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J Immunol 2009; 183:2974-2983; Prepublished online 31 July 2009;
doi: 10.4049/jimmunol.0900495
http://www.jimmunol.org/content/183/5/2974

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/08/05/jimmunol.0900495.DC1

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Antigen Receptor Signals Rescue B Cells from TLR Tolerance

Jayakumar S. Poovassery,* Tony J. Vanden Bush,* and Gail A. Bishop2*†‡

Interactions between innate and adaptive immune receptors are critical for an optimal immune response, but the role played by Ag receptors in modulating innate receptor functions is less clear. TLRs are a family of pattern recognition receptors that play crucial roles in detecting microbial pathogens and subsequent development of immune responses. However, chronic stimulation through TLRs renders immune cells hyporesponsive to subsequent stimulation with TLR ligands, a phenomenon known as TLR tolerance, well characterized in myeloid cells. However, it has not been studied in detail in B lymphocytes. In addition to the BCR, B cells express almost all known TLRs and respond robustly to many TLR ligands. Thus, B cells may receive signals through both TLRs and BCR during an infection and may respond differently to TLR stimulation than myeloid cells. We tested this possibility by stimulating repeatedly through either TLR alone or both TLR and BCR. Prestimulation through TLR7 resulted in reduced B cell proliferation, cytokine production, and IgM secretion upon subsequent TLR7 restimulation. The hyporesponsiveness to TLR7 restimulation was associated with reduced NF-κB and MAPK activation and defective c-Jun phosphorylation. However, simultaneous BCR signaling prevented or reversed TLR7 tolerance in both mouse and human B cells. Importantly, BCR signaling also rescued B cells from TLR7-mediated TLR9 tolerance. Additionally, the reversal of TLR7-mediated JNK activation was dependent on PI3K activation. Together these results present a novel mechanism to prevent and reverse TLR tolerance in B cells. The Journal of Immunology, 2009, 183: 2974–2983.

Signals through innate immune receptors have an important influence upon the outcome of Ag engagement by the BCR (1–3). Of particular interest is a family of pattern recognition receptors called TLR (4). TLRs are expressed on a variety of cell types, where they recognize molecular patterns unique to or enriched in microbes and alert the immune system to the presence of an invading pathogen (5).

However, TLR responses must be tightly regulated because uncontrolled production of cytokines can result in immunopathology. Thus, repeated or chronic stimulation through TLRs can render immune cells unresponsive to the same or different TLR ligands. This phenomenon, known as TLR tolerance, is well characterized in macrophages, especially in the case of TLR4 and its ligand LPS (6–8). However, TLR tolerance is not restricted to TLR4 because prestimulation with other TLR ligands has also been shown to induce refractoriness in immune cells in response to subsequent challenge with the same or different TLR ligands (9–12). The tolerant state in macrophages is associated with reduced activation of NF-κB and MAPK and suppression of various cytokines such as TNF, IL-6, and IL-12 (13). However, other macrophage functions such as bacterial phagocytosis (14) or NO and IL-10 production (15) are not affected in tolerant macrophages. Although a number of mechanisms are thought to be involved in induction of tolerance, such as down-regulation of surface receptors, transcriptional induction of negative regulators such as IRAK-M,3 SOCS-1, and SHIP, and production of anti-inflammatory cytokines such as TGF-β and IL-10 (16), the exact mechanism by which tolerance is induced is not clear. Because not all TLR-mediated functions are affected in tolerant macrophages, a recent study proposed that the regulation could be at the level of individual promoters. According to this model, gene-specific control mechanisms will allow transient inactivation of certain genes following initial TLR stimulation whereas maintaining normal induction of others upon subsequent TLR stimulation (17).

