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Nucleotide receptor P2RX$_7$ stimulation enhances LPS-induced interferon-β production in murine macrophages

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ABSTRACT

Stimulation of P2RX$_7$ with extracellular ATP potentiates numerous LPS-induced proinflammatory events, including cytokine induction in macrophages, but the molecular mechanisms underlying this process are not well defined. Although P2RX$_7$ ligation has been proposed to activate several transcription factors, many of the LPS-induced mediators affected by P2RX$_7$ activation are not induced by P2RX$_7$ agonists alone, suggesting a complementary role for P2RX$_7$ in transcriptional regulation. Type I IFN production, whose expression is tightly controlled by multiple transcription factors that form an enhanceosome, is critical for resistance against LPS-containing bacteria. The effect of purinergic receptor signaling on LPS-dependent type I IFN is unknown and would be of great relevance to a diverse array of inflammatory conditions. The present study demonstrates that stimulation of macrophages with P2RX$_7$ agonists substantially enhances LPS-induced IFN-β expression, and this enhancement is ablated in macrophages that do not express functional P2RX$_7$ or when the MAPK MEK1/2 pathways are inhibited. Potentiation of LPS-induced IFN-β expression following P2RX$_7$ stimulation is likely transcriptionally regulated, as this enhancement is observed at the IFN-β promoter level. Furthermore, P2RX$_7$ stimulation is able to increase the phosphorylation and subsequent IFN-β promoter occupancy of IRF-3, a transcription factor that is critical for IFN-β transcription by TLR agonists. This newly discovered role for P2RX$_7$ in IFN regulation may have implications in antimicrobial defense, which has been linked to P2RX$_7$ activation in other studies. *J. Leukoc. Biol.* 94: 759–768; 2013.

Introduction

Recent immunological studies have revealed an intriguing role for purinergic receptors as danger sensors of immune cells that influence the magnitude of a mounted innate immune response, depending on the concentration and duration of extracellular ATP exposure [1–3]. In particular, extracellular ATP-induced activation of the ionotropic P2RX$_7$ nucleotide receptor has been implicated in the modulation of host immune response to pathogens and inflammatory disease progression [1, 4–6]. Studies conducted in P2RX$_7^{-/-}$ mice found reduced incidence and severity of anticollagen-induced arthritis symptoms compared with littermate controls [7]. Results from further animal studies suggest that targeting P2RX$_7$ may be useful in the treatment of arthritis and spinal cord injury [8–11]. Moreover, it has been observed that patients with rheumatoid arthritis have increased P2RX$_7$ expression on their blood monocytes compared with nonarthritic controls [12]. Furthermore, mutations in P2RX$_7$ have been linked to increased susceptibility to infectious diseases, which has been ascribed, at least in part, to impaired macrophage function [6]. Thus, P2RX$_7$ activation has been linked to numerous diseases, but the intracellular signaling mechanisms that are induced are incompletely understood.

Even though P2RX$_7$ initiates many processes on its own, nucleotide stimulation of P2RX$_7$ also displays a striking capacity to modulate endotoxin/LPS-induced signaling [13–17]. Early purinergic signaling studies found that administration of 2-methylthioadenosine-5′-*O*-triphosphate, a partial P2RX$_7$ agonist, protected mice against lethal endotoxic shock [18]. Moreover, P2RX$_7$ ligation increases LPS-induced ROS, and NO production [15, 19–21] plays a critical role in the processing and

Abbreviations: $^{-/-}$=deficient, BzATP=benzoyl ATP, ChIP=chromatin immunoprecipitation, F=forward, IRF-3=IFN regulatory factor-3, P2RX$_7$=purinergic receptor P2X, ligand-gated ion channel 7, pIRF=phosphorylated IRF, Poly I:C=polyinosinic:polycytidylic acid, q=quantitative, R=reverse, SF=S342F, siRNA=small interfering RNA, TBK-1=TANK-binding kinase-1, TRAM=Toll/IL-1R-domain-containing adaptor-inducing IFN-β-related adapter molecule, TRIF=Toll/IL-1R-domain-containing adaptor-inducing IFN-β

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release of IL-converting enzyme (caspase-1)-dependent cytokines, IL-1β and IL-18, and augments production of TNF-α and IL-6 in macrophages [5, 7, 22]. Interestingly, PBMCs from human subjects that possess a polymorphism that impairs $P2RX_7$ function produced less proinflammatory (e.g., TNF-α) but more anti-inflammatory (e.g., IL-10) cytokines in response to LPS stimulation [23]. Although it is known that stimulation of $P2RX_7$ can potentiate numerous LPS-induced proinflammatory events, particularly in macrophages, the molecular mechanisms underlying this process are not well defined.

It has been suggested that the enhancement of LPS-induced IL-1β and IL-18 release by nucleotides involves the formation of the inflammasome [22]. Although the inflammasome theory explains the release of preformed cytokines within endocytic vesicles [24] or microvesicles [25] in the cell, it does not address the enhancement of LPS-induced mRNA and protein expression after longer stimulation with nucleotides. For instance, activation of $P2RX_7$ increases the mRNA and/or protein expression of TNF-α, iNOS, and COX-2 in LPS-primed macrophages [17, 19, 21, 26]. In terms of intracellular signaling events, it has been observed that costimulation of macrophages with $P2RX_7$ agonists and LPS results in prolonged IκBα degradation and increased NF-κB-binding activity, decreased ERK1/2 activation, and increased JNK and p38 MAPK activation [13]. Although $P2RX_7$ activates multiple transcription factors [27, 28], many of the LPS-induced mediators that $P2RX_7$ has been shown to modulate are not induced by $P2RX_7$ stimulation alone [16, 17]. One explanation for the potentiation of LPS-induced mediator expression following $P2RX_7$ stimulation could be the activation of an enhanceosome, a complex of transcription factors that induce gene expression when stimulated together.

One gene, whose transcription is tightly regulated by enhanceosome formation and is induced by LPS stimulation in macrophages, is IFN-β. Similarly to the actions of $P2RX_7$, type I IFNs (IFN-α and -β), produced by macrophages, play an important role in antimicrobial immunity by acting in an autocrine manner to enhance phagocytosis and the induction of iNOS [29]. IFN-β is the primary type I IFN produced by macrophages following LPS stimulation [30–32]. It is worth noting that $P2RX_7$ stimulation and IFN-β play important roles in inflammatory responses to pathogenic bacteria [18, 33]. Interestingly, IFN-β null mice are completely resistant to LPS-induced septic shock, and inhibition of IFN-β has been proposed as a potential treatment for sepsis [34, 35].

