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Type I IFN Receptor and the B Cell Antigen Receptor Regulate TLR7 Responses via Distinct Molecular Mechanisms

Jayakumar S. Poovassery* and Gail A. Bishop*,†,‡

Toll-like receptor 7 (TLR7) signals to B cells are critically involved in the innate immune response to microbes, as well as pathogenesis of autoimmune diseases, but the molecular mechanisms that normally regulate these responses are incompletely understood. We previously reported that repeated stimulation through TLR7 induces a state of hyporesponsiveness (TLR tolerance) in both human and mouse B cells, characterized by marked inhibition of particular signaling pathways. BCR signals prevent and overcome TLR7 tolerance. Because optimal responses to TLR7 in B cells require type I IFN, we investigated whether BCR-mediated effects on TLR7 tolerance are mediated by type I IFN receptor (IFNAR) signals. Surprisingly, although BCR-mediated reversal of TLR7 tolerance was IFNAR independent, IFNAR signals alone also blocked TLR7 tolerance, despite enhancing TLR7 expression. Both BCR and IFNAR signals restored the phosphorylation of the transcriptional regulator c-Jun, but only BCR signals blocked the tolerance-mediated inhibition of JNK. Both BCR and IFNAR-mediated regulation was dependent on activation of the PI3K/Akt/mammalian target of rapamycin signaling pathway, indicating a central role for this axis in integrating TLR7, BCR, and IFNAR signals in B cells. These new findings reveal distinct and overlapping signaling mechanisms used by BCR and IFNAR in the regulation of TLR7 tolerance and activation. *The Journal of Immunology, 2012, 189: 000–000.

Well-coordinated and tightly regulated signaling through innate and adaptive immune receptors is central to the development of an effective immune response, but dysregulated signaling can lead to excessive inflammation and immunopathology (1, 2). Pattern recognition receptors, including those belonging to the TLR family, are key players in the initiation of innate immune responses (3). TLR-expressing cells of the myeloid lineage, including dendritic cells (DCs) and macrophages, play central roles in innate immunity. However, B cells, the only cell type capable of producing Ab, also express a variety of TLRs (4). B cells produce key inflammatory cytokines such as TNF-α and IL-6 in response to TLRs, and are especially responsive to TLR7 and TLR9 (5-7). TLR stimulation also promotes Ig class-switch recombination and Ab secretion (8). Both Ab-dependent and -independent TLR-stimulated B cell functions play crucial roles in protection against infectious pathogens (9).

TLR signals also interact with signals delivered through a variety of B cell surface receptors. TLR7 signals synergize with BCR and CD40 signals to promote B cell Ag presentation, via upregulation of costimulatory molecules and cytokine production (10). Recent studies reveal a critical role for type I IFN receptor (IFNAR) signals in regulating TLR7-mediated B cell responses (11). In humans, plasmacytoid DC-derived type I IFN is required for TLR7-mediated proliferation of naïve B cells. Type I IFN also enhances memory B cell responsiveness to TLR7-mediated plasmablast differentiation (12, 13). In addition, TLR7-mediated B cell proliferation, cytokine production, and costimulatory molecule expression are defective in IFNAR−/− mice (14). Many synthetic TLR7 ligands are currently under investigation as vaccine adjuvants (15) and antitumor agents (16), and type I IFN also enhances adjuvant activity of such ligands (17).

Although TLR and IFNAR signals play important roles in immune protection, they are also associated with exacerbation of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (18). Both TLR7 and TLR9 are critically involved in recognition of RNA- and DNA-associated autoantigens, respectively, and cooperate with BCR signals in the development of autoantibodies against endogenous nuclear autoantigens (19). In addition, both TLR7 and TLR9 agonists are strong inducers of type I IFN production (20), and IFN-stimulated gene expression in the peripheral blood cells of SLE patients correlates with autoantibodies and disease severity (21). TLR7 is also implicated in the pathogenesis of SLE in several mouse models (22, 23). However, the molecular mechanisms involved in the interactions between TLR7 and IFNAR signals are not understood.

