TLR7/TLR9- and B Cell Receptor-Signaling Crosstalk: Promotion of Potentially Dangerous B Cells

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INTRODUCTION

Toll-like receptor (TLR) responses to nucleic acids (NAs) have been extensively studied in monocytes and macrophages (1). In B cells, TLRs, such as TLR7 and TLR9, have been shown to mediate cell responses to both immunogenic NAs and NA-containing immune complexes (ICs) (2–4). Under normal conditions, B cells can respond immediately to initial microbial insults through NA recognition. B cells can also mount recall responses to previously encountered infectious agents and perpetuate life-long serological memory (5). However, when excessive cellular or tissue damage occurs and B cell responses to endogenous cellular NA are not restrained, autoantibodies and autoimmunity...
are promoted (6). A body of evidence has elucidated cooperative TLR7 or TLR9 (TLR7/TLR9) and B cell receptor (BCR) activation in aberrantly activated B cells. Further understanding of potential molecular synergy between BCR and TLR7/TLR9 pathways in B cells will enable development of agents that can potentially prevent autoimmune states in patients.

**TLR7 AND TLR9 ACTIVATION VERSUS ATTENUATION OF AUTOACTIVE B CELLS**

A number of murine models have been employed to substantiate the roles of TLR9 and/or TLR7 in the production of DNA-associated and RNA-associated autoantibody production, respectively (Table 1). TLR7-deficient autoimmune-prone mice display reduced or absent RNA-associated antibodies, whereas Tlr9-deficient mice have lower amounts of anti-nucleosome and anti-chronatin antibodies (7–9). A pathogenic role for TLR7 was revealed via characterization of the Y chromosome-linked autoimmune accelerating (Yaa) mouse that has known TLR7 overexpression due to gene duplication (10, 11). When Yaa are combined with systemic lupus erythematosus (SLE) mice and the Tlr9 gene knocked out, mice have increased RNA-associated antibodies, exacerbated clinical symptoms, and accelerated mortality (12). Unexpectedly, in all autoimmune-prone mouse models, including MRL/lpr, B6/lpr, Balb/c-Pristane, B6 Nba2 Yaa, B6 Yaa, and Ali5 deficient in TLR9, RNA-associated antibodies are increased, suggesting a more complex role for TLR9 in SLE (8, 9, 12–17). In MRL/lpr, B6/lpr, Balb/c-Pristane, B6 Nba2 Yaa, B6 Yaa, and Ali5 deficient in TLR9, RNA-associated antibodies are increased, suggesting a more complex role for TLR9 in SLE (8, 9, 12–17). In fact, on an autoimmune-prone background, Tlr9 deficiency alone leads to overall increased immune activation, exacerbation of pathogenesis, and in some cases increased mortality (8, 9, 12–15). By contrast, Tlr7-deficiency in autoimmune-prone mice leads to a significant decrease in overall immune activation and disease severity (9, 14). Thus, TLR7 and TLR9 have opposing pathogenic and protective roles, respectively, in autoimmune disease.

Nundel et al. found that TLR9 directly constrains BCR–TLR7-dependent responses, suggesting a B-cell intrinsic protective role for TRL9 (18). By contrast, Tlr7-deficient B cells are not responsive to DNA-containing ICs and have increased death rates. Interestingly, BCR–TLR9-mediated post-proliferative cell death of B cells when TLR7 is absent can be blocked by the TNF family survival cytokine B cell-activating factor (BAFF). Nickerson et al. observed that TLR9 was associated with anti-dsDNA B cell sequestration and deletion, corroborating a protective role for TLR9 (19). The relative contributions of B cell-intrinsic TLR7 and TLR9 on autoimmunity were addressed by Jackson et al. This group generated mixed bone marrow (BM) chimeras by adoptively transferring BM from wild type, Wiskott–Aldrich syndrome (WAS) protein-deficient, Was-deficient–Tlr7-deficient, or Was-deficient-Tlr9-deficient mice with μMT BM (20:80) into lethally irradiated μMT recipient mice (20). In this chimeric WAS model, B cells were the predominant cells rendered WAS-deficient and hyperactive. Since immune dysregulation and autoimmunity was largely confined to the B cell compartment, results suggest that the TLR9 and TLR7 effects were B-cell intrinsic (20). Further studies in B cell-specific knockout models are needed to clarify any impact from the 20% myeloid cells also found in this TLR7/TLR9-deficient chimeric model (20). Together, data highlight a need to better understand the molecular mechanisms that underpin pathological or protective responses of TLR7/TLR9 responses in B cells.