The majority of the signaling events induced by LPS is mediated through TLR4. Stimulation of TLR4 leads to the activation of MyD88- and TRIF-dependent signaling cascades, leading to the activation of NF-κB and IRF-3, respectively, which are both critical transcription factors in the induction of IFN-β expression [30, 36]. Although $P2RX_7$ agonists have been demonstrated to enhance the release of inflammatory cytokines induced via the MyD88-dependent pathway (e.g., IL-1β, TNF-α) [5, 7, 22], the effect of $P2RX_7$ signaling on TRIF-dependent signaling or the induction of type I IFN by LPS is entirely unknown. Considering that $P2RX_7$ is able to modulate other LPS-initiated signaling events and shares many immunological processes with IFN-β, a role for $P2RX_7$ stimulation in the modulation of LPS-induced IFN-β expression was investigated. We found that activation of $P2RX_7$ significantly enhanced the expression and release of LPS-induced IFN-β from macrophages, potentially through the activation of IRF-3.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents for cell culture were purchased from Mediatech (Herndon, VA, USA). All nucleotides and LPS (*Escherichia coli*, serotype 0111:B4) were obtained from Sigma Chemical (St. Louis, MO, USA). Antibodies directed against FosB, β-tubulin, and pIRF-3 (against Ser 396; Cat. #4947) for immunoblotting were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-pIRF-3 (against Ser 386; Cat. #2562-1) for immunofluorescence was purchased from Epitomics (Burlingame, CA, USA); anti-IRF-3 antibody was purchased from ProSci (Poway, CA, USA); Alexa Fluor 488 goat anti-rabbit IgG and ProLong Gold antifade reagent with DAPI were purchased from Invitrogen (Grand Island, NY, USA); anti-β-actin, anti-IRF-3 (for ChIP; Cat. #SC-9082x), and HRP-conjugated anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The active MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI, USA). The p38 inhibitor SB203580 was from Cell Signaling Technology. MRT67307 was a generous gift of Dr. Philip Cohen (University of Dundee, Scotland).

Cell culture

Murine RAW 264.7 macrophages were obtained from American Type Culture Collection (Manassas, VA, USA), whereas $P2RX_7$-defective RAW cells (denoted SF) were generated as described previously [37]. Both RAW 264.7 cell lines were maintained in RPMI, supplemented with 5% cosmic calf serum, 2 mM sodium pyruvate, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. All cells were grown in 10 cm tissue-culture dishes at 37°C in a humidified atmosphere with 5% CO_2.

Isolation and culture of bone marrow-derived macrophages

The bone marrow cells of the tibiae, femurs, pelvic, and humerus bones of C57BL/6 mice were rinsed out with DMEM (Invitrogen) cell maintenance media, collected in a cell strainer, and layered over Histopaque 1083 (Sigma Chemical), twice, to isolate mononuclear cells. Cells were maintained in DMEM media, supplemented with 10% FCS, 20 ng/ml human rM-CSF, or CMG14-12 supernatant (as M-CSF source [38]), and plated in 10-cm Petri dishes at a density of 5×10^6 cells/plate. Three days after isolation, cells were lifted and plated at a density of 2.5×10^5 cells/well in a 12-well plate in CMG14-12-supplemented complete media. Six days after isolation, cells were treated as described.

qPCR

RAW 264.7 macrophages were plated at a density of 7.5×10^5 cells/well in six- or 12-well tissue-culture plates and incubated at 37°C, the day before each experiment. Following treatment and subsequent cell lysis in Trizol (Invitrogen), mRNA was isolated and converted to cDNA, according to the manufacturer's instructions. Primers directed toward murine IFN-β, FosB, or 18S (loading control) were designed using Beacon Design software (Premier Biosoft, Palo Alto, CA, USA). IFN-β primers are: **F**-ACT AGA GGA AAA GCA AGA GGA AAG, **R**-CCA CCA TCC AGG CGT AGC; FosB primers: **F**-TTC CAG ACC AGC CAA GAC, **R**-ACC GAA GAA GTG TAC GAA GG; 18S primers: **F**-GGA CAC GGA CAG GAT TGA CAG, **R**-ATC GCT CCA CCA ACT AAG AAC G. PCR reactions were detected by iQ SYBR Green supermix dye (Bio-Rad, Hercules, CA, USA) and were performed on a MyiQ real-time thermal cycler (Bio-Rad; 57–58°C annealing temp, 40 cycles).

ELISA

RAW 264.7 macrophages were plated at a density of 4×10^5 cells/well in 12-well tissue-culture plates and incubated at 37°C the day before treatment. Following treatment, the supernatants were centrifuged to remove cell debris and analyzed for IFN-β via ELISA, according to the manufacturer's specifications (PBL Laboratories, Piscataway, NJ, USA).

IFN-β luciferase promoter activation assay

The murine IFN-β promoter sequence (+10 from start site to −329) was inserted into a firefly luciferase reporter construct (Promega) and 2 μg of this plasmid was transfected, along with 0.2 μg of a pRL Renilla luciferase control vector (Promega)/2×10^6 RAW 264.7 macrophages/cuvette via Amaxa electroporation. The cells were plated subsequently at 5×10^5 cells/well in a 24-well plate and incubated at 37°C overnight. The day after electroporation, the cells were treated and then lysed to quantify luminescence using the Dual-Glo luciferase assay system (Promega), according to the manufacturer's specifications.

FosB siRNA

RAW 264.7 macrophages were transfected with SMARTpool ON-TARGET siRNA, directed toward FosB or control siRNA (Dharmacon, Lafayette, CO, USA) using Amaxa Nucleofector Kit V and an Amaxa electroporator (Lonza, Walkersville, MD, USA), according to the manufacturer's specifications. Electroporated cells were plated in six-well plates (1×10^6 cells/well), and 24 h after transfection, the cells were treated as specified and lysed in Trizol for mRNA processing.

Immunoblotting

RAW 264.7 macrophages were plated at a density of 3×10^5 cells/well in 24-well tissue-culture plates and incubated at 37°C the day before each experiment. Following treatment and subsequent cell lysis with SDS sample buffer (10 mM Tris, 1 mM EDTA, 0.5 mM Na_3VO_4, 1 mM DTT, 1% SDS, and 10% glycerol), the proteins were resolved on SDS-PAGE gels and transferred to PVDF membranes. Immunoblotting was performed by incubating the membranes with commercially available antisera, according to the manufacturer's protocols, and were visualized using the EpiChemi II darkroom (UVP, Upland, CA, USA), equipped with a 12-bit cooled, charge-coupled device camera.