Although a wealth of information is available on macrophage and dendritic cell TLR responses, B cell-intrinsic TLR functions are only beginning to be appreciated, and it is not clear whether TLR responses are regulated similarly in B cells and myeloid cells. We have previously shown that R848, a synthetic low m.w. compound belonging to the imidazoquinoline family, can induce NF-κB and MAPK activation in B cells, activate B cells to proliferate, produce cytokines and Ab, and express increased levels of costimulatory molecules (1, 18, 19). Additionally, R848 has been shown to cooperate synergistically with the BCR and CD40 to induce increased amounts of cytokines and Ab production (1). Results from our laboratory further showed that the synergistic IL-6 production in B cells stimulated through TLR7 and CD40 is through enhanced JNK signaling and AP-1 activity (20). However, it is not known how these B cell responses are regulated or the effect of repeated or prolonged stimulation through TLR7 to subsequent stimulation through TLR7 or other TLRs, especially TLR9, which recognizes unmethylated CpG motifs of bacterial and viral DNA. This is an important issue to be addressed not only to understand B cell TLR responses, but also because a number of synthetic ligands for this TLR subfamily are under consideration as vaccine adjuvants (21). A better understanding of how B cell responses are regulated following stimulation through TLRs that recognize microbial nucleic acids will be useful in developing...
improved vaccines. In contrast to myeloid cells, B cells express both germline-encoded pattern recognition receptors and clonally rearranged Ag-specific receptors on their surfaces. Thus, it is likely that during an infection B cells will receive signals through both TLRs and the BCR. In this study, we sought to investigate the effects of repeated stimulation through TLR7 on B cell responses and how these responses are modulated in the presence of concurrent BCR signaling. The results suggest a novel mechanism to prevent and reverse TLR7 tolerance in B cells.

Materials and Methods

**Mouse and human B cells**

Resting high density spleen B cells were isolated from C57BL/6 mice, as described previously, with slight modifications (22). Briefly, splenocytes were depleted of T cells by treatment with anti-Thy-1.2 Ab, followed by complement lysis; the resultant cells were subjected to density gradient centrifugation, and high density B cells were recovered from the 65–85% interface. In some experiments, high density resting splenocytes were first isolated by density gradient centrifugation through a 60/65:85% Percoll gradient. Resting lymphocytes at the interface between 65 and 85% were then further purified by negative selection with anti-mouse CD43 magnetic beads (Miltenyi Biotec), according to the manufacturer’s protocols, to obtain resting high density splenic B cells. Cell preparations were >90% pure, as determined by FACS analysis with FITC-anti-B220 staining. Use of normal mouse cells in this study followed a protocol approved by University of Iowa Animal Care and Use Committee. Normal human PBMC were isolated from buffy coats of healthy adult blood donors by ficoll-hypaque density sedimentation. B cells were isolated from PBMC suspensions by negative selection using a B cell isolation kit, according to manufacturer’s instruction (StemCell Technologies). B cell purity as determined by CD19 staining was >95%. The use of normal human B cells in this study was approved by the University of Iowa Institutional Review Board. Cells were cultured in RPMI 1640 with 10% heat-inactivated FCS (Atlanta Biologicals), 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Reagents**

Polyclonal rabbit Abs against phospho-JNK, phospho-p38, p38, c-Jun, and AKT were from Cell Signaling Technology. Rabbit anti-JNK1/2 (FL) Ab was from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-ERK (p44/42 MAPK) and rabbit anti-phospho-Akt (Ser473) Abs were purchased from BioSource International/Invitrogen. Ab specific for phospho-c-Jun was from Upstate Biotechnology. Mouse anti-actin Ab (C4) was purchased from Chemicon International. Affinity-purified (Fab′)2 goat anti-mouse and anti-human IgM (μ-chain specific) Ab, goat IgG (Fab′)2 Ab, and peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG Abs were purchased from Jackson ImmunoResearch Laboratories. LY294002 and BAY11-7082 were obtained from Calbiochem. Anti-mouse and anti-human IL-6 Abs (coating and biotinylated) and anti-TNF Abs (coating and biotinylated) were purchased from e Bioscience. Multiplex assay (mouse 20-plex panel) was from BioSource International/Invitrogen. Recombinant mouse IL-6 and TNF-α were purchased from PeproTech.

**Western blots**

Purified splenic B cells (2 × 10^6) were treated as indicated in figures. The cells were then pelleted by centrifugation, dissolved in 2 × SDS sample loading buffer, and sonicated. Total cell lysates were fractionated by SDS-PAGE and blotted to polyvinylidene difluoride membranes. After blotting with the appropriate Abs, bands were visualized on Western blots using a chemiluminescent detection reagent (Pierce). Images of blots were recorded with a low-light imaging system (LAS-3000, Fujifilm Medical Systems USA). Immunoblots were stripped and reprobed with control Ab to verify equal protein loading in each lane. Chemiluminescence was subsequently quantified using Image Gauge software (Fujifilm). Values were then used to calculate ratios of phosphorylated/total protein (phospho-protein/control protein).

