Molecular abnormalities of the B cell in systemic lupus erythematosus are candidates for functional inhibition treatments

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Introduction: The B cell is a key player in the pathogenesis of systemic lupus erythematosus (SLE). Loss of B cell tolerance resulting in autoantibody production and immune complex formation and deposition are central features of the disease. B cell overactivity is a hallmark of SLE and molecular abnormalities in B cell signaling cascade have been described.

Areas covered: In this review, we will focus on the aberrant phenotype of B cell signaling in patients with lupus. We will also discuss data stemming from the use of small molecules that have recently been recognized to target important steps of the B cell signal transduction pathways with therapeutic implications for SLE.

Expert opinion: Attempts to target the B cell in SLE have been made through depletion, blocking of survival factors and co-receptor inhibition. However, the still unmet need for effective therapy of refractory disease makes the necessity for new drugs impelling.

Keywords: B cell, Bruton’s tyrosine kinase, kinase inhibitor, Lyn, PI3K, spleen tyrosine kinase, systemic lupus erythematosus

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1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with heterogeneous clinical manifestations affecting mostly women of childbearing age. The multisystemic nature of the disease with end organ damage resulting in significant morbidity and mortality, as well as the elusive underlying multifactorial pathogenesis pose a challenge to the clinician [1].

SLE is thought to emerge through loss of self-tolerance resulting in the production of autoreactive cells as well as of autoantibodies against components not only of the cell nucleus (DNA, RNA and nuclear proteins) but of constituents of the cytoplasm, the cell membrane and of soluble substances as well. Failure of tolerance checkpoints to limit autoreactive B cells [2] makes it possible for these aberrantly functioning cells to exert their roles, including autoantibody production. Although the pathogenesis of autoimmune diseases seems to comprise of a complex interaction of innate and adaptive immunity in the context of specific environmental stimuli, B cells have lately been focused on. Experiments in B cell-deficient lupus-prone mice have clearly shown that B cells, but not autoantibodies necessarily, are absolutely required for the appearance of lupus-like systemic autoimmune [3-5]. Defects in B cell maturation into plasma cells have also been reported to be of importance for the induction of autoimmunity. This was evident in the Irf4-deficient B6Δirf4 mice; IRF4 deficiency inhibited the maturation of B cells into plasma cells, and of T cells as well, and inhibited the progression of autoimmunity [6]. Of particular
interest are experiments with lupus-prone animals that do have peripheral B cells but are genetically engineered to be incapable of secreting any antibody (including autoantibodies). Such mice still do develop systemic lupus-like autoimmunity, underscoring the necessity for the presence of B cells and their diverse roles apart from autoantibody production. In such models, the previously established activation of T cells in SLE might be critically mediated by B cells possibly via antigen presentation [5,7]. In humans, linkage analyses, candidate gene studies and more recently genome-wide association studies in populations of different ancestry have identified among others specific SLE susceptibility loci that encode proteins critical for B cell differentiation and proliferation, as well as B cell signaling susceptibility loci. B cell signaling is abnormal in patients with SLE. This was first described in unfractionated lupus B cells exhibiting aberrant early signal transduction events. Augmented cytoplasmic free Ca2+ responses and increased phosphorylation of tyrosine protein residues follows ligation of the BCR with both intact anti-human IgM and anti-human IgD antibodies or antibody fragments. These altered early signaling events were augmented compared to healthy and disease control subjects and were also disease-activity and treatment-status independent [12]. Increased phosphorylation of MAPKs, in particular Erk, Jnk and p38, was found using flow cytometry techniques in the B cells of a limited number of patients with lupus, also implying a hyperactivated B cell phenotype [13]. The altered signaling status of the lupus B cell raises questions about the nature of the B cell abnormality as it could be the result of intrinsic molecular defects or/and the effect of various exogenous factors influencing positive or negative regulators of B cell signaling.

