determine whether the treatment with ustekinumab reduced Tfh responses, and more importantly whether the reduction of Tfh response correlated with the improvement of clinical parameters. Alternatively, treatment with ustekinumab might target other immune cells including Th1 and Th17 cells, which are also proposed to play pathogenic roles in SLE (62, 63). Determining the mode of action of ustekinumab will provide critical insights into the type of pathogenic $CD4^+$ T cells and the pathways in SLE.

Other STAT molecules STAT3 and STAT1 also positively regulate Tfh cell differentiation in mice (4). STAT3 is also critical for human Tfh cell differentiation, as patients with STAT3 *LOF* mutations as well as patients with IL-21 receptor deficiency display significantly less cTfh cells (64). Furthermore, $CD4^+$ T cells from patients with STAT3 *LOF* mutations and IL-21 receptor deficiency produce substantially less IL-21 upon IL-12 stimulation (24), suggesting that the IL-21–STAT3 axis is critical for enhancement of IL-21 expression. Interestingly, despite less IL-21 production, STAT3 *LOF* human $CD4^+$ T cells stimulated with IL-12 express normal levels of CXCR5, ICOS, and Bcl-6 (26). Thus, STAT3 and STAT4 might differently contribute to the expression of each Tfh molecule in human $CD4^+$ T cells, and IL-21 expression might be more dependent on the IL-21–STAT3 axis than the expression of CXCR5, ICOS, and Bcl-6. In this line, it is likely that decreased cTfh cells in STAT3 *LOF* are also due to severely impaired B cell response. STAT3 *LOF* display significantly less memory B cells (65), and the differentiation of STAT3 *LOF* B cells into plasmablast is severely impaired due to failure to respond to IL-6, IL-10, or IL-21 (65). Notably, unlike STAT4, STAT3 has been identified as risk loci in GWAS in autoinflammatory diseases, such as multiple sclerosis, Crohn's disease, and ulcerative colitis, which are less dependent on autoantibodies (53). STAT1 signals seems to play less significant roles in Tfh cell differentiation in humans than in mice, as patients with both STAT1 *LOF* and gain-of-function (*GOF*) mutations display normal frequency of cTfh cells (24, 58, 64). No GWAS on autoimmune diseases have identified STAT1 as a risk allele. These observations suggest that genetic variants of STAT1 and STAT3 do not contribute much to pathogenic Tfh and GC responses in autoimmune diseases. However, this notion does not preclude the involvement of STAT3 mutations in human autoimmune diseases. Patients with STAT3 *GOF* mutations show massive multiorgan autoimmunity, autoimmune cytopenia, lymphoproliferation, and immunodeficiency, likely due to impaired Treg differentiation and functions (66–68). Whether STAT3 *GOF* increases Tfh response or not is currently unclear. However, many patients display hypogammaglobulinemia (68), and thus that autoimmunity in STAT3 *GOF* seems to be caused by dysregulation of antibody response rather than a general increase of Tfh response.

IRF5

IRF5 is another transcription factor whose haplotype is highly associated with an increased risk of SLE, RA, and Sjogren's syndrome (53). IRF5 is expressed by a broad range of APCs, including monocytes, macrophages, DCs, plasmacytoid DCs, and B cells, and is critically involved in the production of inflammatory cytokines, including IL-12, IL-23, IL-6, and type I IFN (69–71). These cytokines are enriched in inflamed tissues in autoimmune diseases, and such inflammatory cytokine milieu causes a positive-feedback loop for the expression of IL-12 and IL-23 by promoting the generating M1 macrophages that highly express IRF5 (69). Thus, it is possible that IRF5 risk haplotype is linked to the enhanced production of Tfh-promoting cytokines in subjects with autoimmune diseases traits. IRF5 is required in normal human B cell proliferation and differentiation into plasmablasts (72), and therefore, IRF5 risk allele may also intrinsically affect B cells. The importance of IRF5 for lupus pathogenesis was also demonstrated by the attenuation of the disease in mouse models deficient of IRF5, which was accompanied with decreased activated $CD4^+$ T cells and autoantibodies (73, 74).

TNFSF4

TNFSF4 encodes Ox40L, a TNF ligand family molecule that delivers signals promoting human Tfh cell differentiation (11) as described earlier. *TNFSF4* has been identified as another prominent risk allele in SLE and RA (53). In active SLE, blood $CD14^+$ monocytes and tissue $CD11c^+$ myeloid cells upregulate the expression of Ox40L (11). *TNFSF4* risk allele might enhance signals *via* Ox40 and contribute to enhanced Tfh responses. The importance of the Ox40–Ox40L axis for antibody response (75–77) and autoimmune disease was also demonstrated in studies with mouse models (18, 78, 79). *TNFSF4* is also found as a risk allele in GWAS on allergies, such as asthma, hay fever, and eczema; an observation consistent with the importance of Ox40–Ox40L pathway for Th2 response (80).

In contrast, it is somewhat surprising that ICOS, another key co-stimulatory molecule for Tfh cell differentiation in humans and mice, and its ligand ICOS-ligand (encoded by *ICOSLG*) are not among the risk loci identified in GWAS on autoantibody mediated autoimmune diseases. *ICOSLG* was instead found to be highly associated with Crohn's disease and ulcerative colitis (53). Thus, while ICOS and ICOS-ligand are involved in pathogenic

Tfh response, as demonstrated in Roquin$^{san/san}$ mice, the haplotype *ICOS* and *ICOSLG* themselves *per se* do not seem to contribute to pathogenic Tfh responses.

CONCLUDING REMARKS

Although the mode of actions remains to be established, a positive outcome of ustekinumab in active SLE patients provides a new proof of principle that T cells can be a therapeutic target for autoantibody-mediated autoimmune diseases. Another evidence came from a clinical trial performed some 15 years ago with anti-CD40L (BG9588) on lupus nephritis. Although the trial was prematurely terminated due to the development of thrombotic events and associated fatality in some patients, the early results were encouraging and the treatment reduced serum anti-dsDNA titers, increased serum complement levels, and reduced nephritis score (81). Thus, targeting B helper T cells can be beneficial for treatment of autoimmune diseases. It will be important to define the differentiation mechanisms and the functions of Tfh and Tfh-like cells that directly contribute to the production of autoantibodies in lymphoid organs as well as inflamed tissues. Determining how risk loci in autoimmune diseases are associated with the molecular mechanisms for exaggerated Tfh responses is also of great importance. These studies will enhance our understanding in human autoimmune diseases and also might provide novel therapeutic targets.

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HU and SH, have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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