**Results**

**Effect of repeated stimulation through TLR7 on B cell cytokine production**

We have previously shown that R848, a synthetic TLR7 ligand, is a potent activator of B cells. R848 stimulation can initiate B cell proliferation, up-regulation of costimulatory molecule expression, and Ab production, and this synthetic compound is currently under consideration as a vaccine adjuvant (1, 18). Although B cell-activating properties of R848 have been well characterized, how these responses are regulated or how B cell functions are modulated in the context of repeated or chronic stimulation through TLR7 is not clear. To address this, high density resting B cells isolated from mouse spleen were stimulated with R848 for 24 h. Cells were washed extensively and restimulated with R848 for another 24 h. Cell culture supernatants were harvested and cytokines were measured to determine the effect of dual stimulation on tolerance induction.

In these experiments, after 24-h stimulation, cells were washed and restimulated with R848 and anti-IgM Ab were used to determine the effect of dual stimulation on tolerance induction. In these experiments, after 24-h stimulation, cytokine production was greatly reduced in B cells prestimulated with R848 or R848 and anti-IgM Ab for 24 h were labeled with CFSE (0.5 μM), following manufacturer’s instructions. Cells were then stimulated with or without R848 in the presence or absence of anti-IgM Ab for 4 days. Proliferation was determined by CFSE dilution via flow cytometry.

Mouse or human primary B cells (1 × 10^6/ml) were stimulated, as described previously, with R848 and anti-IgM Ab for 24 h. Cytokine levels in the culture supernatants were measured by sandwich ELISA, using mAb from e Biosciences following the manufacturer’s instructions. Plates were read at 450 nm by a SpectraMax 250 Reader ( Molecular Devices). Data were analyzed with SoftMax Pro software ( Molecular Devices). Luminescence-based multiplex (mouse 20-plex) was performed according to the manufacturer’s protocol. Mouse IgM- specific ELISA was performed according to the protocol provided by Southern Biotechnology Associates. Plates were read at 405 nm by a SpectraMax 250 Reader. Data were analyzed with SoftMax Pro software ( Molecular Devices).

**CFSE assays**

Naive high density resting splenic B cells isolated from mouse spleen or B cells prestimulated with R848 or R848 and anti-IgM Ab for 24 h were labeled with CFSE (0.5 μM), following manufacturer’s instructions. Cells were then stimulated with or without R848 in the presence or absence of anti-IgM Ab for 4 days. Proliferation was determined by CFSE dilution via flow cytometry.

**ELISA**

Naive primary B cells (1 × 10^6/ml) stimulated, as described previously, with R848 and anti-IgM Ab for 24 h were labeled with CFSE (0.5 μM), following manufacturer’s instructions. Cells were then stimulated with or without R848 in the presence or absence of anti-IgM Ab for 4 days. Proliferation was determined by CFSE dilution via flow cytometry.

**B cell stimulation**

Mouse high density spleen B cells or human peripheral blood B cells (1 × 10^6/ml) stimulated with R848 for 24 h were washed and restimulated with R848 or R848 and anti-IgM for another 24 h. Cell culture supernatants were collected and stored at −20°C until used for ELISA to determine cytokine production. Control B cells were incubated for 1 h (mouse splenic B cells) or for 24 h (human peripheral blood B cells) in medium and then stimulated with R848 for 24 h. Goat IgG (Fab′)2 Ab stimulation was used as a control to confirm the specificity of the anti-IgM response. Mouse high density resting spleen B cells prestimulated with R848 and anti-IgM Ab were used to determine the effect of dual stimulation on tolerance induction. In these experiments, after 24-h stimulation, cells were washed and restimulated with R848 alone for another 24 h.