3. Altered B cell signaling in SLE

B cell signaling is abnormal in patients with SLE. This was first described in unfractionated lupus B cells exhibiting aberrant early signal transduction events. Augmented cytoplasmic free Ca2+ responses and increased phosphorylation of tyrosine protein residues follows ligation of the BCR with both intact anti-human IgM and anti-human IgD antibodies or antibody fragments. These altered early signaling events were augmented compared to healthy and disease control subjects and were also disease-activity and treatment-status independent [12]. Increased phosphorylation of MAPKs, in particular Erk, Jnk and p38, was found using flow cytometry techniques in the B cells of a limited number of patients with lupus, also implying a hyperactivated B cell phenotype [13]. The altered signaling status of the lupus B cell raises questions about the nature of the B cell abnormality as it could be the result of intrinsic molecular defects or/and the effect of various exogenous factors influencing positive or negative regulators of B cell signaling.

**Article highlights.**

- Loss of B cell tolerance resulting in increased numbers of autoreactive B cells is an important feature of SLE.
- BCR-triggered signaling is abnormally hyperactive in patients with SLE.
- Molecular abnormalities exist in the B cells of patients with SLE, such as reduced expression of kinase Lyn and Lyn mRNA, perhaps due to a multitude of mechanisms.
- Targeting key effector molecules of the B cell signaling apparatus is feasible nowadays with small molecule inhibitors. Some of these inhibitors are already approved or in clinical trials regarding patients with B cell malignancies, delivering promising outcomes in terms of efficacy and tolerability.
- Syk, PI3K and Btk inhibition are examples of a crop of novel, encouraging results from the study of lupus-prone animal models, clearly depicting amelioration of manifestations such as nephritis, skin disease and autoantibody production.
- The proper design of trials that will address the issue of small molecule-mediated inhibition of key B cell signaling mediators in patients with SLE is a challenge.

This box summarizes key points contained in the article.
B cell signaling [14]. In the attempt to clarify the mechanisms underlying the aberrant B cell status, important data have emerged from the study of genetically engineered animal models and small molecule inhibitors (Table 1).

3.1 Targeting Lyn kinase

Lyn is a src family kinase expressed in all hematopoietic cells except T cells. It is the predominant kinase of B cells featuring unique properties with both positive and negative roles in the B cell signaling pathways [15]. Lyn is believed to have a prominent role in the initiation of signaling events after BCR antigen linking, primarily by phosphorylating the Ig-α/Ig-β ITAM domains of the BCR complex. It also enhances B cell responses by mediating CD19 phosphorylation. While this positive regulation of Lyn can be substituted by other src family kinases, the negative roles of Lyn appear to be indispensable [10,15]. Lyn also phosphorylates immunoreceptor tyrosine-based inhibitory motif domains of key inhibitory receptors such as FcγRIIB and CD22. This results in the recruitment of tyrosine phosphatases, such as SHIP and SHP-1, that function in order to downregulate the BCR signal.

Lyn-deficient mice are an excellent example of the essential role of this kinase as a negative regulator of BCR responses, as Lyn−/− mice are characterized by a hyperactive B cell phenotype and the production of autoantibodies [16]. Interestingly, autoimmune aberrations are also present in the gain-of-function Lyn mutant mice. The enhanced positive signaling manages to prevail over the continuous phosphorylation of negative BCR complex regulators, thus leading to autoantibody development and severe glomerulonephritis [17]. In a recent study, Lyn heterozygote mice were generated with one normal and one mutant non-functioning allele, exhibiting immune system aberration suggesting that Lyn haploinsufficiency can also lead to autoimmunity [18]. All Lyn mutant animal models have phenotypic aspects quite reminiscent of SLE. A fragile equilibrium exists for Lyn kinase, which appears to be in the spotlight regarding proximal B cell signaling.

