

Toll-like receptor function in primary B cell defects

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1. ABSTRACT

Primary immunodeficiency diseases include more than 150 different genetic defects, classified on the basis of the mutations or physiological defects involved. The first immune defects to be well recognized were those of adaptive immunity affecting B cell function and resulting in hypogammaglobulinemia and defects of specific antibody production; more recently, novel defects of innate immunity have been described, some involving Toll-like receptors (TLRs) and their signaling pathways. Furthermore, it is increasingly evident that the innate and adaptive pathways intersect and reinforce each other. B cells express a number of TLRs, which when activated lead to cell activation, up-regulation of co-stimulatory molecules, secretion of cytokines, up-regulation of recombination enzymes, isotype switch and immune globulin production. TLR activation of antigen presenting cells leads to heightened cytokine production, providing additional stimuli for B cell development and maturation. Recent studies have demonstrated that patients with common variable immunodeficiency (CVID) and X-linked agammaglobulinemia (XLA) have altered TLR responsiveness. We review TLR defects in these disorders of B cell development, and discuss how B cell gene defects may modulate TLR signaling.

2. INTRODUCTION

About 20% of serum proteins are immunoglobulins (Ig) which collectively contain all the antibody types that a human needs to be protected against most infections. Aside from the fact that immunizations received in childhood are often sufficient for decades of protection, vaccinations are essential public health strategies against emerging pathogens. In spite of the reliance of the medical field on a healthy humoral immune system, exactly how these large quantities of desirable antibodies are made and continuously replenished while the production of auto-antibodies is prohibited remains largely a mystery. Antibodies that circulate are the end product of a number of steps that include continuous reconfiguration of genes for antigen receptors and the elimination of 90% of B cells along the way. Murine models have illustrated the most basic principles of B cell biology, but what is most solidly known for humans is based on studies of primary immune defects.

There are a number of primary B cell immunodeficiencies, associated with either identified or as yet unidentified genetic defects. These impair B cell physiology at many stages of development, from maturation in the bone marrow to progression into Ig-

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secreting plasma cells. Historically, the study of X-linked agammaglobulinemia (XLA) permitted the elucidation of the X chromosome encoded cytoplasmic tyrosine kinase Bruton's tyrosine kinase (BTK), which is necessary for signaling from the B cell receptor (BCR) and crucial for maturation of B cells (1). The X-linked Hyper IgM syndrome revealed that CD40 ligand is required for class switching, germinal center formation, and the development of B cell memory. Other Hyper IgM defects illustrate the additional requirements for Ig class switch and somatic hypermutation, and appropriate expression of activation induced cytidine deaminase (AID) and uracil-DNA glycosylase (UNG) (2, 3). However, the most common primary immune defect of B cells is common variable immunodeficiency (CVID), which is a group of heterogeneous defects with a range of impairments. While the gene mutations for the majority of patients remain unidentified, for a few, some interesting defects have been elucidated, each of which has furthered our understanding of normal B cell physiology.

For the past decades the majority of studies of primary immune deficiencies have centered on defects of the adaptive immune system. More recently, mutations in genes of the innate immune system, for example, the Toll-Like Receptor (TLR) signaling pathways (IRAK4, MyD88, UNC93B, and TLR3) as reviewed (4, 5), have illustrated that these defects lead to selected immune impairments and characteristic infectious disease susceptibilities. Further studies have revealed focal defects in TLR signaling in CVID and XLA, suggesting a possible role for these innate receptors in dysfunctional development of humoral immunity.

3. TLR AND B CELL ACTIVATION

While B cells are traditionally considered key players in adaptive immunity due to their ability to produce antibodies, activation of innate immune receptors on B cells appears to provide a co-stimulatory effect that promotes both the function and survival of B cells (6). In mammals, at least 13 TLRs have been described; nine of which have been shown to be functional receptors that are able to coordinate innate and adaptive immune signals resulting in a wide range of cellular responses. TLRs are expressed in various cell types, including monocytes, phagocytic cells, dendritic cells and B cell subsets. TLR1, 2, 4, 5, and 6 are expressed primarily on the extracellular surface and recognize microbial surface components including LPS, lipopeptides, and flagellin. TLR3, 7, 8, and 9 are primarily expressed in the endosomal compartment; this location makes these receptors poised to recognize viral- and bacterial-derived DNA and RNA breakdown products. With the exception of TLR3, all TLRs share structurally similar pathways that utilize the MyD88 adaptor protein as part of the intracellular signaling pathway leading to NF-kappaB and MAP kinase activation.