**Discussion**

The induction of R848-mediated tolerance was dependent on the concentration of the ligand as well as duration of the initial stimulation. Pretreatment with as little as 0.01 μg/ml R848 was able to induce tolerance in B cells (supplemental Fig. 1A). Additionally, tolerance was induced only after pretreatment with R848 for greater than 12 h (supplemental Fig. 1B).
Effect of TLR7 tolerance on B cell proliferation and IgM secretion

R848 has also been shown to induce B cell proliferation, up-regulation of costimulatory molecules, and Ab secretion (19). So, we next tested whether TLR7 tolerance is associated with a defect in any of the above-mentioned B cell functions. To determine the effect of TLR7 tolerance on B cell proliferation, naive B cells isolated from mouse spleen were then washed and restimulated with R848 for 24 h. Cell culture supernatants were assayed for IL-6, TNF-α, and MIP-1α production by ELISA/Multiplex assay. Naive B cells incubated in medium for 1 h and then stimulated with R848 for 24 h were used as controls. Results are representative of three independent experiments for IL-6 and TNF-α, and two independent experiments for MIP-1α.

Defective MAPK activation in TLR7-tolerant B cells

MAPK activation, one of the earliest events initiated by R848 stimulation in B cells (18), has been shown to be important for many B cell functions, including IL-6 production (24). Thus, we next studied whether the reduced IL-6 production in TLR7-tolerant B cells is associated with any defects in MAPK activation. Mouse primary B cells were stimulated with R848 for 24 h, and after washing, were stimulated with R848 for indicated time points. Cellular extracts were then subjected to Western blotting and stained with Abs specific for phosphorylated forms of JNK, ERK1/2, and p38 kinase. Cellular extracts from naive B cells stimulated with R848 for indicated time points were used as controls. R848 stimulation induced robust MAPK activation in control B cells with phosphorylation of JNK, ERK1/2, and p38 evident as early as 5 min in control B cells (Fig. 3) and peak response at ~15 min. In contrast, MAPK activation was severely impaired in R848-prestimulated B cells in response to subsequent R848 stimulation. The effect was even more striking for JNK. Phosphorylation of JNK was almost completely absent in TLR7-prestimulated B cells in response to second R848 stimulation. Phosphorylation of ERK1/2 and p38 was also greatly reduced in prestimulated B cells compared with control B cells.

NF-κB activation and c-Jun phosphorylation in TLR7-tolerant B cells

Defective NF-κB activation is a well-known feature of LPS tolerance in macrophages (13). To test whether NF-κB activation is
affected in TLR7-tolerized B cells, IκB-α phosphorylation and degradation were determined in R848-pretreated and control B cells in response to subsequent R848 stimulation. As shown in Fig. 4A, R848 induced IκB-α phosphorylation in naive B cells as early as 2 min with peak response at 5 min after R848 stimulation. R848 also induced IκB-α degradation in naive B cells, evident by 15 min. IκB-α phosphorylation or degradation was markedly reduced in B cells pretreated with R848 in response to subsequent R848 stimulation.

B cell activation properties of R848 and CD154/CD40L are in some ways remarkably similar (1), and CD40-mediated IL-6 production in B cells has been shown to be dependent on factors downstream of JNK signaling (24). Because R848-mediated JNK phosphorylation was severely impaired in TLR7-tolerant B cells, we investigated whether phosphorylation of its substrate, c-Jun, is also affected. Indeed, phosphorylation of c-Jun was severely impaired in B cells pretreated with R848 in response to a second R848 stimulation (Fig. 4B). Naive B cells stimulated with R848 exhibited a progressive increase in c-Jun phosphorylation. These results indicate that repeated stimulation through TLR7 can induce a state of refractoriness in B cells characterized by severe defects in major signaling pathways.

Effect of combined BCR and TLR7 stimulation on TLR7 tolerance in B cells

Most of our understanding about TLR tolerance to date is from studies conducted using myeloid cells. However, an important difference between myeloid and B cells is the presence of a specific Ag receptor on the surface of the latter. Thus, it is possible that B cells may receive signals simultaneously through both the BCR and TLR. However, the effect of combined signaling through BCR and TLR7 on TLR7 tolerance is not known. To address this, B cells were stimulated either with R848 alone or with R848 and anti-IgM Ab for 24 h, after which cells were washed extensively and stimulated with R848 for another 24 h, and cell culture supernatants were tested for IL-6 production. Consistent with above findings, R848 stimulation induced high amounts of IL-6 production by naive B cells and markedly reduced IL-6 production in TLR7-tolerant B cells (Fig. 5A). Interestingly, B cells pretreated with both R848 and anti-IgM Ab produced markedly higher amounts of IL-6.
following subsequent R848 stimulation, suggesting that BCR signals can prevent TLR tolerance in B cells. Importantly, control IgG Ab treatment was not effective in preventing TLR7 tolerance. Additionally anti-IgM Ab stimulation alone did not induce any IL-6 production (data not shown).