These data warrant efforts to explain immune aberrations encountered in SLE via the study of Lyn expression and function. In SLE, genetic studies have been attempted in populations of different ancestries aiming at identifying potential susceptibility polymorphisms in the Lyn kinase gene [19]. At the protein level, lupus B cells are deficient in Lyn. This defect is encountered in two-thirds of patients with lupus (n = 21) analyzed; again, this defect was disease-activity and treatment-status unrelated [20]. Two different groups reproduced these data in ethnically different populations of patients with SLE [21,22]. In an effort to mechanistically explain Lyn deficiency of the lupus B cell, increased ubiquitination has been reported [21]. However, this post-translational modification may not efficiently explain the finding of reduced Lyn mRNA levels that accompanies
reduced Lyn protein levels \cite{20,22}. Recently, an interesting post-transcriptional mechanism defect has been reported according to which increased expression of microRNA-30a (miR-30a) could lead to decreased Lyn protein in lupus B cells \cite{22}.

The dual role of Lyn makes this molecule a rather difficult direct therapeutic target since the results of inhibition or gain of function cannot be readily predicted. Indirect modification of Lyn expression could however be considered in SLE. The aforementioned data point to miR-30a as a possible target molecule. Moreover, B cell-specific Lyn knockout mice have recently been engineered, showing similar autoimmune phenotypic aspects as the prototypic model. This group provided novel data on mechanism(s) underlying Lyn-deficiency-induced autoimmunity by depicting a critical role for B cell MyD88 signaling pathway in the expression of the lupus phenotype in these mice. The additional deletion of MyD88 in their B cell-specific Lyn-/- model counteracted/corrected autoimmune manifestations and abrogated the development of lupus-like systemic autoimmunity \cite{23}. Therefore, MyD88 inhibition might represent a potential future therapeutic approach to target B cell defects in SLE.

### 3.2 Targeting spleen tyrosine kinase

Syk is widely expressed in different immune and non-immune cells. It is a critical regulator of immune cell signaling having a close association with BCR and TCR complexes. Syk binds via its SH2 domains to phosphorylated tyrosines of the BCR and TCR complex. Such binding results in activation of Syk itself leading to the subsequent phosphorylation of its own substrates; such activated tyrosine-phosphorylated Syk substrates induce downstream events like mobilization of intracellular Ca\(^{2+}\) and eventually gene regulation \cite{24}.

SLE B and T cells exhibit an activated phenotype \cite{12,25} while SLE T cells are known to actually overexpress Syk \cite{26}. Thus, Syk inhibition appears to be an attractive therapeutic target in SLE in an effort to modulate lymphocyte function. Fostamatinib is a Syk inhibitor that has already been tested in clinical trials involving patients with rheumatoid arthritis with encouraging and acceptable results in terms of efficacy and safety, yet not promising enough for the sponsoring companies to continue further trials \cite{27-29}. Other Syk inhibitory molecules are also currently under development.

RO9021 is a novel selective Syk inhibitor found to suppress anti-human IgM-induced BCR signaling in a human B cell lymphoma cell line as this was reflected on the Ca\(^{2+}\) flux and the phosphorylation of Btk, PLC\(_{\gamma}2\), Akt and Erk. It was also suggested that Syk inhibition with RO9021 was able to dampen toll-like receptor 9 (TLR9) mediated responses in human B cells. More specifically, B cell proliferation and plasmablast differentiation as well as IgM, IgG and IL-6 production were inhibited when using a TLR9 agonist along with concomitant use of RO9021 \cite{30}. Considering the emerging role of TLRs in SLE pathogenesis \cite{31}, Syk