Peripheral blood memory B cells express constitutively high levels of TLR1, 6, 7, 8, 9, and 10 whereas naïve B cells express lower levels of TLRs (6-10). It is notable that unlike murine B cells, human B cells bear

very little TLR4 although it may be up-regulated in states of activation (11). These differences between memory and naïve B cells have suggested that the expression patterns are associated with different adaptive functions. One of the best studied TLR signaling responses is that of TLR9 on B cells which is triggered by oligodeoxynucleotides (ODN) derived from bacterial and viral DNA containing unmethylated CpG motifs (CpG-ODN) (12). Of the structural types of CpG ODN that have been described, type B ODN is the most potent stimulator of B cell activation. The functional outcomes of TLR9 signaling on human B cells are broad, and include cell activation, up-regulation of co-stimulatory molecules including CD23, CD25, CD40, CD54, CD80, CD86 CD69 and HLA-DR, secretion of IL-6 and IL-10, T-independent isotype switch, germline C(gamma)1, C(gamma)2, and C(gamma)3 gene transcription, somatic hypermutation, and B cell receptor (BCR)-independent immune globulin production (10, 13-17). Ligation of TLR9 on memory B cells further up-regulates its own expression, increasing cell sensitivity to activation, independently of the stimulating antigen, while concurrently reinforcing specific antibody response (18, 19). While memory B cells are more responsive to TLR signals, activation of naïve B cells under the appropriate circumstances also leads to maturation and Ig secretion (20). Appropriate CpG-containing DNA motifs also direct B cells to "Th1-like" Ig isotypes (IgG2a, IgG2b, and IgG3) while suppressing Th2 isotypes (IgG1 and IgE); for human B cells, activation by CpG-ODN counteracts IgE production induced by IL-4 (14, 21).

TLR7 and TLR8, which share similar ligand specificity and are expressed alongside TLR9 in the B cell endosomal compartment, share overlapping signaling pathways with TLR9. TLR7 agonists such as single-stranded RNA products, synthetic guanosine analogs, such as imiquimod or loxoribine, and imidazoquinoline derivatives, such as CL097, can activate both memory and naïve human B cells (22). As for TLR9, TLR7 activation leads to both cellular differentiation and Ig production (23, 24). Interestingly for TLR7-mediated stimulation, removal of plasmacytoid dendritic cells (pDCs) reduces Ig production, demonstrating that either direct or indirect interaction with pDCs is required. Since the addition of IFN-alpha restores Ig secretion, this cytokine appears essential for antibody production in normal B cell cultures (23). Both *in vivo* and *in vitro* experiments suggest that B cell switching to IgG isotypes requires the simultaneous presence of at least two signals alongside BCR engagement: TLR activation, CD40 engagement, and/or IFN-alpha (25). These observations led to studies suggesting that TLR activation might provide the long term stimuli important for the maintenance of memory B cell proliferation and differentiation into mature antibody-secreting cells which is initially induced by BCR and T cell help (10, 26).

However, the hierarchical role of TLRs in B cell biology is not clear: are these receptors required for the development of some facets of normal humoral immunity or is TLR stimulation an adjuvant for existing functions? MyD88 knockout mice, lacking the TLR adaptor critical