Because TLR7 tolerance was associated with reduced MAPK activation in B cells, we next investigated whether combined BCR and TLR7 stimulation can ameliorate the impaired R848-mediated MAPK activation seen in TLR7-tolerant B cells (Fig. 5B). Combined BCR and TLR7 stimulation induced enhanced JNK phosphorylation in naïve B cells, and more importantly preserved R848-mediated JNK phosphorylation in prestimulated B cells. Simultaneous BCR ligation also rescued defective ERK1/2 and p38 phosphorylation in TLR7-tolerant B cells (data not shown). Furthermore, B cells prestimulated with R848 and anti-IgM Ab exhibited higher amounts of c-Jun phosphorylation compared with B cells prestimulated with R848 alone in response to subsequent R848 stimulation (Fig. 5, C and D). To our understanding, this is the first report of prevention of TLR7 tolerance by a physiological Ag receptor signal. Importantly, Ag signals are frequently delivered to B cells together with TLR signals during an infection or vaccination.

To determine whether TLR tolerance can be reversed if BCR signals are provided during the second stimulation, B cells were prestimulated with R848 for 24 h to induce TLR7 tolerance. After washing, cells were restimulated with R848 or R848 and anti-IgM Ab for another 24 h. B cells prestimulated with R848 produced significantly higher amounts of IL-6 in response to second R848 stimulation if simultaneously triggered through BCR compared with B cells restimulated with R848 and IgG control, showing that BCR signals cannot only prevent, but also rescue B cells from TLR7 tolerance (Fig. 6A). We also found that as little as 1 μg/ml anti-IgM can reverse TLR7 tolerance in B cells (supplemental Fig. 2). BCR signals also reversed defective JNK activation (Fig. 6B) and c-Jun phosphorylation (Fig. 6C) in TLR7-tolerant B cells.

Effect of repeated TLR7 stimulation on human peripheral blood B cells

Because human and mouse B cells can respond differentially to some TLR ligands, we investigated whether repeated stimulation with R848 also induced tolerance in human peripheral blood B cells, and whether BCR signals can rescue human B cells from TLR7 tolerance. To address this, CD19+ B cells isolated from the peripheral blood of healthy volunteers by negative selection were...
stimulated with R848 for 24 h. After extensive washing, cells were
stimulated either with R848 alone or with R848 and anti-IgM Ab
or R848 and IgG control. Human B cells prestimulated with me-
dium robustly produced IL-6 in response to subsequent R848
stimulation (Fig. 7). In contrast, IL-6 production was markedly reduced
in R848-prestimulated B cells when restimulated with R848. Thus,
as in the case of mouse B cells, repeated stimulation through TLR7
induced tolerance in human B cells. Importantly, B cells prestimulated through TLR7 exhibited a marked reduction
in IL-6 production in response to subsequent CpG stimulation,
suggesting the induction of heterotolerance as described in mac-
rophages. Importantly, BCR signals were able to reverse TLR7-
mediated hyporesponsiveness to TLR9 stimulation in B cells.
Because TLR7 stimulation has also been shown to affect subsequent
TLR4 responses in macrophages (26), we also tested the effect of
prestimulation through TLR7 upon subsequent restimulation
through TLR4 in B cells. However, IL-6 production in TLR4-
stimulated naive B cells was either very low or absent, and TLR7
prestimulation had no effect on subsequent TLR4-mediated IL-6
production (data not shown).