An important regulatory control of TLR responses is TLR tolerance, a temporary hyporesponsiveness of immune cells to repeated stimulation through the same or different TLRs (24). We showed that TLR tolerance is induced in mouse and human B cells by ligands to TLR7 and TLR9 (25). TLR7 tolerance in B cells is characterized by severe defects in MAPK and NF-κB activation, and associated reduction in cytokine and Ab production. TLR7 tolerance can be prevented and overcome by providing prior or simultaneous signals through the BCR (25). Because certain B cell TLR7 responses are regulated by IFNAR signals (12, 14), we investigated the role of IFNAR-mediated regulation of TLR7...
tolerance in B cells. We found that BCR-mediated signals that mitigate against TLR7 tolerance were not dependent on type I IFN. However, IFNAR signals themselves blocked TLR7 tolerance in both mouse and human B cells. Notable differences, as well as important converging pathways, contributed to the strategies used by BCR and IFNAR in regulating TLR7-mediated B cell activation and tolerance.

Materials and Methods

Cells and reagents

Resting high-density splenic B cells were isolated from C57BL/6 mice, as described previously (25). Spleen B cell purity determined by CD19 staining was >90%. Use of normal mouse cells in this study followed a protocol approved by the University of Iowa Animal Care and Use Committee. Normal human PBMCs were isolated by Ficoll-Hypaque density gradient sedimentation from discarded leukocyte reduction system cones obtained from DeGowin Blood Center, University of Iowa (Iowa City, IA). The Blood Center has University of Iowa Institutional Review Board approval to provide these unidentified samples to investigators. B cells were purified from PBMC suspensions by negative selection using a B cell isolation kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer’s instructions. B cell purity as determined by CD19 staining was >95%. Cells were cultured in RPMI 1640 medium with 10% heat-inactivated FCS (Atlanta Biologicals, Atlanta, GA), 10 μM 2-ME (Invitrogen, Grand Island, NY), 2 mM L-glutamine, penicillin, and streptomycin (BCM-10). Polyclonal rabbit Abs against phosphorylated and native forms of the kinases pJNK (catalog no. 9251), p38 (catalog no. 9215 and 9212), p70S6K (catalog no. 9205 and 2708), the JNK substrate c-Jun phosphorylated at serine 73 (catalog no. 9164), and MyD88 (catalog no. 4283) were from Cell Signaling Technology (Danvers, MA). Rabbit anti-JNK1/2 Ab (catalog no. sc-571) was from Santa Cruz Bio-technology (Santa Cruz, CA). Rabbit anti- p-Akt (Ser473) Ab (catalog no. 46-621G) was from Invitrogen. Mouse anti-actin Ab (catalog no. MAB1501) was purchased from Millipore (Billerica, MA). Affinity-purified Fab(α′)2 goat anti-mouse and anti-human IgM (μ-chain–specific) Ab and peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG Abs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). PI3K inhibitor LY294002, 38 PAK inhibitor SB203580, MEK inhibitor U0126, and the mammalian target of rapamycin (mTOR) inhibitor rapamycin were obtained from Calbiochem (Philadelphia, PA). Anti-mouse and anti-human IL-6 (MP5-20F3 and MP5-52C11) biotin-conjugated and MQ-13A5 and MQ293C3 biotin-conjugated), anti-mouse TNF-α (1F4/74 and 1F4/72) biotin-conjugated, anti-mouse IL-10 mAbs (JES5-16A3 and JES5-2A5 biotin-conjugated), and anti-TLR7 Abs were purchased from eBioscience (San Diego, CA) for ELISAs. Recombinant mouse IL-6 (rmIL-6), rmTNF-α, rmIFN-γ, rmIL-2, rmIL-12 (p70), and rmIL-10 were purchased from PeproTech (Rocky Hill, NJ). Streptavidin-HRP was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). ELISA tetramethylbenzidine peroxidase substrate was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Synthetic CpG-containing oligonucleotide 1826 (TCCATGACGTTCCTGACGGT) was obtained from Integrated DNA Technologies (Coraville, IA). The TLR7 agonist R848 was purchased from Alexa Biosciences/Enzo Life Sciences (Farmingdale, NY). Recombinant human and mouse IFN-α (rhIFN-α and rmIFN-α, respectively), rhIFN-β, and rmIFN-β were from PBL IFN Technologies (Piscataway, NJ) or from Cell Sciences (Canton, MA). Abs specific for IFN-α and IFN-β were from HyCult (Plymouth Meeting, PA).