Immunocytochemistry

RAW 264.7 cells (2×10^5/4 ml media) were plated in 60-mm dishes containing 20 mm × 20 mm coverslips and incubated at 37°C for 24 h. Cells were then stimulated as described. After stimulation, the media were removed, and the coverslip was rinsed with 0.1 M phosphate buffer (pH 7.4). The cells were fixed for 20 min with 4% PFA/PBS and then permeabilized with PBS containing 0.2% Triton X-100. To detect pIRF-3, the coverslips were blocked with 10% goat serum in 0.2% Triton X-100 PBS for 1 h and then incubated overnight with 1:400 anti-pIRF-3 antibody, diluted in 1% BSA-PBS. The coverslips were washed three times with 0.2% Triton X-100 PBS and incubated with 1:2000 Alexa Fluor 488 goat anti-rabbit IgG in 1% BSA-PBS for 1 h. The samples were washed three times with PBS and mounted on slides with ProLong Gold antifade reagent with DAPI. Staining was visualized with a Nikon 80i upright confocal microscope under 60× magnification. The antibody specificity of the anti-pIRF-3 antibody for immunocytochemistry was verified using macrophages from IRF-$3^{-/-}$ mice [39].

EMSA

RAW 264.7 cells were plated at 1.5×10^6 cells/well in six-well tissue-culture plates and incubated for 18–24 h before the start of the experiment. After the cells were treated, nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Biotin-labeled complementary oligonucleotides of the murine IFN-β promoter (5′-ATG ACA GAG GAA AAC TGA AAG GGA GAA CTG AAA GTG GGA AAT TCC T-3′; nt −151 to −106) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Binding reactions were performed as described previously [40] using the LightShift Chemiluminescent EMSA kit (Thermo Scientific). Reactions were loaded onto native (6%) polyacrylamide gels and transferred to nylon (Biodyne B; Pierce, Thermo Scientific) membranes (45 min at 100 V). The migration of the labeled oligonucleotide was detected using HRP-conjugated streptavidin (LightShift Chemilu-

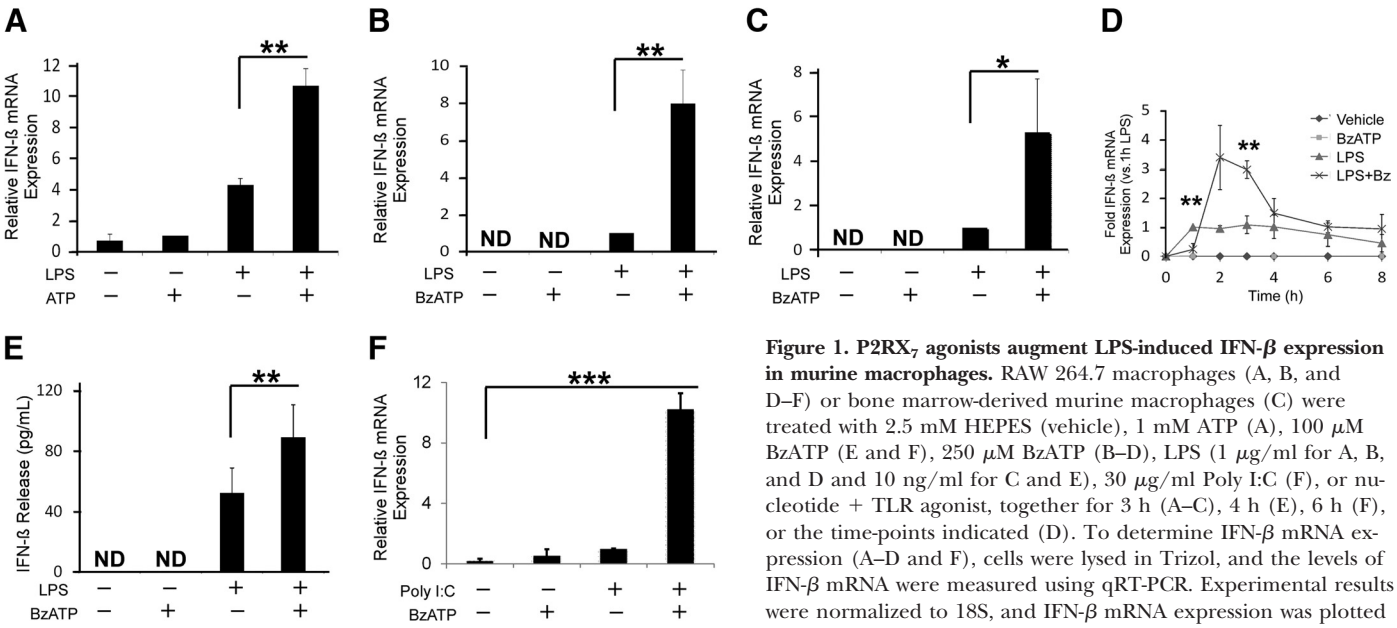

Figure 1. P2RX$_7$ agonists augment LPS-induced IFN-β expression in murine macrophages. RAW 264.7 macrophages (A, B, and D–F) or bone marrow-derived murine macrophages (C) were treated with 2.5 mM HEPES (vehicle), 1 mM ATP (A), 100 μM BzATP (E and F), 250 μM BzATP (B–D), LPS (1 μg/ml for A, B, and D and 10 ng/ml for C and E), 30 μg/ml Poly I:C (F), or nucleotide + TLR agonist, together for 3 h (A–C), 4 h (E), 6 h (F), or the time-points indicated (D). To determine IFN-β mRNA expression (A–D and F), cells were lysed in Trizol, and the levels of IFN-β mRNA were measured using qRT-PCR. Experimental results were normalized to 18S, and IFN-β mRNA expression was plotted as an average of experimental replicates (mean±SEM). (E) Supernatants were spun down and analyzed for IFN-β via ELISA. The results from four independent experiments were summarized and represent relative IFN-β protein expression (mean±SEM). ND, Not detected; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. BzATP+PolyI:C vs. all other conditions. Each panel represents data from at least three independent experiments.