### Table 1. Inhibition of kinases found in the B cell in lupus-prone animal models.

| Ref.           | Kinase      | Drug     | Model                  | Outcome                                                                 |
|----------------|-------------|----------|------------------------|--------------------------------------------------------------------------|
| Bahjat et al.  \cite{32} | Syk         | Fostamatinib | NZB/NZW mice          | Renal disease amelioration, improved survival                           |
| Deng et al.  \cite{33} | Syk         | Fostamatinib | MRL/Ipr mice           | Skin and renal disease/lymphadenopathy amelioration                     |
| Deng et al.  \cite{33} | Syk         | Fostamatinib | BAK/BAX double-knockout mice | Skin disease/lymphadenopathy amelioration                               |
| Barber et al.  \cite{39} | PI3K\(_{\gamma}\) isoform | AS605240 | MRL/Ipr mice           | Renal disease amelioration, improved survival, reduced autoantibody titers |
| Winkler et al.  \cite{40} | PI3K\(_{\gamma}/\delta\) isoforms | IPI-145 | NZBWF1/J mice          | Renal disease amelioration, reduced anti-dsDNA titers                   |
| Honigberg et al.  \cite{48} | Btk         | Ibrutinib | MRL/Ipr mice           | Renal disease amelioration, decreased spleen size, reduced autoantibody titers |
| Hutcheson et al.  \cite{49} | Btk         | Ibrutinib | B6.Sle.Ipr3 mice (pre-diseased) | Renal disease amelioration, reduced IgG anti-dsDNA titers, reduced splenic plasma cell numbers |
| Mina-Osorio et al.  \cite{50} | Btk         | RN486    | NZB/NZW mice          | Renal disease amelioration, reduced IgG anti-dsDNA antibodies, reduced spontaneous germinal center formation and splenic plasma cell numbers |
| Rankin et al.  \cite{51} | Btk         | PF-06250112 | NZB/NZW mice          | Renal disease amelioration, reduced IgG anti-dsDNA antibodies, reduced spontaneous germinal center formation and splenic plasma cell numbers |
inhibition may represent an attractive potential therapeutic modality in lupus because of its capacity to inhibit B cells via more than one signaling pathways.

Fostamatinib has already been used in murine lupus models with remarkable results. In NZB/NZW lupus-prone mice fostamatinib prevented, if given in pre-diseased mice, the progression of renal damage and prolonged overall survival. Perhaps more importantly, similar results in terms of proteinuria, azotemia, kidney histopathology and survival were also reported in a dose-dependent manner in mice with already established proteinuria before treatment initiation. It is remarkable that Syk inhibition with fostamatinib had no effect regarding autoantibody titers [32]. In the MRL/lpr and the BAK/BAX double-knockout murine lupus models, skin lesions among other manifestations are part of the lupus phenotype. A group of investigators evaluated the effects of fostamatinib treatment in these mice. In the MRL/lpr model significant improvement was reported in established skin and kidney disease and established lymphadenopathy. Again, serum levels of anti-DNA antibodies were not affected. In BAK/BAX double-knockout mice, data presented imply amelioration of skin disease and lymphadenopathy [33].

### 3.3 Targeting PI3Ks

PI3Ks are lipid kinases that phosphorylate the 3’-hydroxyl group of phosphatidylinositol and its phosphorylated derivatives within the plasma membrane and intracellular compartments. They are divided into three classes with the class I PI3Ks being the most studied. Class I PI3Ks are comprised of a regulatory (SH2 domain-containing) subunit and a catalytic (p110) subunit. There are four isoforms of the catalytic subunit, namely, p110α, p110β, p110γ and p110δ. The PI3Kγ and PI3Kδ isoforms are enriched in leucocytes and are considered key signaling molecules in neutrophils, macrophages, dendritic cells, T cells, B cells and mast cells. The class I PI3Ks generate the important second messenger molecule PIP3, which can recruit pleckstrin homology (PH)-domain containing effectors like Akt (also known as Protein Kinase B) and Btk [34].

Patients with SLE frequently exhibit (70%) increased activity of the PI3K/Akt pathway in their PBMCs as shown by the levels of phosphorylated Akt (p-Akt). In vitro PI3K assays in PBMCs from patients with lupus attribute this activation to increased PI3Kδ, but not PI3Kγ, isoform activity. In this study, researchers mainly refer to T cells as the source of the aberrantly expressed p-Akt levels. However, they also present data from the study of B cells from a small number of lupus patients which show increased presence of p-Akt in the cell membrane compartment compared to healthy controls using an immunofluorescence technique [35]. It can thus be concluded that aberrant PI3K/Akt pathway activity characterizes both the B and the T cell compartments in human SLE.