B cell stimulation

Mouse high-density splenic B cells or human peripheral blood B cells (1 × 10^7/ml in 24- or 48-well tissue culture plates in a total volume of 1 or 0.5 ml/well) were stimulated with R848 for 24 h. R848 was used at a concentration of 1 μg/ml, unless otherwise noted. To induce TLR7 hyporesponsiveness, we washed and restimulated cells with R848 for another 24 h. Cell culture supernatants were collected and stored at −20°C until used to determine cytokine production by ELISA as described later. Unstimulated B cells were incubated for 1 h (mouse splenic B cells) or for 24 h (human peripheral blood B cells) in BCM-10, then stimulated with R848 for 24 h. Mouse high-density resting splenic B cells prestimulated with R848 and rmIFN-α or rmIFN-β (300 IU/ml) or with goat anti-mouse IgM Ab (10 μg/ml) for 24 h were used to determine the effect of dual-receptor stimulation on tolerance induction. In these experiments, after 24 h of stimulation, cells were washed and restimulated with R848 alone for another 24 h. B cells initially stimulated with R848 alone for 24 h, to induce tolerance, were washed and restimulated with R848 alone or with R848 and rmIFN-α or rmIFN-β for 24 h to determine the effect of type I IFNs on reversal of B cell TLR7 tolerance.

Western blots

Western blotting was performed as described previously (25). In brief, mouse splenic B cells (2 × 10^6) stimulated with R848 for 24 h were washed and restimulated for indicated time points with R848 in the presence or absence of rmIFN-β (1000 IU/ml). Control B cells were stimulated with R848 or rmIFN-β for times indicated. Inhibitor treatments were performed as indicated in the figures. Cells were treated as indicated in the figures. Bands were visualized on Western blots using a chemiluminescent detection reagent (Pierce, Rockford, IL). Images of blots were recorded with a low-light imaging system (LAS3000; Fujifilm Medical Systems Stamford, CT). Immunoblots were stripped and reprobed with Abs to control proteins to verify equal protein loading in each lane.

ELISAs for cytokine detection

Mouse or human primary B cells (1 × 10^7/ml) were stimulated as described in the figures. Supernatants were collected and stored at −20°C. Cytokine levels in the culture supernatants were measured by quantitative sandwich ELISA, using mAbs described earlier, following the manufacturer’s instructions. After development of enzyme reactions, optical densities of microwells were read at 450 nm by a SpectraMax 250 Reader (Molecular Devices, Sunnyvale, CA). Data were analyzed with SoftMax Pro software (Molecular Devices).

Statistical analysis

Statistical significance of differences in mean values of triplicate samples was determined by Student t test (GraphPad Prism, San Diego CA). The p values are indicated in the figures above bar graphs by asterisks: *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.0001. Data are presented as mean ± SEM of replicate samples.

Results

Effect of type I IFN on TLR7/TLR9 tolerance in mouse and human B cells

We showed previously that repeated stimulation through TLR7 induces hyporesponsiveness to subsequent stimulation through TLR7 or TLR9 in mouse and human B cells, a state called TLR7 tolerance (25). Simultaneous signals through the BCR and TLR7 prevent and reverse TLR7 tolerance (25). B cells coordinate signals from a variety of receptors essential for appropriate regulation of B cell responses (26). TLR7-mediated proliferation and IL-6 production by both human and murine B cells are dependent on signals through the IFNAR (12, 14). Because BCR signals restore optimum B cell activation in TLR7-tolerant B cells (25), we hypothesized that TLR7 tolerance may reflect defective type I IFN production, and BCR-mediated reversal of TLR7 tolerance may act by restoring IFNAR signaling in tolerant B cells. Although TLR7 stimulation induces type I IFN pathway activation in B cells, the small amount of type I IFN protein directly produced by B cells is difficult to measure reliably (27). Thus, to address the potential role of type I IFN in BCR-mediated regulation of the TLR7 response, we first examined whether exogenously added type I IFNs prevented TLR7 tolerance in B cells.