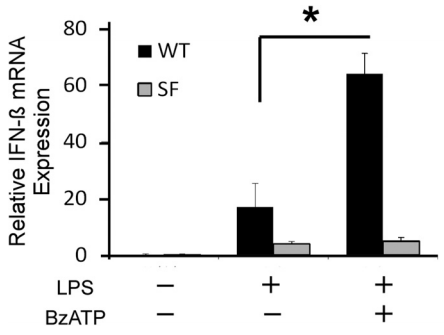

Figure 2. BzATP-induced enhancement of LPS-induced IFN-β mRNA requires functional $P2RX_7$. RAW 264.7 macrophages endogenously expressing WT or nonfunctional $P2RX_7$ (SF) were treated with vehicle, 1 μg/mL LPS, or 1 μg/ml LPS + 300 μM BzATP for 3 h. IFN-β and 18S mRNA levels were assessed by qPCR. The results from at least five independent experiments were averaged and represent relative IFN-β mRNA expression (mean±SEM); $*P = 0.01$.

minescent EMSA kit), visualized on the EpiChemi II darkroom, and image processing and analyses were performed using ImageJ 1.33u.

ChIP

RAW 264.7 macrophages were plated at a density of 6×10^6 cells/well in 10-cm tissue-culture plates and incubated at 37°C, 24 h before treatment. After treatment, cells were processed using the EZ-Magna ChIP A kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Protein/DNA complexes were immunoprecipitated with anti-IRF-3 (Santa Cruz Biotechnology; Cat. #SC-9082x), and purified, pulled-down DNA was amplified and quantified using qPCR with the following murine IFN-β primer sequence: **F**-GAA AGG GAG AAC TGA AAG, **R**-AGT GAG AAT GAT CTT CCT. IFN-β levels were normalized to input, and normal rabbit IgG (supplied in kit) was used as a control for nonspecific immunoprecipitation.

Statistical analysis

The Student's two-tailed paired *t*-test or Tukey-Kramer method (ANOVA) was used to calculate the statistical differences between samples. Significance levels were set at P values < 0.05.

RESULTS

$P2RX_7$ agonists augment LPS-induced IFN-β expression in murine macrophages

$P2RX_7$ signaling and IFN-β have been implicated in inflammatory responses to bacterial infection. Even though $P2RX_7$ is known to regulate other LPS-induced inflammatory cytokines, the effect of purinergic receptor signaling on type I IFN production has not been described previously. To test the hypothesis that extracellular adenine nucleotides exert an influence on LPS-induced IFN-β in macrophages, we used a well-characterized murine macrophage cell line RAW 264.7. These cells were costimulated with LPS and ATP or the potent $P2RX_7$ agonist BzATP, and IFN-β mRNA induction was quantified using real-time qPCR (**Fig. 1A and B**). In the presence of extracellular nucleotides and LPS, RAW 264.7 macrophages produced significantly higher levels of IFN-β mRNA than in response to LPS alone, supporting a role for $P2RX_7$ in the augmentation of LPS-induced IFN-β expression. $P2RX_7$ stimulation alone did not induce detectable IFN-β expression. The transcriptional effect of purinergic receptor signaling on IFN-β appeared relatively cytokine-specific, as BzATP did not augment mRNA for IL-6 or TNF-α mRNA production during this time frame (data not shown). The ability of BzATP to potentiate LPS-induced IFN-β mRNA expression was also observed in primary murine bone marrow-derived macrophages (Fig. 1C). To determine the kinetics of IFN-β mRNA expression, a time course was performed. After 1 h of treatment, BzATP attenuates LPS-induced IFN-β expression (P=0.006), but by 2 h, BzATP augments LPS-induced IFN-β mRNA expression, and this synergy is sustained up to 4 h (Fig. 1D). To examine whether the ability of $P2RX_7$ to enhance LPS-induced IFN-β mRNA reflects increases in IFN-β protein production, IFN-β release was monitored. As shown in Fig. 1E, BzATP significantly increased the amount of IFN-β released from macrophages in response to LPS. To determine if $P2RX_7$ activation is able to potentiate the expression of IFN-β induced by other TLRs, RAW 264.7 macrophages were stimulated with the TLR3 synthetic agonist Poly I:C in the absence or presence of BzATP. Although neither stimulus alone induces robust IFN-β, the combination of Poly I:C and BzATP induces significant IFN-β expression in macrophages (Fig. 1F), without causing cell death (data not shown).

BzATP-induced enhancement of LPS-induced IFN-β mRNA requires functional $P2RX_7$

Macrophages express the mRNA for many other purinergic receptors, in addition to $P2RX_7$ [26]. Although BzATP is a potent agonist of $P2RX_7$, BzATP also activates several other P2Rs at high concentrations (e.g., $P2RX_1$ and $P2RY_{11}$) [41]. Therefore, to ascertain further whether the enhancement of LPS-induced IFN-β mRNA expression by BzATP is mediated

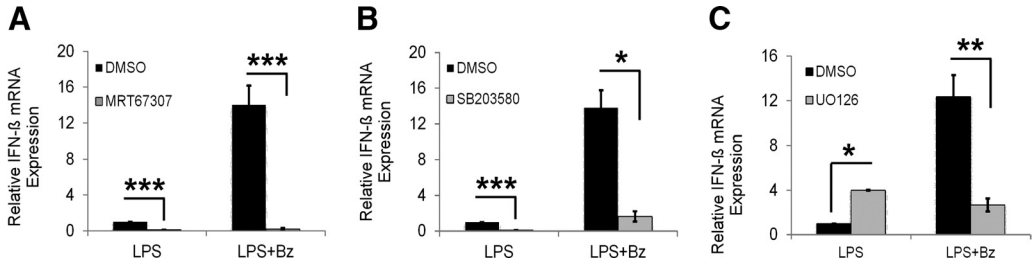

Figure 3. Effect of kinase inhibition on $P2RX_7$ agonist-potentiated, LPS-induced IFN-β expression. RAW 264.7 macrophages were pretreated for 30 min (A) or 15 min (B and C) with vehicle (DMSO) or the indicated kinase inhibitor (10 μM). The cells were subsequently treated with vehicle (2.5 mM HEPES), 250 μM BzATP, 1 μg/ml LPS, or BzATP + LPS together for 3 h. IFN-β and 18S mRNA levels were assessed by qPCR. The results of at least three independent experiments were collated and are represented as relative IFN-β mRNA expression (with LPS treatment set to 1; mean±SEM); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

through endogenous P2RX$_7$, a nonfunctional P2RX$_7$ cell line was used. Specifically, "SF" RAW 264.7 macrophages possess an endogenous mutant P2RX$_7$ gene containing a serine-to-phenylalanine mutation in the second transmembrane domain (SF) that confers attenuated P2RX$_7$ protein expression and function [15, 37, 42, 43], while retaining the ability to respond to other stimuli to the same extent as the P2RX$_7$ WT-expressing cells. As shown in **Fig. 2**, BzATP treatment of macrophages containing the SF P2RX$_7$ mutant gene did not enhance LPS-induced IFN-β mRNA expression (P=0.62). Of note, LPS-induced IFN-β mRNA expression trended lower in SF versus WT P2RX$_7$-expressing macrophages, but this difference was not statistically significant (P=0.26). These mutant P2RX$_7$ RAW cells were not generally defective in responding to LPS, as they produce as much TNF-α in response to LPS as RAW macrophages expressing WT P2X$_7$ (data not shown).