Role of PI3K in BCR-mediated reversal of TLR7 tolerance
The molecular basis for TLR7 tolerance induction and BCR-me-
diated reversal of TLR tolerance is not clear. It is possible that
Because B cells are known to express TLR9 and respond robustly
to CpG stimulation (2), we next investigated whether prestimu-
lation through TLR7 has any effect on B cell IL-6 production upon
subsequent restimulation through TLR9. Naive B cells stimulated
through TLR9 produced large amounts of IL-6 (Fig. 8). However,
B cells prestimulated through TLR7 exhibited a marked reduction
in IL-6 production in response to subsequent CpG stimulation,
suggesting the induction of heterotolerance as described in mac-
rophages. Importantly, BCR signals were able to reverse TLR7-
mediated hyporesponsiveness to TLR9 stimulation in B cells.
Because TLR7 stimulation has also been shown to affect subsequent
TLR4 responses in macrophages (26), we also tested the effect of
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through TLR4 in B cells. However, IL-6 production in TLR4-
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Role of PI3K in BCR-mediated reversal of TLR7 tolerance
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Effect of prestimulation through TLR7 on B cell IL-6 production
upon subsequent TLR9 stimulation
Prestimulation through TLR7 has also been shown to affect sub-
sequent responses to other TLR ligands in myeloid cells (25, 26).
initial TLR7 signaling may induce the expression of negative regulators shown to play roles in TLR tolerance induction (16) and BCR-mediated reversal of TLR tolerance could be through down-regulation of these negative regulators. Because induction of TLR7 tolerance requires >12 h, this suggests a potential role for newly synthesized proteins in development of tolerance. Unfortunately, various inhibitors of protein synthesis used to test this possibility were highly toxic to B cells over the period of time required to induce TLR7 tolerance (data not shown). We also investigated the expression of various negative regulators of TLR tolerance, such as MyD88s, IRAK-M, and SOCS-3 in tolerant B cells, and observed no induction of any of these molecules (data not shown). The expression of IRAK-4 did not differ between B cells prestimulated through TLR7 alone or through both TLR7 and BCR (data not shown).

PI3K is one of the key signaling molecules activated early during BCR signaling (27). PI3K activation has also been reported in myeloid cells in response to a variety of TLR ligands. Selective inhibition of PI3K increases TLR-mediated IL-12 production in dendritic cells and macrophages, suggesting a potential role for PI3K as a negative regulator of TLR-mediated inflammatory responses in these cells (28). However, the responses of B cells vs myeloid cells to various TLR stimuli do not always coincide. To address the role of PI3K in B cells, mouse high density resting B cells were stimulated with R848 for 24 h. After washing, cells were treated with different concentrations of the PI3K inhibitor LY294002 and then stimulated with R848 or R848 and anti-IgM Ab for time points indicated. As shown in Fig. 9A, treatment with PI3K inhibitor also blocked BCR-mediated reversal of ERK1/2, p38, and IκB-α phosphorylation and IκB-α degradation in TLR7-tolerant B cells (data not shown). Because BCR cross-linking can also trigger NF-κB signaling, we investigated whether BCR-mediated NF-κB activation plays a role in reversal of TLR7 tolerance. For this, TLR7-prestimulated B cells were stimulated with R848 and anti-IgM in the presence or absence of two different concentrations of an IκB inhibitor for 24 h. However, the concentrations of IκB inhibitor used did not block the anti-IgM-mediated reversal of IL-6 production in TLR7-tolerant B cells (supplemental Fig. 3).

Discussion

TLR tolerance is a well-characterized phenomenon in cells of myeloid lineage and is defined as a temporary hyporesponsiveness of immune cells to TLR ligands following repeated or chronic stimulation through the same or different TLRs (12). However, our understanding of TLR tolerance in B cells is fragmentary. In addition to the clonally rearranged Ag-specific receptor that they use to recognize, bind, and internalize specific Ags, B cells also express almost all known TLRs and respond robustly to many TLR ligands (2, 29–31). Accordingly, B cells can receive signals simultaneously through both BCR and TLRs, and thus may respond differently to TLR stimulation compared with myeloid cells. In this study, we tested this possibility by stimulating B cells repeatedly through TLR alone or through both TLR and BCR.