Consistent with our previous findings (25), resting B cells robustly produced IL-6 in response to primary TLR7 stimulation, but B cells prestimulated with the TLR7 ligand R848 produced markedly reduced amounts of IL-6 in response to subsequent restimulation (TLR7 tolerance). Interestingly, B cells prestimulated with R848 in the presence of either IFN-β (Fig. 1A) or IFN-α (Supplemental Fig. 1A) produced IL-6 in amounts comparable with B cells stimulated with R848 alone, demonstrating that exogenous type I IFN prevented TLR7 tolerance in B cells. To determine whether type I IFN also reversed TLR7 tolerance, we first
stimulated B cells with R848 alone for 24 h to induce tolerance. Cells were then restimulated with R848 in the presence or absence of IFN-α or IFN-β. Again, TLR7-tolerant B cells restimulated in the presence of IFN-β (Fig. 1B) or IFN-α (Supplementary Fig. 1B) produced significantly higher amounts of IL-6 compared with B cells restimulated with R848 alone. Importantly, IFN-α or IFN-β alone did not induce B cell IL-6 production. We conclude that direct signals through the B cell IFNAR both prevented and reversed TLR7 tolerance.

Because TLR responses can vary between mouse and human B cells (26), it was important to investigate whether IFNAR-mediated reversal of TLR7 tolerance is also applicable to human B cells. Human peripheral blood CD19+ B cells were stimulated with R848 for 24 h, then washed and restimulated with R848 for 24 h; both stimulations were performed in the presence or absence of rhIFN-β. IFN-β prevented (Fig. 1C) and reversed (Fig. 1D) impaired IL-6 production in TLR7-tolerant B cells, demonstrating that the IFNAR regulates TLR7 tolerance similarly in mouse and human B cells.

Prestimulation through TLR7 also induces hypersensitiveness to subsequent stimulation through TLR9 (TLR7/9 heterotolerance) in B cells (25). IFNAR signals also prevented (Supplemental Fig. 1C) and reversed (Supplemental Fig. 1D) TLR7/9 heterotolerance. It has been shown that IFN-γ can prevent endotoxin tolerance in macrophages (28). Consistent with its effects on macrophages, IFN-γ restored IL-6 production in TLR7-tolerant B cells (Fig. 2A). However, other cytokines such as IL-2 and IL-12 failed to reverse TLR7 tolerance in B cells (Supplemental Fig. 2), suggesting that not all cytokines can reverse TLR7 tolerance in B cells. To determine the duration of TLR7 tolerance in B cells, we prestimulated splenic B cells with R848 for increasing times. Cells were then washed and restimulated with R848 for another 24 h, and cell culture supernatants were tested for cytokine production by ELISA. B cells prestimulated with R848 for as long as 120 h also exhibited markedly reduced cytokine production in response to subsequent restimulation, suggesting that TLR7 tolerance lasts for >5 d in B cells (Supplemental Fig. 3A).

Independence of IFNAR and BCR-mediated reversal of TLR7 tolerance

Because IFNAR signals restored optimum B cell TLR7 responses in tolerant B cells, we considered the possibility that TLR7 tolerance is a consequence of defective B cell type I IFN production and/or IFNAR signaling. As discussed earlier, direct measurement of B cell-produced IFN protein is problematic, although TLR7 clearly induces IFNAR-mediated signaling pathways in B cells (27). Thus, to address the hypothesis that BCR signals reverse TLR7 tolerance by restoring type I IFN production, we performed experiments in which BCR signals were delivered in the presence or absence of IFNAR blocking Ab for 24 h.

Consistent with our previous report (25), BCR signals reversed TLR7 tolerance in B cells (Fig. 2A), but restoration of IL-6 production by the BCR signal was undiminished in the presence of anti-IFNAR blocking Ab, demonstrating that BCR-mediated regulation of TLR7 tolerance was independent of IFNAR signaling. The effectiveness of the IFNAR blocking Ab in abrogating direct IFNAR-mediated reversal of TLR7 tolerance was validated by data presented in Fig. 2B. An anti–IFN-α Ab also blocked IFNAR-mediated reversal of TLR7 tolerance (Supplementary Fig. 3B). IFNAR signals did not reverse TLR7 tolerance by upregulating ILR7 expression, as this was actually increased by R848 stimulation, as was MyD88 expression (Fig. 3).