**TABLE 1 | TLR7/TLR9 responses have substantiated roles in both autoantibody production and autoimmunity, especially in B cell receptor (BCR)-activated B cells.**

| TLR7 and TLR9 functions in B cell autoimmunity | Reference |
|-----------------------------------------------|-----------|
| RNA-associated antigen recognition            | (11)      |
| RNA-associated autoantibody production        | (9)       |
| Pathogenic role in development of autoimmunity| (7, 14, 18, 20) |

1. Increased IgG production
2. Increased immune (B and T) cell activation
3. Promoted survival of plasmablast/antibody forming cells
4. Increased systemic lupus erythematosus (SLE)-related mortality and pathogenesis

**Functional synergy of BCR–TLR7/TLR9 pathways**

| BCR–TLR7/TLR9 autoimmune responses |
|------------------------------------|
| Syk inhibition of B cells blocked the CpG response | (40) |
| Btk and Syk mediate TLR9 crosstalk | (38, 41, 42) |
| Btk is dispensable for TLR7 and 9 (ligands and immune complex) proliferation | (39) |
| Lyn negatively regulates: |
| 1. Both anti-RNA and anti-dsDNA antibody production (both global deletion and B-cell specific) |
| 2. IgG class-switching |
| 3. B cell activation |
| 4. Cytokine production (pro-inflammatory) |
| 5. Autoimmune pathology |

**TLR7– AND TLR9–BCR RESPONSES ARE LIMITED BY AVAILABILITY AND TRAFFICKING OF NA LIGAND**

TLR7 and TLR9 are located in endosomal compartments and as a consequence, are usually sequestered away from...
NA-associated ligands. Immunogenic NA is derived from microbes or from damaged or dying cells located in the extracellular matrix (21). In both the physiological and autoimmune settings, endogenous NAs are more likely to form complexes with proteins or antibodies. As depicted in Figure 1A, TLR7/TLR9 ligands like NA-bound proteins can be brought into the B cell via several potential mechanisms. Endocytosis of NA-bound protein and diffusion of a synthetic agent (e.g., imiquimod/R848 or CpG) are known examples. Alternatively, NA or NA-ICs can be recognized and internalized by BCRs or Fc receptors and then presented to endosomal TLR7 or TLR9 for subsequent activation (6, 21). Trafficking of TLR7 and TLR9 from the endoplasmic reticulum to endosomal compartments is tightly regulated by the chaperone protein, UNC93B1 (22).

The balance of TLR7:TLR9 determines downstream effector function in part because of outcompetition of TLR9 binding to UNC93B1 (23, 24).

Dual engagement of BCR and activation of TLR7/TLR9 were first shown in seminal papers by Marshak-Rothstein's group (25, 26). These investigators employed transgenic (Tg) mice that express rheumatoid factor (RF) AM14 BCR. AM14 BCR specifically binds with low affinity to IgG2a that is bound to endogenous or synthetic, highly purified NA. These IgG-NA ICs are “dual specific” and bind to BCR and various forms of NA (chromatin, dsDNA, RNA, SnRNPs). A series of studies using this unique set of tools has now substantiated a requirement for BCR-IC internalization in TLR7/TLR9-mediated autoantibody production (25–27).
TLR7/TLR9 ACTIVATION OF B CELLS RELIES ON BCR ACTIVATION IN CERTAIN CONTEXTS

The role of the BCR is not simply to internalize and present NA antigen. After BCR activation, both total and endosomal TLR9 levels increase, suggesting that BCR directly regulates TLR9 (28, 29). Several signaling molecules downstream of BCR operate in concert with TLR pathways to modulate TLR responses (30, 31). In the healthy state, dual BCR and TLR7/TLR9 engagement confer synergistic responses, including cytokine production, antibody production, and class-switch recombination (32, 33). In autoimmune disease, synergistic BCR–TLR7/TLR9 activation by NA-IC results in increased B cell proliferation and autoantibody production (25–27). For full activation of autoreactive RF-B cells, combined signals from the BCR and either TLR7/TLR9 are required (30, 34). Dual engagement of BCR and TLR9 by chromatin-IC leads to distinct functional outcomes (29). BCR activation can operate with TLR7 to attenuate peripheral B cell tolerance (35). Conversely, BCR–TLR9 synergy either TLR7/TLR9 are required (30, 34). Dual engagement of BCR and TLR9 by chromatin-IC leads to distinct functional outcomes (29). BCR activation can operate with TLR7 to attenuate peripheral B cell tolerance (35). Conversely, BCR–TLR9 synergy rather than to the level of activation of individual B cells (39). Lyn, a src kinase molecule associated with the positive and negative regulation of the BCR pathway (43) has been shown to negatively regulate TLR7/TLR9 activation (44–46). Lyn-deficient or B cell-specific Lyn-deficient mouse models had increased NA-associated autoantibodies, cytokine production (including IL-6 and IL-10), and autoimmune pathology (44–46). The exact mechanism of this negative regulatory role in TLR signaling has not been defined in B cells, although it is well established that Lyn is required to phosphorylate and activate CD22, an important negative regulator of BCR signaling (43). While the mechanistic role for Btk remains somewhat contradictory, roles for Lyn and Syk are more defined.