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for TLR7, 8, and 9 signaling have reduced serum levels of IgM, IgG1, IgG2a and IgG3 in comparison to wild type mice (27). Antigen specific IgM and IgG1 responses are reduced and IgG2 responses abolished to T-dependent antigens. These studies suggest a requirement for TLR signaling for optimum response, potentially via B cells directly but also via TLR-mediated DC maturation and T_H activation (28). Both TLR7^{-/-} and MyD88 deficient mice exposed to influenza A have significantly reduced levels of influenza-specific IgG2a and IgG2b, fail to develop bone marrow plasma cells and do not maintain long-term serum anti-viral antibodies (29, 30). However, the requirement of TLRs for optimum B cell activation is challenged by other work that showed that MyD88^{-/-} mice had robust antibody responses to T cell-dependent antigens given with an adjuvant (31). In addition, MyD88^{-/-} mice have retained TLR-independent antibody responses, although the degree of response may be reduced (32). One current view is that TLR signaling enhances IgM antibody responses in mice, but is not essential for long-term serologic memory responses (33). Interestingly, mutations in IRAK4 and MyD88 in humans do not lead to clearly identified defects in antibody responses (34, 35). Taken together, these studies imply that these TLR signaling pathways may provide a secondary stimuli to B cell development, however other molecular mechanisms could compensate for defective signaling through these innate receptors.

4. CVID

CVID is the most common clinically significant primary antibody deficiency due to the medical complications which develop and the need for life-long immune globulin replacement. The incidence is estimated at 1:25,000 to 1:50,000 (36, 37). The hallmarks include reduced levels of serum Ig due to lack of normal B cell differentiation (36-38). Although most subjects with CVID have normal numbers of peripheral B cells, the immunologic abnormality observed in a majority of patients is the reduced numbers of circulating CD27⁺IgD⁻ (isotype-switched) memory B cells and the absence of plasma cells in tissues (39-41). Since specific exogenous signals are required to differentiate naïve B cells into antibody secreting cells, many studies have examined *in vitro* Ig synthesis in CVID to dissect the nature of this collection of defects. These studies show that B cells of some CVID subjects retain a capacity for Ig synthesis *in vitro* while B cells of others do not. Although the pathogenesis for this group of disorders has not been clearly delineated, mutations in several genes associated with B cell development, including autosomal recessive mutations in BAFF-R, CD20, CD19, CD81, CD21, and ICOS, have been found in a small subset of patients (42-46). Mutations in the gene transmembrane activator and calcium modulating cyclophilin ligand interactor (TACI, *TNFRSF13B*), found in 8-10% of CVID patients (47-49) are not considered disease-causing as immune-competent first degree relatives, and rare normal controls have the same mutations. However, TACI mutations are significantly more common in CVID than healthy controls (50), and are significantly associated with both autoimmunity and lymphoid hyperplasia (51, 52).

4.1. TLRs and CVID B cells

As TLR agonists are powerful activators of human B cells, we have examined the effects of TLR agonists in CVID. We first noted that one of the most active of the phosphorothioate oligonucleotides that stimulate human B cells, the antisense to HIV rev gene, caused B cells of only some CVID subjects to produce immune globulin as seen in normal B cells (53). Subsequently this was explained when we found that CpG DNA activation of CVID B cells, alone or in conjunction with a BCR agonist, did not result in the up-regulation of CD86, a surface co-stimulatory molecule, nor did it enhance B cell proliferation, up-regulate AID mRNA expression, or enhance secretion of Ig (54). All of these outcomes are typically seen in TLR9-stimulated normal B cells. Coupling bacterial extracts from *S. pneumoniae* and *H. influenzae* with TLR9 ligands B cell activation also showed significantly reduced responses in CVID (55). No mutations or polymorphisms in TLR9 have been identified in CVID, suggesting that these functional differences are not due to defects in this receptor (54).

Examination of TLR7-mediated memory B cell responses revealed that TLR7 and TLR8 were also significantly impaired in CVID (56). When stimulated with loxoribine, isolated CVID CD27⁺ B cells demonstrated blunted proliferation, poor Ig secretion, and failure of CD27⁻ naïve B cells to up-regulate CD27 and shed IgD, the maturational phenotype associated with isotype switch. As seen in TLR9 studies, TLR7 and TLR7/8 activation also did not up-regulate AID mRNA expression or stimulate Ig secretion in CVID B cells, further indicating an impairment of the class-switch mechanism in CVID (56). The greater proportion of CD27⁺ memory B cells from healthy donors could presumably account for more robust TLR responses; in contrast, there are generally greater numbers of CD27⁻ naïve peripheral blood B cells in CVID rather than the more TLR-sensitive CD27⁺ memory B cells. However, additional studies on isolated CD27⁺ memory and CD27⁻ naïve B cells revealed that neither the naïve or memory CVID B cell subset proliferated or up-regulated AID mRNA upon TLR7 or TLR9 stimulation, showing that this defect was not restricted to the naïve B cell compartment (unpublished data). When CVID patients were further evaluated according to their peripheral memory B cell repertoire, CVID Group 1 subjects, who possess the lowest percentage of CD27⁺IgD⁻ peripheral switch memory B cells (<0.55% of peripheral blood mononuclear cells, (PBMCs)), appear to be the most deficient in their functional responses to both TLR7 and TLR9 activation. CVID Group 2 subjects (>0.55% CD27⁺IgD⁻ B cells) had higher TLR7 and TLR9 mRNA expression, which was also further up-regulated by their respective TLR ligands (unpublished data).