The finding that IFNAR signals directly prevented and reversed B cell TLR7 tolerance via a mechanism distinct from that used by the BCR prompted us to investigate how signals through TLR7 interact with IFNAR signals, and to compare the signaling path-
inhibitors. TLR-tolerant B cells restimulated with IFN-γ-inhibitor (10 μM; SB203580) (B) or ERK1/2 inhibitor (20 μM; U0126) (C) were restimulated with R in the presence or absence of rmIFN-β for 24 h. Cell culture supernatants were tested for cytokine production by ELISA. Results are representative of two independent experiments. Student t test. *p ≤ 0.05, **p ≤ 0.001, ***p < 0.0001.

FIGURE 4. Role of the PI3K pathway in IFNAR-mediated regulation of TLR tolerance

BCR-mediated reversal of TLR tolerance in B cells is dependent on activation of PI3K and its downstream pathways (25). In addition to activating the p38 MAPK pathway, signals through IFNAR also activate PI3K/Akt signaling pathways (32). To determine whether IFNAR-mediated regulation of TLR7 tolerance is dependent on PI3K activation, we restimulated tolerant B cells with R848 alone or with R848 and IFN-β in the presence or absence of LY294002, a specific PI3K inhibitor. LY294002 almost completely blocked IFN-β-mediated restoration of IL-6 production in both human and mouse B cells (Fig. 6A, 6B). The observed reduction in IL-6 production was not due to nonspecific toxic effects of the inhibitor on B cells, as IL-10 production was either intact or only slightly reduced (Supplemental Fig. 4A). Consistent with our previous findings (25), LY294002 treatment also blocked BCR-mediated reversal of IL-6 production by both human and mouse B cells (Fig. 6C, 6D). Because no inhibitor is absolutely specific, to further confirm the role of the PI3K pathway in IFNAR-mediated regulation of TLR7 tolerance, we examined the effect of IFN-β on phosphorylation of Akt, a substrate of PI3K, in tolerant B cells. As shown previously (25), phosphorylation of Akt was greatly reduced in tolerant B cells (Fig. 7). Importantly, IFN-β reversed the

Differential induction of proinflammatory and anti-inflammatory cytokines by BCR and IFNAR signals in TLR7-tolerant B cells

The production of cytokines is one of the most important roles of TLR7-mediated activation of immune cells (26). We thus investigated whether regulation of TLR7 tolerance via BCR versus IFNAR had similar or distinct impacts on the production of proinflammatory and anti-inflammatory cytokines. Although both BCR and IFNAR signals restored TNF-α production by tolerant B cells, BCR was a much more efficient stimulus for TNF-α restoration than was IFNAR (Fig. 5A). Conversely, IFNAR signals induced much higher amounts of IL-10 in tolerant B cells, compared with BCR signals (Fig. 5B), which may be contributing to the reduced TNF-α production by type I IFN-stimulated cells. Interestingly, the BCR and IFNAR showed similar ability to restore IL-6 production in tolerant B cells (Fig. 5C).

IFNAR regulation of TLR7-mediated MAPK activation

Signals through the IFNAR activate JAK/STAT (29) and MAPK signaling pathways, particularly the MAPKs p38 (30) and ERK1/2 (31). MAPK activation is greatly reduced in TLR-tolerant B cells (Fig. 4A) (25), and IL-6 production in response to TLR7 is dependent on pathways downstream of MAPKs. B cells restimulated with R848 in the presence of IFN-β exhibited markedly higher amounts of p38 and ERK1/2 phosphorylation compared with tolerant B cells (Fig. 4A). But in contrast with BCR-mediated regulation of TLR7/9 tolerance (25), IFNAR did not reverse the reduced JNK phosphorylation observed in tolerant B cells. Phosphorylation of p38, ERK1/2, and JNK was either minimal or undetectable in B cells stimulated through IFNAR alone. To directly test whether IFNAR-mediated reversal of IL-6 production was dependent on MAPK activation, we restimulated tolerant B cells with IFN-β in the presence or absence of various MAPK inhibitors. TLR-tolerant B cells restimulated with IFN-β in the presence of a p38 inhibitor exhibited a significant reduction in IL-6 production (Fig. 4B), indicating that p38 MAPK activation was required for IFNAR-mediated restoration of IL-6 production. Although an ERK1/2 inhibitor also blocked this tolerance reversal, it was less efficient compared with p38 inhibition (Fig. 4C). Thus, p38 and ERK1/2 may be downstream of IFNAR regulation of TLR7-mediated MAPK activation.
impaired phosphorylation of Akt in tolerant B cells (Fig. 7). These results highlight an important role for the PI3K signaling pathway in IFNAR-mediated reversal of TLR7 tolerance in B cells and a feature of TLR tolerance regulation that is shared with the BCR.