BCR AND TLR7/TLR9 SIGNALING CASCADES: CROSSTALK AND POTENTIAL ABERRANT B CELL ACTIVATION

Figure 1B is a simplified depiction of the major molecular components TLR7/TLR9 and BCR signaling. As depicted on the left in Figure 1B, TLR7 and TLR9, unlike BCR, signal in a MyD88-dependent fashion. After ligand associates with TLR7 or TLR9 in the endosomal compartment, TLR monomers dimerize and recruit the adaptor protein MyD88 to the intracellular domains. MyD88 then binds the kinase interleukin-1 receptor-associated kinase (IRAK)-4, promoting its autoprophosphorylation. IRAK4 subsequently associates with and phosphorylates IRAK1 (47). The resultant multimeric MyD88–IRAK4–IRAK1 complex (often referred to as the “Myddosome”) is critical for downstream effector signaling. Phosphorylation of IRAKs is required for recruitment of the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) to the complex (48). TRAF6, together with two other ubiquitin-conjugating enzymes (not depicted), itself becomes ubiquitinated before translocation into the cytosol where it activates transforming growth factor-beta-activated kinase 1 (TAK1) (48). TAK1 activation results in the phosphorylation and subsequent activation of MAPKs and/or NFkB. NFkB activation leads to phosphorylation of downstream transcription factors, including interferon regulatory factors (IRFs)—IRF3, IRF5, or IRF7. As shown on the right in Figure 1B, BCR activation incites signaling through activation of proximal BCR molecules Syk, Lyn, and Btk. When soluble antigen ligates the BCR or when BCR is cross-linked by anti-IgM surrogate antigen, several src kinases including Lyn are rapidly activated. In turn, immuno-receptor tyrosine-based activation motifs (ITAM) within the cytoplasmic domains of the CD79a/CD79b heterodimer complex of the BCR are phosphorylated by Lyn (43). Dual phosphorylation of ITAM tyrosine residues allows the association and subsequent activation of Syk tyrosine kinase (51). Syk can then associate with and activate a number of other kinases, including Btk and adaptor proteins such as B cell linker protein (BLNK) and B cell adaptor for phosphoinositide 3-kinase (BCAP) (51). Another adaptor protein, B cell scaffold protein with ankyrin repeats 1 (BANK1) is primarily activated by B-lymphoid tyrosine kinase (Btk) (52). Adaptor proteins, BANK1 and BCAP, lack kinase activity but function as scaffold proteins in the formation of macromolecular complexes that enable efficient effector signal transduction. Subsequent downstream signals include PLCγ2 activation, calcium mobilization, MAPK, NFkB pathways, and BCAP–PI3K-mediated pathways (52–54).

Improved understanding of distinct molecular mediators of BCR–TLR crosstalk in normal versus aberrant B-cell signaling is emerging. As shown in Figure 1B, BCR-proximal kinases Lyn and Syk have been specifically implicated in molecular BCR–TLR7/
TLR9 crosstalk. Lyn has been shown to negatively regulate TLR activation in vivo (44–46). While not yet studied directly in B cells, the molecular mechanism may be similar to that found in dendritic cells, where Lyn directly associates with IRF5 and in doing so, inhibits the ability of TRAF6 to associate with and activate the transcription factor (55). Syk is a positive regulator of TLR signaling (40, 41). Syk activation has been associated with TRAF6 expression in B cells from patients with SLE (38). The association between Syk and TRAF6 suggests an important point of crosstalk in the context of autoimmune disease, suggesting a potential mechanism for how Syk blockade attenuates the TLR9 responses (40).

B cell adaptor proteins, BCAP and BANK1, are also potential components of BCR-endosomal TLR signaling crosstalk (Figure 1B). BCAP negatively regulates inflammatory responses mediated by TLRs 4, 7, and 9 by linking TLR–PI3K pathways (56–58). This has been shown to occur through a hidden TIR domain in the full-length BCAP protein, which allows its direct association with TLR adaptor proteins (58, 59). Recently, Halabi et al. published that BCAP binds directly with TLR adaptor proteins to facilitate PLCγ2- and PI3K-mediated depletion of the cell membrane phospholipid component of macrophages (59). Without these phospholipid substrates, the TLR adaptor proteins could not associate with the cell membrane. This potentially results in inhibition of subsequent signal transduction (59). Similar mechanisms downstream of endosomal TLR7/TLR9 may be utilized by B cells, but this requires further investigation. B cell adaptor proteins may also positively regulate TLR7/TLR9. BANK1 in B cells has been shown to be involved in B cell responses (40). BANK1-deficient mice crosses with B6Sle1.Y aa mice (the TLR7/TLR9- and BCR-Signaling Crosstalk model has been shown to be TLR-dependent (69). Importantly, DOCK8 was not required for initial BCR signaling. This suggests a pivotal role for the integration of DOCK8 and TLR–BCR signaling cascades in BCR activation by low affinity antigen, although studies examining simultaneous BCR and TLR9 activation are required to address this. Thus, in the physiological context NA-ICs when low affinity BCR is activated before NA is presented to TLR9, DOCK8, Syk, and Lyn may all play even more significant roles in integration of BCR–TLR9 signaling.