4.2. TLRs and CVID plasmacytoid dendritic cells (pDC)

While TLR defects were characteristic of CVID B cells, these defects are not global in this disease, as TLR activated CVID PBMCs produced normal amounts of IL-6, IL-12, and TNF-alpha; in addition, TLR3-activated CVID fibroblasts produced the same amounts of IFN-beta as control fibroblasts (56). However, while TLR7 or TLR9 activated pDCs constitutively express high levels of TLR7

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and TLR9 and normally secrete copious amounts of IFN-alpha (23, 57, 58), which further potentiates TLR7-mediated B cell responses (23). CVID pDCs produced significantly reduced amounts of IFN-alpha in response to TLR ligands (56). Upon TLR7 and TLR9 triggering, pDCs also stimulate monocytoid dendritic cells (mDCs) to produce cytokines to activate B cells (59).

The mechanisms by which IFN-alpha and other type I IFNs can augment normal B cell development are complex and exerted at a number of levels. IFN-alpha induces B cell activation, amplifies the BCR signal, increases B cell proliferation and enhances B cell survival (60). Type I IFNs can also trigger mDCs to up-regulate expression of potent B cell activators, such as B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) (61), promoting specific antibody production. When pDCs as the main producers of IFN-alpha are removed from influenza-stimulated cultures of human PBMCs, the B cells in these cultures lose the capacity to secrete specific antibody to the virus and fail to mature into plasma cells (62). For naïve B cells of normal donors, IFN-alpha enhances the *C. albicans*-specific IgM responses, promotes differentiation of memory B cells and plasma cells, enhances MyD88 mRNA expression, and augments the production of IL-6, IL-10, TNF-alpha, and IL-1-beta (63).

Since IFN-alpha stimulates normal B cells and augments B cell TLR activation, we examined if adding IFN-alpha to CVID B cell cultures could restore some aspects of B cell function. These experiments showed that adding IFN-alpha to TLR-activated B cells of some CVID subjects significantly improved both cellular proliferation and isotype switch. In some cases, Ig production was also enhanced, suggesting that the lack of this cytokine is a contributing factor to the failure of CVID B cells to mature and function normally. In contrast, the enhancing, but potentially deleterious, effects of type I IFNs on humoral immunity are well known in autoimmune disease, where excess IFN-alpha plays a pathogenic role, leading to the activation and proliferation of auto-reactive B cells (64-67). In this regard, we do not know if the addition of IFN-alpha to CVID B cell cultures might lead to the expansion and isotype-switching of B cells which recognize environmental antigens or of self-reactive B cells.

4.3. TLRs, clinical complications, and immunophenotypes in CVID

As CVID is a heterogeneous disease with highly variable clinical course, much effort has been devoted to identifying clinically relevant biomarkers to stratify patients by immunologic phenotypes to predict clinical outcomes and suggest medical management strategies. Classification systems, such as the EUROClass study, have been designed to define subgroups of this complex disease by their immunologic parameters (40, 68, 69). These and other studies show that fewer switched memory B cells are associated with an increased risk for the development of granulomas, autoimmunity, and splenomegaly (70, 71). Lower numbers of memory B cells may also be correlated with more severe respiratory and intestinal complications

(72, 73). In our studies, CVID subjects with greater numbers of isotype switched memory B cells were more likely to have retained TLR responses and heightened sensitivity to added IFN-alpha, suggesting that TLR responsiveness could be potentially related to clinical phenotypes.