The importance of PI3Ks and in particular the p110δ subunit in B cell function has been shown from the study of mice lacking a functional enzyme, such as the p110δ mutant and knockout mice [36,37]. A recent study reports that the presence of a mutated, catalytically inactive p110δ isoform, in the genome of the Lyn-deficient mouse model of autoimmunity, led to improved overall survival and attenuation of the lupus-like manifestations such as autoantibody production and kidney pathology [38].

Inhibition of the p110γ and p110δ isoforms has shown promising results in murine lupus models. The intraperitoneal administration of the PI3Kγ inhibitor AS605240 in MRL-lpr mice improved glomerulonephritis histopathologic findings, autoantibody titers and survival. It also resulted in reduction of the size of spleen and lymph nodes. These results were evident when treatment was initiated both in prediseased as well as in 3.5-month old mice with already established disease. The comparison of dexamethasone- and AS605240-treated mice provided similar results in terms of efficacy while an increased susceptibility to infections was seen only in the dexamethasone-treated group [39]. IPI-145, an oral inhibitor of both PI3Kγ and PI3Kδ isoforms, was administered in NZBWF1/J mice after disease onset, starting at 23 weeks of age. IPI-145 treatment resulted in significantly reduced anti-dsDNA antibody titers and proteinuria and amelioration of lupus nephritis histopathologic parameters in a dose-dependent manner [40]. Taking into account that oral PI3Kδ inhibitors, such as GS-1101, have already been used in humans in clinical trials for B cell malignancies with promising results [41], one may assume that PI3K inhibition may represent a plausible way of dampening the active B cell phenotype in patients with SLE.

### 3.4 Targeting Btk

Btk is a TEC family non-receptor protein kinase that is essential for B-lymphocyte development, differentiation and signaling [42]. It is expressed in B cells and other hematopoietic cells but not in plasma cells and T cells [43]. Btk activation and recruitment to the BCR complex follows the recruitment of Lyn and Syk in the order of the signaling cascade. Btk molecule bears a PH phospholipid binding domain and its activation requires the generation of specific phospholipids by PI3Ks.

The importance of this kinase in B lymphocyte maturation, longevity and proper signaling is demonstrated by the effect of Btk mutations in humans. Btk mutations cause a severe immunodeficiency disease state called X-linked agammaglobulinemia. Similarly, point mutations in the PH domain of Btk generate X-linked immunodeficiency (Xid) in mice [44].

Btk inhibitors are novel small molecules that appear promising in the therapeutic of B cell malignancies. Among them, ibrutinib (PCI-32765) is tested in ongoing Phase III trials in mantle cell lymphoma and chronic lymphocytic leukemia with remarkable clinical efficacy and tolerability [45]. Even though, to our knowledge, Btk has not been directly studied in human SLE, the following data from murine models provide rationale for targeting this molecule. Mice with B-cell-specific transgenic overexpression of Btk appear to have increased plasma cell numbers and enhanced spleen germinal center formation, exhibiting autoimmunity features...
such as autoantibody production and kidney damage [46]. Interestingly, congenic NZB/NZW mice homozygous for the Xid mutation, therefore lacking functional Btk expression, fail to develop renal disease as is expected in this animal model [47]. Furthermore, the use of Btk inhibitors in lupus animal models provides interesting findings. Treatment of 8-week-old MRL-Fas (lpr) mice with daily oral ibrutinib for 12 weeks resulted in statistically significant reduction of proteinuria and blood urea nitrogen as well as a nonsignificant trend towards amelioration of glomerulonephritis in kidney histopathologic evaluation [48]. Ibrutinib has also been used to treat B6.Sle1.Sle3 lupus-prone mice. Pre-diseased 4-month-old female mice treated with ibrutinib displayed amelioration of renal pathology and decreased infiltration of kidney with B220+ B cells as well as decreased spleen size. A significant reduction of both IgM and IgG anti-histones and anti-ribose autoantibodies was also seen. No data are provided however for B6.Sle1.Sle3 mice with full-blown disease [49]. RN486, another selective inhibitor of Btk, has been tested in NZB/NZW mice with already established proteinuria. RN486 treatment induced an impaired production of autoantibody and immune complex-dependent activation of monocytes. Kidney functional markers, such as proteinuria, and histopathologic markers were significantly improved in the RN486-treated mice compared to controls. IgG but not IgM anti-DNA antibodies as well as splenic plasma cell numbers were also significantly lower in the treated group [50]. Consistent with these findings were also the results of another group that used a novel Btk inhibitor (PF-06250112) in the same mouse model. PF-06250112 was found to potently inhibit BCR-initiated signaling and B cell proliferation and when given to mice with established kidney disease it significantly reduced glomerular injury as well as IgG and complement deposition. In addition, it was shown that spontaneous germinal center formation, splenic plasma cell numbers and serum IgG anti-dsDNA antibodies were significantly reduced in the treated group [51].