FUTURE STUDIES: TLR–BCR-MEDIATED AUTOIMMUNITY IN HUMANS

We now know there are a number of molecular links between BCR and TLR7/TLR9 signaling. Further studies are required to determine distinct pathologic signaling pathways so that agents can be used to block aberrant TLR/BCR signaling in patients. Future studies should address mechanisms that restrain potentially dangerous responses to antigen in autoimmune-provoking environments. To do this, more physiological research tools are required to further define mechanisms of TLR–BCR signaling, particularly for studies of human B cells. Many studies use anti-IgM surrogate antigen for the BCR–TLR activation component, which does not precisely recapitulate the more physiological setting of BCR–TLR activation by ICs that requires internalization. Additional technical challenges need to be addressed. Potentially pathogenic B cells are already in an activated state, hampering the ability to stimulate and delineate meaningful mechanistic studies ex vivo without inducing cell death. Gene knockdown is also challenging in primary disease-state B cells. Until these technical and logistical barriers are overcome, studies of synergistic BCR–TLR signal transduction in the physiological setting in human B cells remain challenging.

Understanding B-cell intrinsic BCR–TLR signaling and activation in the context of human disease will also require investigation of extrinsic factors involved in the promotion of autoreactive B cells. BAFF plays a pivotal role in B cell development and the maintenance of B cell homeostasis (64). Elevated BAFF levels have been implicated in breaking B cell tolerance in systemic autoimmune diseases including SLE and Sjögren’s syndrome (SS) (65). Elevated BAFF levels have been correlated with circulating autoantibodies, disease progression, and anti-dsDNA antibodies in SLE patients (66, 67) and with autoantibody levels in SS patients (68). One mechanism by which BAFF breaks this tolerance in a lupus-like disease model has been shown to be TLR-dependent (69). Data suggest a model whereby excess BAFF expands autoreactive B cells, and BAFF signals directly promote TLR activation and internalization of dsDNA or NA-IC autoreactive BCRs. BAFF also increases TLR7/TLR9 expression, and TLR7/TLR9 signaling promotes BAFF receptor expression, thus providing a positive feedback loop (69). Further study will elucidate how the extrinsic factor BAFF dysregulates intrinsic BCR–TLR B cell signals and promotes aberrant B cell activation and pathogenesis.
IMPLICATIONS FOR A DISEASE THAT DEVELOPS IN AN AUTOIMMUNE-PROVOKING ENVIRONMENT: ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HCT)

Current evidence as summarized above compels examination of aberrant B cells in patients with autoimmune pathology. This includes patients who develop chronic graft-versus-host disease (cGVHD) that develops after HCT. cGVHD is a B-cell mediated autoimmune disease-like state that is unacceptable debilitating and difficult to treat. Persistently altered B cell homeostasis in patients with cGVHD is potentially perpetuated by global B cell depletion strategies (70–72). In cGVHD, intrinsic abnormalities in the proximal BCR machinery of B cells are being defined. Thus, we and others are interested in developing ways to target only B cells from patients with cGVHD that are hyperactivated and primed for survival in vivo via BAFF- and BCR-associated pathways (73). Based on murine and human studies that demonstrated a role for BCR-activated B cells in the pathophysiology of cGVHD (74, 75), the novel application of signaling pathway inhibitors is being tested in clinical trials.

After HCT, B cells are recovering in an NA and alloantigen (76) rich environment that may promote pathological B cells. Circulating monocytes in cGVHD patients upregulate gene pathways involved in innate cellular damage responses (77). Some of these genes include TLR7, BAFF and Type 1 interferons. No definitive examination of TLR7/TLR9 in cGVHD has yet been performed, but studies suggest that there is a muted signaling response to TLR9 agonists by plasmablast-like cells that normally regulate immune responses via the production of cytokines including IL-10 (78). We conclude that studies of cGVHD addressing TLR9 and TLR7 signaling of BCR-activated B cells after HCT are warranted. Such studies will inevitably lead to further understanding of human B cell tolerance and will likely compel the expanded use of targeted therapeutic agents in patients.

AUTHOR CONTRIBUTIONS

SS and AS both researched the topic, wrote and edited the manuscript, and made the table and figure for this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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