Consistent with previous reports (1, 20), naive B cells isolated from mouse spleen responded robustly to TLR7 stimulation by producing high amounts of IL-6, TNF-α, and the chemokine MIP1-α. However, prestimulation with R848 resulted in a drastic reduction in the ability of B cells to produce these cytokines upon subsequent restimulation through TLR7. Additionally, TLR7 tolerance also impacted B cell proliferative responses and their ability to secrete IgM Ab in response to subsequent R848 restimulation. This state of refractoriness was associated with reduced MAPK and NF-κB activation and c-Jun

**FIGURE 9.** Role of PI3K activation in BCR-mediated reversal of JNK activation in TLR7-tolerant B cells. Mouse high density resting B cells stimulated with R848 (1 μg/ml) for 24 h were washed and restimulated with R848 (1 μg/ml), and R848 and anti-IgM at 10 μg/ml for indicated time points. B cells incubated at 37°C for 1 h in medium and then stimulated with R848 for indicated times were used as controls. Cells were exposed to different concentrations of PI3K inhibitor, LY294002, or diluent control DMSO starting 60 min before addition of stimuli. Cells were lysed, and whole-cell extracts were subjected to Western blot analysis for phospho-JNK (A and B) or pAkt (C and D). Blots were stripped and reprobed for total JNK (A and B) or Akt (C and D). Results shown are representative of two comparable experiments.
phosphorylation. These findings are consistent with TLR7 tolerance reported in macrophages and in human B-chronic lymphocytic leukemia cells (23, 32). Reduced proinflammatory cytokine production associated with defective MAPK signaling and impaired activation of transcription factors NF-κB and AP-1 are also well-documented features of endotoxin-tolerant macrophages (13). However, B cell IL-10 production or costimulatory molecule expression was not altered in TLR7-tolerant B cells, which was again consistent with similar reports in TLR-tolerant macrophages and dendritic cells of selective inhibition of certain functions while preserving others (14, 15, 33).

IL-6 production in B cells following CD40 or combined TLR7 and CD40 stimulation was shown to be dependent on JNK activation and phosphorylation of the transcription factor c-Jun (20, 24). Additionally, the TNF-α promoter contains both AP-1 and NF-κB sites (34). Thus, defective activation of MAPK and transcription factors that play crucial roles in governing the expression of various cytokines could be contributing to the defective cytokine production in TLR7-tolerant B cells. Because both IL-6 and TNF-α play important roles in B cell differentiation, the reduced IgM production in TLR7-tolerant B cells could be due to defective IL-6 and TNF-α production by these cells (35).

Although the dampened B cell responses to repeated TLR7 stimulation could be a potential regulatory mechanism to control immunopathology, this may also make individuals more susceptible to infection, as reported in septic shock patients (36). Although macrophages and dendritic cells are the major cell types affected in endotoxin tolerance (37, 38), tolerance can be induced through other TLRs, and this may affect the functions of other cell types, especially B cells that express almost all known TLRs (39), although this has not been studied in detail. Triggering TLR7/8 responses by R848 or HIV ssRNA has been shown to prevent HIV replication in lymphoid tissues, and this anti-HIV activity was absent when B cells were depleted from lymphoid tissues (40), suggesting a potential role for B cells activated through TLR7 in preventing HIV replication. Furthermore, a recent study showed that both naive and memory B cells from HIV-infected individuals are defective in their proliferative responses to TLR9 stimulation (41). In this context, it is interesting to note that in our study, TLR9 response, as determined by IL-6 production, was also defective in TLR7-prestimulated B cells. Thus, it is possible that in HIV-infected individuals, repeated stimulation through TLR7 by HIV ssRNA could be making their B cells unresponsive to subsequent TLR9 stimulation. Cross-tolerance between various TLR ligands has been demonstrated in macrophages (10), and concurrent TLR7 signaling has been shown to negatively regulate TLR9-mediated IFN-α production by plasmacytoid dendritic cells (25, 33).

Although the TLR7 tolerance phenotype in B cells resembled that reported in macrophages, B cell responses were entirely different when TLR7 stimulation was combined with simultaneous BCR ligation. Although anti-IgM alone was not effective in inducing IL-6 production in naive or R848-prestimulated B cells, when BCR ligation was combined with R848 stimulation, it greatly enhanced IL-6 production in naive B cells and, more importantly, prevented and reversed TLR7 homotolerance and TLR7-mediated TLR9 tolerance in B cells. It is important to note that BCR stimulation reversed TLR7 tolerance in human as well as mouse B cells. Because human and mouse B cells are known to respond differently in some situations to certain TLR agonists, species-specific differences must be considered when interpreting TLR responses (42).