TLR dysregulation and/or abnormalities have long been implicated in the development of autoimmunity. Because microbial DNA and RNA patterns have some overlap with human DNA and RNA, host nucleotide fragments may inadvertently induce autoimmune responses. Immune complexes containing self/human RNA and DNA may inappropriately trigger TLR7 and TLR9 activation of autoreactive cells in systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) (74). One report described TLR7 and TLR9 defects in a SLE patient whose disease subsequently evolved into CVID with regression of clinical lupus, suggesting that previously intact TLR7 and TLR9 signaling may have contributed to the development of autoimmunity (75). Interestingly, while TLR-activated CVID pDCs produce decreased amounts of IFN-alpha, approximately 20% of patients still develop autoimmune complications, with immune thrombocytopenic purpura and hemolytic anemia occurring most commonly (36, 38). Possible explanations included TLR stimulation of a predominantly naïve population containing self-reactive B cells, or otherwise abnormal TLR signaling which disrupts the regulation of other activation pathways. In this regard, patients with mutations in MyD88, UNC-93B, or IRAK4 have increased numbers of circulating autoreactive B cells in the periphery (76). On the other hand these patients do not demonstrate heightened levels of auto-antibodies in the blood, nor do they have increased autoimmune disease, potentially because the existing TLR activation defects are also protective against secretion of auto antibodies (34, 35).

5. TLR SIGNALING AND BTK

Bruton's tyrosine kinase (BTK), a cytoplasmic kinase encoded by the X chromosome, lies downstream of the B-cell receptor and is essential for B cell development in humans, (77-81). Mutations in *BTK* lead to X-linked agammaglobulinemia (XLA), a classic primary immunodeficiency in which patients have absent or nearly absent B cells, agammaglobulinemia and recurrent pyogenic infections. These infections largely disappear after replacement Ig therapy is initiated (82). While BTK is essential for B cell survival, BTK is also expressed by all leukocytes with the exception of T-cells and plasma cells (83).

5.1. TLR Signaling in *Xid* mice

The first suggestion that BTK might be involved in TLR signaling came from work in *Xid* mice, in which a point mutation in the pleckstrin homology domain of BTK leaves the kinase non-functional (84). Early work showed that *Xid* mice produced less TNF-alpha and IL-1beta in response to systemic LPS treatment; in addition, isolated *Xid* macrophages and neutrophils had an impaired production of reactive oxygen intermediates (85). Other

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studies in human and mouse cell lines showed that activation of TLRs 2, 4, 7, 8 and 9 resulted in phosphorylation of BTK (86). In addition, BTK was found to co-immunoprecipitate with MyD88, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP, the MyD88 adaptor-like protein, or Mal), and Interleukin-1 receptor-associated kinase 1 (IRAK1) (86-88). These observations led to further investigation of the role of BTK in TLR signaling, and yeast-2-hybrid studies found a direct interaction between BTK and the cytoplasmic Toll/Interleukin-1 receptor (TIR) domains of TLR4, 6, 8 and 9 (86). LPS was found to induce PI3K in the plasma membrane, establishing the PIP₃ gradient thought to recruit BTK to activated TLRs (89, 90). Confirming a functional role, TLR4 or TLR9 stimulated BTK deficient murine B cells and macrophages produced increased amounts of the pro-inflammatory cytokines, TNF-alpha and IL-6 (91, 92).

5.2. BTK in X-linked agammaglobulinemia

While the mouse studies suggested that BTK could in some sense regulate inflammatory cytokine production, the *Xid* mouse is only partially useful for studying the human counterpart, XLA. While antibody responses to thymus-independent antigen are impaired, *Xid* mice still retain a sizeable B cell population and produce Ig (81, 93-95).

TLR signals are important for monocyte, macrophage, neutrophil, and dendritic cell function; thus, if BTK is integral to these pathways, in what way does the loss of BTK affect the functions of these cells? Along with a susceptibility to severe bacterial infections, XLA patients are in some cases initially neutropenic. Others, generally not yet on Ig replacement, have had enteroviral meningitis. Some investigators have suggested that these complications could be related to TLR-dysfunction due to the loss of BTK. However, once on Ig therapy patients are typically healthy, suggesting these complications can be ascribed to the underlying humoral deficiency (82, 96-98). However, to explore this question, various studies in XLA have been performed. First, several studies noted that *in vitro* monocyte-derived macrophages and dendritic cells from subjects with XLA had selective impairments in TLR2, 4 and 8 induced cytokine production (87, 99-101). On the other hand, other studies of non-differentiated XLA mononuclear cells showed either normal or increased responsiveness to TLR ligands (102, 103).