Inhibition of the MEK1/2 pathway prevents the P2RX$_7$ agonist from enhancing LPS-induced IFN-β expression

To begin investigating the mechanism by which P2RX$_7$ activation augments LPS-mediated IFN-β expression, kinase inhibitor experiments were conducted to elucidate further the signal transduction pathways that distinguish LPS-induced IFN-β from BzATP-enhanced, LPS-induced IFN-β. Induction of IFN-β by TLR4 agonists, such as LPS, requires the activation of the TRIF/TRAM pathway for complete activation of the enhanceosome [44]. To ascertain if the TRIF/TRAM pathway was involved in P2RX$_7$ agonist-induced, increased IFN-β expression, we chose to inhibit IKKε and TBK-1, as the TRIF/TRAM complex activates IKKε/TBK-1 directly, and these kinases are known to regulate IRF-3 activation and subsequent IFN-β expression in response to LPS [45]. As expected, pretreatment of RAW 264.7 macrophages with the IKKε/TBK-1 inhibitor MRT67307 [46] inhibited LPS-induced IFN-β mRNA expression (**Fig. 3A**), as well as BzATP + LPS-induced IFN-β expression.

It is known that LPS and P2RX$_7$ agonists are able to induce the activation of p38 MAPK, and there is evidence that p38 activation is involved in bacterially induced IFN-β expression [47]. Thus, we examined the effect of the p38 inhibitor SB203580 on IFN-β expression. We found that p38 inhibition was able to significantly attenuate LPS-induced and LPS + BzATP-induced IFN-β expression (Fig. 3B).

Treatment of macrophages with P2RX$_7$ agonists leads to the activation of the MAPKs, MEK1/2 and subsequently, ERK1/2 [37]. To determine if the MEK/ERK MAPK pathway participates in augmenting IFN-β expression after P2RX$_7$ activation, the pharmacological MEK1/2 inhibitor, U0126, was used. U0126 previously has been shown to attenuate viral-induced IFN-β release via antagonism of IRF-3 activation [48]. When macrophages were pretreated with U0126, the ability of BzATP to enhance LPS-induced IFN-β expression was ablated (Fig. 3C). Interestingly, U0126 actually slightly enhanced LPS-induced IFN-β production, which has been observed previously [49, 50]. These data suggest that P2RX$_7$ stimulation augments LPS-induced IFN-β via a MEK1/2-dependent pathway, external or accessory to canonical TLR4-IFN-β signaling.

Potentiation of LPS-induced IFN-β production by the P2RX$_7$ agonist is observed at the promoter level

To decipher further how P2RX$_7$ activation is enhancing the ability of LPS to induce IFN-β expression, the role of P2RX$_7$ in activating the IFN-β promoter was investigated. Macrophages were transfected with a luciferase reporter construct, driven by the IFN-β promoter and treated with LPS ± BzATP for 6 h. As shown in **Fig. 4A**, treatment of macrophages with BzATP significantly enhances LPS-induced luciferase activity, supporting a role for P2RX$_7$ in amplifying IFN-β expression at

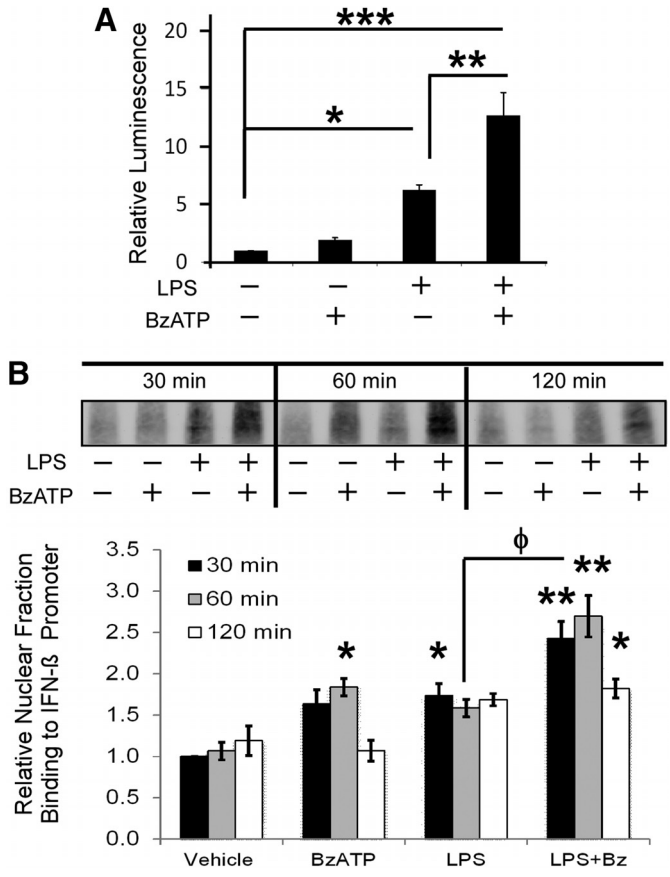

Figure 4. Potentiation of LPS-induced IFN-β production by P2RX$_7$ agonist is observed at the IFN-β promoter level. (A) The IFN-β promoter sequence was inserted into a luciferase reporter construct and transfected into RAW 264.7 macrophages. Cells were subsequently treated with vehicle (2.5 mM HEPES), 250 μM BzATP, 10 ng/ml LPS, or BzATP+LPS for 6 h. After treatment, cells were lysed, and luminescence was quantified. Data from three independent experiments were combined and graphed ± SEM, with the luminescence values for the vehicle-treated samples set to 1; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (B) RAW 264.7 macrophages were treated with vehicle (2.5 mM HEPES), 250 μM BzATP, 1 μg/ml LPS, or BzATP+LPS for the times indicated, and nuclear fractions were collected for nonradioactive EMSA, using the biotin-labeled IFN-β promoter sequence to cause a gel shift. A representative gel shift is shown, and data from four independent experiments were collated and are represented as relative nuclear protein binding to the IFN-β promoter (with 30 min vehicle treatment set to 1; mean±SEM); *$P < 0.05$; **$P < 0.005$ compared with time-matched, vehicle-treated band density; ϕ$P < 0.005$ between LPS and LPS + Bz-treated band density.