To further delineate the molecular mechanisms involved in PI3K-dependent reversal of TLR7 tolerance in B cells, we examined the effect of simultaneous signals through IFNAR on signaling molecules activated downstream of PI3K in B cells. mTOR is an important IFNAR-activated PI3K downstream signaling molecule (32), but its role in B cell activation after TLR7 stimulation is not known. TLR7-tolerant B cells were restimulated with R848 alone or with R848 + IFN-β in the presence or absence of rapamycin, an mTOR inhibitor. IFN-β-mediated reversal of IL-6 production was blocked by rapamycin (Fig. 6E), supporting a role for mTOR in IFNAR-mediated reversal of inhibited IL-6 production. Importantly, TLR7 stimulation induced phosphorylation of p70S6K, a downstream target of mTOR, and phosphorylation of p70S6K was almost completely absent in tolerant B cells. Restimulation with R848 in the presence of IFN-β restored phosphorylation of this signaling molecule (Fig. 6F). BCR-mediated reversal of IL-6 production was also blocked in the presence of rapamycin (Supplemental Fig. 4B), further supporting the importance of this signaling pathway in both BCR and IFNAR-mediated reversal of TLR7 tolerance.

### Relationship between MAPK and PI3K pathways in IFNAR-mediated effects on TLR tolerance

Activation of the MAPKs p38 and ERK1/2 was required for IFNAR-mediated reversal of inhibited IL-6 production (Fig. 4A–C). We thus addressed the hypothesis that PI3K functions to restore IL-6 production in TLR7-tolerant B cells through altering MAPK activation. TLR7-tolerant B cells were restimulated with R848 alone or with R848 and IFN-β in the presence or absence of LY294002, or rapamycin for indicated times. Cells were lysed and whole-cell extracts were subjected to Western blot analysis for p-p70S6K. Naïve B cells stimulated with R alone for 24 h to induce tolerance. After washing, cells were pretreated with rapamycin for 3 h, then stimulated with R alone or with R + IFN-β for indicated time points. Cells were lyzed and whole-cell extracts were subjected to Western blot analysis for p-p70S6K. Naïve B cells stimulated with R alone for indicated time points were used as controls. Blots were stripped and reprobed for actin. Results are representative of two comparable experiments. Statistical analysis was conducted using Student t test. *p ≤ 0.05, **p < 0.001, ***p ≤ 0.0001.

FIGURE 5. Differential effects of BCR and IFNAR signals on cytokine production by TLR7-tolerant B cells. Mouse high-density splenic B cells were stimulated with R848 (R) for 24 h, washed, and restimulated with R in the presence or absence of rmIFN-β or anti-IgM Ab for 24 h. Cell culture supernatants were tested for TNF-α (A), IL-10 (B), and IL-6 (C) by ELISA. Results are representative of two independent experiments. Statistical analysis was conducted using Student t test. **p ≤ 0.001, ***p ≤ 0.0001.