4. Expert opinion

SLE is a systemic autoimmune disease with incompletely understood pathogenesis. Because of this, currently implemented therapeutic strategies are still far from ideal. Some of our everyday practices rely on broad immunosuppression; although mortality and morbidity seen in the past are restrained, they are not diminished. Lupus patients with refractory disease, severe drug-related toxicities and quality-of-life issues still exist in a disease population consisting mostly of young women.

B cells are a keystone in the autoimmunity of SLE. In this review, we presented data from the study of B cell biology in patients with lupus that are indicative of a hyperactive B cell phenotype owing perhaps to a multitude of B-cell-intrinsic molecular abnormalities. Consistent with the aforementioned data, animal models with genetically engineered alterations in components of B cell signaling pathways have been shown to exhibit lupus-like manifestations. In a reverse manner, the use of small molecules that inhibit more or less specifically enzymes of the B cell signaling apparatus in models abrogated the predictable lupus-like manifestations.

Biologic agents that target B cells have already been used in SLE. Although rituximab, a B cell depleting monoclonal antibody against CD20, failed to meet primary endpoint criteria in two large clinical trials involving patients with SLE [52,53], it is widely used in refractory SLE cases in everyday clinical practice with promising results, as open label studies have shown. Moreover, belimumab, a monoclonal antibody targeting the critical B cell survival factor BAFF, has gained regulatory approval for the treatment of some forms of active SLE.

Understanding the B cell signaling pathways along with their lupus-relevant molecular aberrations identified may allow for more targeted and rational interventions. Taking into account the successful implementation of small molecule-mediated inhibition in hematologic malignancies, the idea of providing a specific, tailored to the patient’s ‘molecular identity’ and possibly less toxic therapeutic agent in patients with lupus appears attractive. In order to avoid unnecessary effects on other cells, thus risking to create just another unspecific immunosuppressant, it would be ideal to target molecules that are B cell specific. In light of this, Lyn and Btk modulation appears to be more rational. The study of small molecules that inhibit specific B cell signaling enzymes/mediators in lupus-prone animal models offers an important insight and also provides the background to attempt similar trials in humans.

Traditional treatment options seem to have reached a therapeutic plateau in the case of SLE. Biological agents and/or small molecule-mediated inhibition of specific intracytoplasmic enzymes could be an area of discussion and interest in the near future. Ongoing research efforts towards understanding the molecular abnormalities that drive immune system aberrations and the targeted development of drugs which can abrogate critical components of cell signaling in a refined way may represent a novel basis for individualized therapy in lupus. B cells remain a critical player in the pathogenesis of SLE and agents that target B cell signaling pathways are likely to be expected in future clinical trials. The identification of subgroups of patients characterized by specific pathogenic components as well as the rational design of clinical trial endpoints could improve the outcome of our future treatments. Promising novel molecules that functionally inhibit specific instead of non-specific aspects of the lupus B cell pathophysiology, alone or in combination with traditional immunosuppressive drugs and/or biological agents, could therefore emerge as potential choices in the treatment of patients with SLE.

Declaration of interest

The authors have no competing interests to declare and have received no funding in preparation of the manuscript.
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