To further clarify the question of the role of BTK in TLR signaling in non-differentiated human cells, our group assessed TLR responsiveness of neutrophils, monocytes, mDCs and pDCs isolated from blood of XLA patients. We found TLR-induced signaling through NF-kappaB and MAP kinase pathways to be present in neutrophils and mononuclear cell populations. We further found that TLR4- and TLR7/8-induced neutrophil effector functions were not impaired and TLR stimulation prolonged neutrophil survival in a manner similar to control cells, suggesting TLR defects are not likely to be the cause of the neutropenia occasionally found in XLA patients with active infection (104). Interestingly, we found that XLA monocytes and mDCs produced significantly higher

amounts of TNF-alpha, IL-6, and IL-10 as compared to control cells. In addition, TLR7, 8 and 9 stimulated pDCs from XLA patients produced comparable amounts of IFN-alpha as control cells, suggesting no loss of this cytokine to account for susceptibility to enteroviral infections ((54) and unpublished data). These studies suggest a potentially increased cytokine potential in XLA; on the other hand XLA patients on IVIG are generally well with few inflammatory complications.

TLR pathways appear to not only play a central role in the innate immune response, but also play an integral role in potentiating B cell responsiveness, as discussed above; as such, modulation of these pathways might serve as an ideal target for anti-inflammatory pharmacological intervention, particularly in processes involving auto-antibodies (74, 105, 106). The initial human studies that demonstrated BTK-deficient XLA cells to have dampened TLR responsiveness, led to the suggestion that BTK-inhibitors such as LFM-A13, initially developed as oncologic chemotherapeutics, could have a role in targeted treatment of inflammatory and auto-immune disorders. However, we have found there to be an increase in inflammatory response by XLA cells treated with LFM-A13, which suggests the possibility of an undesired pro-inflammatory response (unpublished data). Furthermore, studies of normal B cell physiology, the *Xid* mouse and other primary immune deficiencies including CVID suggest TLRs play a key role in maintaining and potentiating B cell responses, and if BTK plays an inhibitory role in these pathways, inhibition of this signaling intermediate may potentiate B cell responsiveness to these ligands, and promote a possible pathogenic activation of autoimmune B cells. As XLA patients lack B cells, the role the BTK plays in the TLR signaling in human B cells remains unclear, and a greater understanding of TLR signaling modulation is necessary before the incorporation of BTK-inhibitors into clinical treatments.

6. SUMMARY

CVID and XLA are primary immunodeficiencies affecting B cell development, activation, and production of antibody in response to antigen. It is becoming increasingly evident that invariant receptors of the innate immune system, particularly TLRs, play a key role in regulating the humoral immune system. Furthermore, studies of both these disorders suggest the possibility that the genetic abnormalities hindering B cell development also may affect normal TLR activation outside the lymphoid compartment. These studies further our understanding of the intricate interplay between the innate and adaptive immune systems, and through the study of primary immunodeficiency, we are widening our understanding of key regulatory steps in the inflammatory response. A better comprehension of the regulatory steps in TLR signaling will allow for targeted development of pharmacotherapies for autoimmune and inflammatory diseases linked to aberrant TLR and/or B cell activation.

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Abbreviations: AID: activation-induced cytidine deaminase, APRIL: a proliferation inducing ligand, BAFF: B cell activating factor, BCR: B cell receptor, BTK: Bruton's tyrosine kinase, CVID: common variable immunodeficiency, Ig: immunoglobulin, IFN: interferon, mDC: monocytoid dendritic cell, ODN: oligodeocynucleotide, PBMC: peripheral blood mononuclear cell, pDC: plasmacytoid dendritic cell, SLE: systemic lupus erythematosus, TLR: toll-like receptor, TNF: tumor necrosis factor, XLA: X-linked agammaglobulinemia

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