FIGURE 6. Roles of PI3K and mTOR regulation of cytokine production in mouse and human TLR7-tolerant B cells. B cells from human peripheral blood (A, C) or mouse spleen (B, D) were stimulated with R848 (R) for 24 h to induce TLR7 tolerance. Tolerant B cells were incubated with the PI3K inhibitor (2 μM), LY294002 (LY), or DMSO control for 1 h and restimulated with R in the presence or absence of species-specific rIFN-β (A, C) or anti-IgM Ab (B, D) for 24 h. IL-6 in cell culture supernatants was assayed by ELISA. Results are representative of experiments performed with B cells from four different human donors or (for mouse B cells) of two independent experiments. (E) Mouse B cells were stimulated with R for 24 h to induce TLR7 tolerance. Tolerant B cells were incubated with the mTOR inhibitor (100 nM), rapamycin (Rap), or DMSO for 3 h and restimulated with R in the presence or absence of rmIFN-β for 24 h. IL-6 production in culture supernatants was assayed by ELISA. (F) Mouse B cells were stimulated with R for 24 h to induce tolerance. After washing, cells were pretreated with rapamycin or DMSO for 3 h, then stimulated with R alone or with R + rmIFN-β for indicated time points. Cells were lyzed and whole-cell extracts were subjected to Western blot analysis for p-p70S6K. Naïve B cells stimulated with R alone for indicated time points were used as controls. Blots were stripped and reprobed for actin. Results are representative of two independent experiments. Statistical analysis was conducted using Student t test. *p ≤ 0.05, **p < 0.001, ***p ≤ 0.0001.
establishing that PI3K is activated upstream of both Akt and mTOR in TLR7-stimulated B cells. As shown in Fig. 4A, IFN-β was not effective in reversing JNK phosphorylation defects in tolerant B cells. However, both LY294002 and rapamycin blocked the cells’ residual JNK phosphorylation.

Although PI3K inhibitor blocked ERK activation, PI3K and mTOR inhibitors were not effective in blocking IFNAR-mediated restoration of p38 phosphorylation in TLR7-tolerant B cells. IFNAR-mediated effects on c-Jun phosphorylation were greatly reduced in the presence of rapamycin and LY294002, indicating that an important mechanism by which the PI3K/Akt/mTOR pathway mediates downstream restoration of IL-6 production in TLR7-tolerant B cells is through activation of c-Jun. Thus, IFNAR signals reversed impaired c-Jun phosphorylation in TLR7-tolerant B cells. IFNAR-mediated effects on c-Jun phosphorylation were greatly reduced in the presence of rapamycin and LY294002, indicating that an important mechanism by which the PI3K/Akt/mTOR pathway mediates downstream restoration of IL-6 production in TLR7-tolerant B cells is through activation of c-Jun. These results are consistent with our previous findings that IL-6 production in B cells is dependent on AP-1 activation (10, 33), and indicate that in contrast with BCR-mediated effects, the IFNAR contributes to c-Jun phosphorylation in a JNK-independent manner.

Discussion

Many B cell functions are modulated by costimulatory signals received through cytokine receptors (34). It has been shown that IFNAR signals play important roles in regulating TLR7 responses in both human and mouse B cells, contributing to the roles played by TLR7 in both normal immunity and autoimmunity (12–14). In this study, we demonstrated a novel role for the IFNAR in regulating TLR7 responses in B cells. IFNAR signals prevented and reversed B cell TLR7 tolerance, a property shared with BCR signals (25). However, there were both interesting similarities and striking differences in the molecular mechanisms by which the BCR and IFNAR mediated regulation of TLR tolerance; these are summarized in Fig. 8B.

A number of mechanisms are thought to be involved in negative regulation of TLR signaling, including downregulation of surface

![FIGURE 7](image-url) Relationship between MAPK and PI3K pathways in IFNAR-mediated effects on TLR tolerance. Mouse B cells stimulated with R848 for 24 h were washed and incubated in the presence or absence of LY294002 (20 μM) for 1 h or rapamycin (500 nM) for 3 h. Cells were then stimulated with R848 in the presence or absence of mIFN-β for indicated time points. Naive B cells stimulated with R848 alone were used as controls. Cells were lysed and whole-cell extracts were subjected to Western blot analysis for pERK1/2, p-p38, pJNK, pAKT, and p-p70S6K. Blots were stripped and reprobed for ERK1/2, p38, JNK, AKT, and p70S6K. Results are representative of two comparable experiments.

![FIGURE 8](image-url) A Schematic representation of distinct and overlapping signaling pathways used by BCR and IFNAR signals to regulate B cell TLR7 tolerance. TLR7 tolerance is associated with defective MAPK and PI3K/mTOR activation, and defective c-Jun phosphorylation. BCR signals restore impaired JNK, p38, and ERK1/2 activation in tolerant B cells. However, IFNAR signals are effective in restoring only p38 and ERK1/2 activation, indicating that other kinases are involved in c-Jun activation. Both BCR and IFNAR signals restore activation of the PI3K/Akt/mTOR signaling pathway, c-Jun activation, and cytokine production.
TLR expression (35). In B cells, IFNAR signals are shown to induce increased TLR7 expression (14). However, our findings show that TLR7 expression was not decreased in tolerant B cells. In addition, not all B cell functions were defective in TLR7 prestimulated B cells. IL-10 production in response to subsequent R848 stimulation was unimpaired in tolerant B cells. Consistent with previous findings (14), IFN-β either alone or in combination with R848 induced increased TLR7 expression in B cells. Interestingly, B cells restimulated with R848 and IFN-β exhibited a sustained high level of TLR7 expression, which may contribute to the enhanced responses observed in dual-stimulated “rescued” B cells.