the transcriptional level. To investigate the effect of $P2RX_7$ activation on the transcriptional enhancement of IFN-β expression, EMSAs were performed. Cotreatment of RAW 264.7 macrophages with LPS and BzATP significantly enhances nuclear protein binding to the IFN-β promoter (Fig. 4B). These data introduce the possibility that $P2RX_7$ alters LPS-induced promoter activity through modulation of the *ifnb1* enhanceosome.

Costimulation of murine macrophages with LPS and a $P2RX_7$ agonist induces robust FosB expression, but it is not related to IFN-β augmentation

We discovered recently that activation of $P2RX_7$ in macrophages leads to the robust expression of the AP-1 family transcription factor FosB, and this expression was downstream of the MEK/ERK signaling cascade [43]. Considering that the promoter of IFN-β has an AP-1-binding site that is important for the assembly of the enhanceosome, we investigated if $P2RX_7$ agonist-induced FosB was upstream of IFN-β expression. We first examined the expression of FosB mRNA and protein after $P2RX_7$ agonist and LPS treatment. As shown in **Fig. 5A**, treatment of macrophages with BzATP or LPS induces FosB mRNA expression, and the combination of BzATP and LPS induces an enhanced induction of FosB mRNA expression compared with either treatment alone. This enhanced expression of FosB after BzATP + LPS treatment was also observed at the protein level (Fig. 5B). To determine if this induction of FosB was upstream of IFN-β expression, siRNA directed against FosB (or control siRNA) was transfected into macrophages, followed by treatment with BzATP and LPS. Although the FosB siRNA and not the control siRNA was able to knock down BzATP/LPS-induced FosB expression at mRNA (Fig. 5B) and protein levels (data not shown), FosB knockdown had no effect on BzATP/LPS-induced IFN-β expression (Fig. 5C).

Activation of IRF-3 is up-regulated after $P2RX_7$ activation

IRF-3 is a transcription factor critical for the activation of the IFN-β enhanceosome [51, 52]. Although the ability of LPS to induce IRF-3 activation is documented [52, 53], the ability of the $P2RX_7$ agonists to activate this transcription factor is unknown. To detect IRF-3 activation, macrophages were treated with LPS ± BzATP, and pIRF-3 was monitored. In **Fig. 6A**, treated macrophages were subjected to immunoblotting using an antibody against the S396 pIRF-3, which at S396 was not reliably detectable after BzATP treatment. However, BzATP treatment was able to significantly enhance LPS-induced pIRF-3 (P=0.009; n=3; Fig. 6A). To determine whether $P2RX_7$ agonist-induced enhancement of pIRF-3 translated into increased IRF-3 occupancy on the IFN-β promoter, ChIP was performed. As shown in Fig. 6B, treatment of macrophages with LPS + BzATP induces increased IRF-3 occupancy on the IFN-β promoter over LPS treatment, alone after 1 h and 2 h of treatment. The results demonstrate for the first time that stimulation of $P2RX_7$ is able to activate IRF-3 and may explain the enhanced IFN-β expression.

To determine which signaling mediators were upstream of BzATP-mediated IRF-3 activation, macrophages were pretreated with MRT67307, SB203580, and U0126 before BzATP treatment, and S386 pIRF-3 was monitored by immunofluorescence. Interestingly, BzATP and LPS treatment appeared to enhance pIRF-3 at this residue (**Fig. 7A**). Furthermore, BzATP-induced pIRF-3 S386 was decreased in the presence off all of the inhibitors. When these same inhibitors were used to measure the phosphorylation of S396 by immunoblotting, MRT67307 was able to significantly attenuate LPS and LPS + BzATP-induced pIRF-3, but SB203580 was not able to inhibit either. Consistent with Fig. 3C, U0126 was only able to attenu-

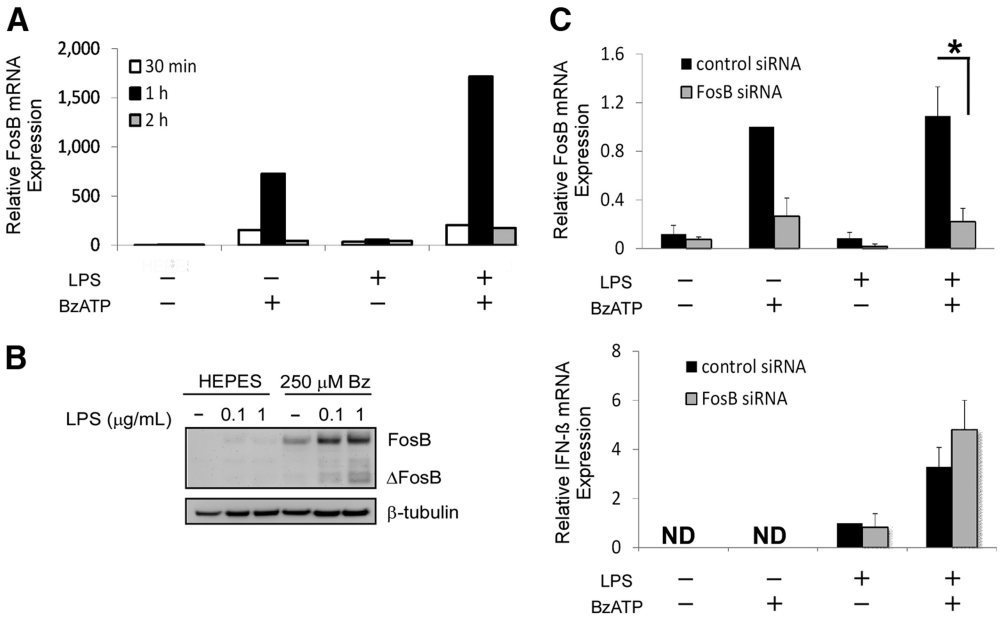

Figure 5. Costimulation of murine macrophages with LPS and a $P2RX_7$ agonist induces FosB expression but is not related to IFN-β release. RAW 264.7 macrophages were treated with 2.5 mM HEPES (vehicle), 250 μM BzATP, 1 μg/ml (and 0.1 μg/ml for B), LPS, or BzATP + LPS together for the time-points indicated. (A) Cells were lysed, and FosB mRNA levels were measured using qPCR. Experimental results were normalized to 18S. Data are representative of two independent experiments. (B) Cells were lysed in sample buffer after treatment and immunoblotted for FosB and anti-β-tubulin (loading control). Data are representative of three independent experiments. (C) Twenty-four hours prior to 3 h treatment with LPS ± BzATP, cells were transfected with control siRNA or siRNA directed toward murine FosB, and levels of FosB and IFN-β mRNA were measured. The results of three independent experiments were collated and are represented as relative FosB or IFN-β mRNA expression; *P = 0.01.