Prevention or reversal of endotoxin tolerance by certain cytokines has been demonstrated in human monocytes (43) and is thought to be mediated through modulating IRAK expression and its association with MyD88 in tolerant cells (44). However, type 1 IFNs were not effective in reversing TLR7 tolerance in B-chronic lymphocytic leukemia cells (32). Reversal of TLR tolerance by adaptive immune receptor signals has not been reported before. Interestingly, we found that reversal of TLR tolerance by signaling through an ITAM-containing receptor is not unique to B cells. Our preliminary results showed that TLR7 tolerance can be prevented or reversed in a macrophage cell line, RAW 264.7, by simultaneous cross-linking of FcγR (data not shown). We are currently investigating whether stimulation through FcγR can prevent endotoxin tolerance in macrophages.

Although it was known for several years that TLR7 can synergize with Ag-specific signals (1), how these two signaling pathways interact is not clear. To begin to address the molecular mechanisms involved in BCR-mediated reversal of tolerance, we investigated the role of PI3K, a key signaling molecule activated following BCR engagement that is also thought to be involved in TLR signaling (45, 46). Engagement of Ag receptor has been shown to initiate a multitude of signaling pathways (47), and PI3K activation is one of the earliest events (48, 49). PI3K may function as an important initiator of downstream pathways through recruitment to the membrane of a number of pleckstrin homology domain-containing signaling molecules (50, 51). The PI3K/Akt pathway has also been shown to play an important role in TLR3-mediated IRF-3 phosphorylation and activation (52). Additionally, B cells deficient in PI3K subunits have been shown to exhibit reduced proliferation following LPS stimulation (53). Our findings indicate that although PI3K activation was not required for R848-mediated JNK activation in naive B cells, PI3K activation was essential for BCR-mediated reversal of TLR7 tolerance. Although PI3K has been shown to play a role in BCR-mediated ERK phosphorylation and activation (54), its role in JNK activation following BCR cross-linking is not clear. It is possible that PI3K may interact with signaling molecules upstream of JNK that are initiated following triggering through TLR7. Interestingly, PI3K has been shown to interact with MyD88 following LPS stimulation in macrophages and is shown to play a role in LPS-mediated IL-1β production (55). Moreover, anti-IgM stimulation has been shown to increase MyD88 protein and gene expression in B cells (56). Although none of the chemical inhibitors are absolutely specific, treatment of B cells with LY294002 completely inhibited anti-IgM or R848-mediated phosphorylation of Akt in R848 or R848 and anti-IgM-stimulated B cells. Additionally, the drastic difference in JNK activation between control and LY294002-treated cells further argues for a potential role for this molecule in BCR-mediated reversal of TLR tolerance.

It has been shown that CpG-mediated TLR9 signals can synergize with BCR signals in enhancing p38 and JNK phosphorylation and NF-κB activation (57) and that the BCR recruits TLR9-containing endosomes to autophagosomes, where hyperactivation of MAPKs occurs (58). Interestingly, the recruitment of TLR9 to autophagosome-like compartments was not observed in B cells stimulated in the presence of PI3K inhibitors (58). In the study presented in this work, we have also observed enhanced JNK, p38, and ERK1/2 phosphorylation in B cells stimulated through both R848 and anti-IgM. Similar to TLR9, TLR7 is localized to endosomes (59), and it is possible that BCR stimulation may recruit TLR7 to autophagosomes. We are currently investigating whether BCR cross-linking recruits TLR7 to autophagosomes and whether signaling from different intracellular compartments in R848 vs
R848 and anti-IgM-stimulated cells could explain the reversal of tolerance observed in BCR-stimulated cells.

In conclusion, we present a novel mechanism to prevent or reverse TLR tolerance in mouse or human B cells. Identifying the molecular mechanisms involved in B cell-intrinsic TLR responses and how they are modulated in the context of simultaneous signaling through adaptive immune receptors could greatly improve our ability to develop better vaccines. Because BCR-TRL7 synergy has also been shown to play a role in autoimmune disorders, a better understanding of the interaction between these two receptors as well as the regulation of their responses will be helpful when identifying potential therapeutic targets.

Disclosures

The authors have no financial conflict of interest.

References