One of the earliest events initiated after TLR7 ligation in B cells is MAPK activation, and TLR7 tolerance in B cells is associated with marked reduction in JNK, p38, and ERK activation (25). One of the notable differences between BCR and IFNAR-mediated reversal of TLR tolerance was their differential effects on MAPK activation. Although BCR signals restored JNK activation in tolerant B cells, IFNAR signals failed to do so. However, both BCR and IFNAR signals restored tolerance-associated impaired phosphorylation of the JNK substrate, c-Jun. Thus, BCR and IFNAR signaling pathways use distinct mechanisms to converge at this important step in the tolerance-abrogating pathway.

Both p38 and ERK can also phosphorylate c-Jun (36), and IFNAR signals restored p38 and ERK activation in tolerant B cells. Although the biological effects associated with type I IFN were originally thought to be mediated solely through the classical JAK/STAT pathway (29), it is now clear that many other signaling cascades, including MAPK and PI3K/Akt pathways, are activated either independently or in parallel after IFNAR ligation, and cooperation between pathways is essential for complete transcriptional activation of target genes (32). Both pharmacological inhibition and gene ablation studies show a critical role for p38 pathways in transcriptional regulation of type I IFN-stimulated genes (37, 38). The MEK/ERK pathway is also activated downstream of IFNAR signals (39), and type I IFN-induced AP-1 activation in primary human microglia requires ERK activation (39). Although IFNAR-mediated ERK phosphorylation was not detectable in naïve B cells, IFNAR signals restored impaired TLR7-mediated ERK phosphorylation in tolerant B cells. Interestingly, only ERK1/2, but not p38, activation downstream of IFNAR signals in tolerant B cells was dependent on PI3K activation (Fig. 7). Thus, LY294002-mediated blockade of c-Jun phosphorylation observed in B cells restimulated through IFNAR and TLR7 (Fig. 8A) may be partly mediated through inhibiting the effects of ERK1/2 on c-Jun. However, inhibition of c-Jun phosphorylation was also observed in the presence of rapamycin (Fig. 8A), which was also observed in the presence of rapamycin (Fig. 8A), which makes B cells hyporesponsive to subsequent TLR9 stimulation.

Type I IFN has potent immunomodulatory effects on a variety of cell types, and its production is normally tightly regulated. Repeated stimulation through TLR7 limits type I IFN production by plasmacytoid DCs (20). Thus, TLR7 tolerance may restrain B cell innate immune responses and prevent immunopathology associated with uncontrolled proinflammatory cytokine production. However, in an autoimmune setting, type I IFN produced by DCs in response to autoantigen-containing immune complexes may prevent or reverse tolerance induction, and promote autoimmunity (18, 23). Conversely, tolerance induction upon repeated administration of TLR ligands may contribute to the limitation of success obtained by systemic use of TLR7 ligands as antitumor agents (52). Identification of novel methods to regulate tolerance could assist in the development of improved therapeutic strategies to treat cancer and autoimmune diseases. A better understanding of the signaling pathways involved in regulation of tolerance would be helpful in developing small-molecule drugs that could be used to target key signaling molecules involved in autoimmune disease development.

We demonstrated a novel role for type I IFN in regulating TLR7 responses in B cells. IFNAR signals both prevented and reversed TLR7 tolerance. However, clear and interesting similarities and differences between BCR and IFNAR-mediated effects (Fig. 7B) indicate that the regulation of TLR responses by additional acti-
vating signals is multifaceted and complex. Understanding the overarching and distinct mechanisms by which TLR tolerance is regulated and at the molecular and functional levels can enhance the ability to manipulate TLR responses.

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Disclosures

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