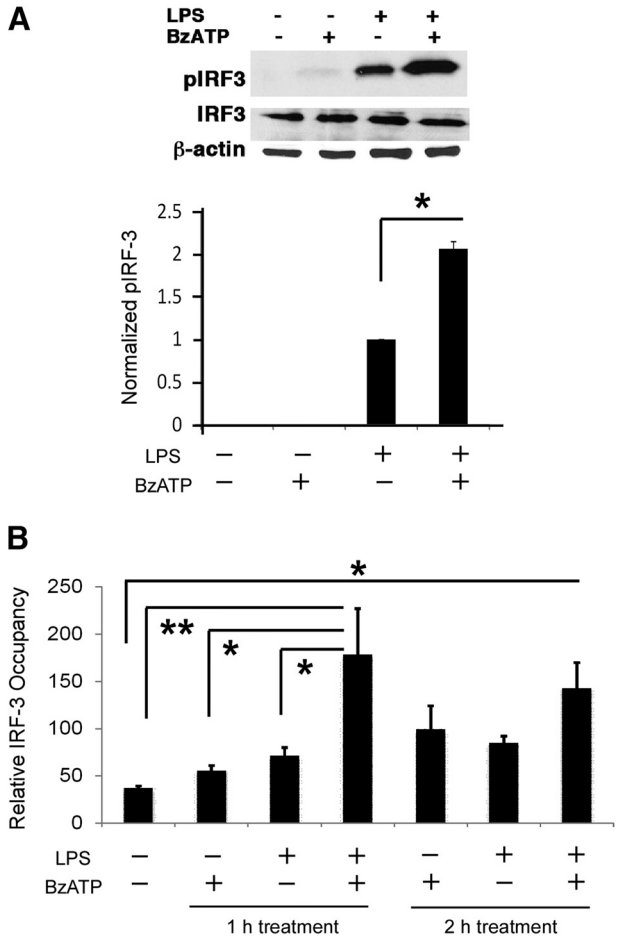

Figure 6. Treatment of RAW 264.7 macrophages with a P2RX$_7$ agonist promotes pIRF-3. RAW 264.7 macrophages were treated with 2.5 mM HEPES (vehicle), 250 μM BzATP, 1 μg/ml LPS (A), 100 ng/ml LPS (B), or BzATP + LPS together for 2 h (A) or the times indicated (B). (A) Lysed cells were immunoblotted for pIRF-3 and total IRF-3 (loading control). A representative immunoblot displaying pIRF-3/IRF-3 is shown. The results of three independent experiments were collated and are represented as relative pIRF-3 (normalized to IRF-3; mean±SEM); *$P < 0.01$. (B) Cross-linked protein/DNA complexes were immunoprecipitated with an IRF-3 antibody, and the collected DNA was amplified with primers against the IFN-β promoter. IFN-β levels were normalized to their respective input control, and the results from three independent experiments were averaged and plotted (mean±SEM); *$P < 0.05$; **$P < 0.005$.

ate LPS + BzATP but not LPS-induced pIRF-3 (Fig. 7B). Cell viability assays were performed as described previously [43] to confirm that these effects were not a result of toxicity (data not shown). Together, these data suggest that P2RX$_7$ signals through MAPK pathways and TBK-1/IKKε to activate IRF-3 and thus, augment LPS-induced IFN-β expression.

DISCUSSION

This present study is the first to demonstrate that LPS-induced IFN-β is enhanced in macrophages upon activation of P2RX$_7$, at the mRNA and protein level. The potentiation of IFN-β expression was not exclusive to TLR4 agonists, as the induction of IFN-β expression by a TLR3 agonist was also augmented in the presence of the P2RX$_7$ agonist BzATP. These data suggest that the P2RX$_7$ agonist-induced enhancement of LPS-triggered IFN-β expression occurs at the promoter level of the IFN-β gene and is related to an increase in active IRF-3.

Type I IFNs can exert biological effects at low levels of expression [54]. For instance, low levels of IFN-α/β prime cells to respond to other cytokines, such as IFN-γ and IL-6, possibly by recruiting commonly used signaling molecules to the cell membrane in proximity to other cytokine receptor complexes [54]. Therefore, the increased IFN-β released from macrophages after LPS and P2RX$_7$ cotreatment is likely to have a biological impact. It has been shown that IFN-β plays a role in mediating anti-inflammatory IL-10 expression in macrophages [55]. To begin to determine potential endpoints that could be downstream of LPS + P2RX$_7$ agonist cotreatment, the induction of IL-10 mRNA expression in macrophages was measured. Although LPS and BzATP alone were able to induce increased IL-10 mRNA expression, the combination of these treatments was additive at most and not synergistic compared with the induction of IFN-β (Supplemental Fig. 1).

This study demonstrates that P2RX$_7$ agonist-enhanced, LPS-induced IFN-β expression is abrogated by multiple kinase inhibitors (Fig. 3), but the effect of the MEK1/2 antagonist U0126 (Fig. 3C) distinguished LPS-induced versus LPS + P2RX$_7$ agonist-induced IFN-β expression, suggesting that the MEK/ERK1/2 cascade is uniquely involved in the ability of P2RX$_7$ to up-regulate the expression of IFN-β in macrophages. It is known that treatment of macrophages with LPS leads to the activation of ERK1/2, and this has been shown to be MyD88- but not TRIF-dependent [56]. As TLR4-induced IFN-β expression occurs via a MyD88-independent pathway, it is not surprising that LPS-induced ERK activation is not involved in IFN-β expression (Fig. 3C) and has been described by others [57].

As a result of the importance of the AP-1 component of the IFN-β enhanceosome and our recent observation that P2RX$_7$ stimulation induces a robust activation of the AP-1 protein FosB (which is downstream of P2RX$_7$-induced MEK/ERK activation), we first hypothesized that FosB may be involved in the increased IFN-β expression observed after LPS treatment. Although our data show that cotreatment of macrophages with the LPS and P2RX$_7$ agonist induces FosB mRNA and protein expression (Fig. 5), our RNA interference data do not support a role for FosB in IFN-β expression. Therefore, our data raised the possibility that another U0126-dependent transcription factor is contributing to P2RX$_7$-LPS synergistic IFN-β induction.

It has been noted previously by our group [13] and others [27, 58] that P2RX$_7$ stimulation leads to the activation of NF-κB, and this could also be a mechanism by which nucleotide signaling enhances LPS-induced IFN-β expression, as there are NF-κB-binding elements (for a p50/p65 NF-κB heterodimer) in the enhanceosome region of the IFN-β promoter [59]. In macrophages, LPS induces the formation of p65/p50 NF-κB heterodimers, whereas ATP induces p65 NF-κB homodimers [58, 60]. Intriguingly, p50 and p105 NF-κB subunits, which lack transcriptional activation domains, inhibit LPS-induced

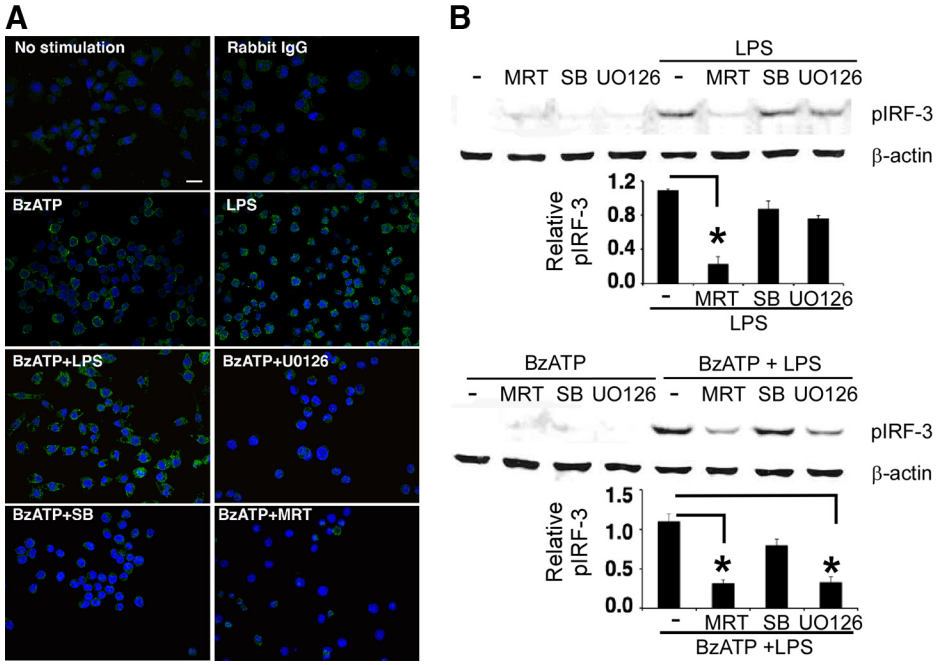

Figure 7. Effect of kinase inhibition on pIRF-3 expression. RAW 264.7 macrophages were pretreated for 30 min [for MRT67307 (MRT)] or 15 min [for SB203580 (SB) or U0126] with vehicle (DMSO) or 10 μM of the indicated kinase inhibitor. The cells were subsequently treated with vehicle (2.5 mM HEPES), 250 μM BzATP, 1 μg/ml LPS, or BzATP + LPS together for 2 h. (A) Cells were fixed and stained for pIRF-3 (green); DAPI stain (nuclei; blue). (B) Cells were lysed in sample buffer after treatment and immunoblotted for pIRF-3 and total β-actin (loading control). A representative immunoblot displaying pIRF-3 is shown. The results of at least three independent experiments were collated and are represented as relative pIRF-3 (normalized to β-actin; mean$\pm$SEM); $^*P < 0.05$.

IFN-β expression, purportedly by activating the MEK/ERK signaling cascade and preventing IRF-3 recruitment to the IFN-β promoter [60, 61]. We observed previously that U0126 pretreatment of macrophages leads to a sustained loss of IκBα expression in response to LPS [13]. As IκBα degradation is necessary for NF-κB translocation into the nucleus and subsequent gene activation, the sustained loss of IκBα expression suggests that NF-κB is transcriptionally active longer, and this may account for the increase in LPS-induced IFN-β expression upon U0126 pretreatment, shown in Fig. 4. However, as U0126 ablated the enhancement of LPS-induced IFN-β expression induced by $P2RX_7$ activation, these data suggest that the U0126-sensitive portion of $P2RX_7$ signaling may not proceed via NF-κB to enhance IFN-β expression.

Our study is the first to demonstrate that $P2RX_7$ activation alone induces the pIRF-3 at serine 386, which is essential for the IRF-3 interaction with the transcription coactivator CREB-binding protein and subsequent transcriptional activation [62]. Our Western blot data also support a role for $P2RX_7$ in enhancing LPS-dependent phosphorylation of serine 396 on IRF-3. IRF-3 is known to have two activation clusters, with one cluster including S386 and the other including S396, and there are data to support a role for phosphorylation of both of these serines in IRF-3-initiated gene transcription [63–65]. IRF-3 plays a critical role in the activation of the IFN-β enhanceosome induced by bacterial and viral stimuli [36, 66]. Therefore, it is plausible that the enhancement of IFN-β by $P2RX_7$ agonists is attributed to an increase in pIRF-3. Increased IRF-3 occupancy on the IFN-β promoter under LPS and BzATP costimulation (Fig. 6B) further supports a role for enhanced IRF-3 transcriptional activation in elevated LPS-induced IFN-β expression after $P2RX_7$ activation in macrophages. The differential sensitivity of LPS- and BzATP-induced pIRF-3 to the MEK1/2 inhibitor U0126 suggests that $P2RX_7$ stimulation supplements TLR4-triggered IFN-β induction by MAPK-dependent activation of IRF-3.

Previous studies have supported a role for purinergic receptor stimulation and IFN-β in inflammatory responses to pathogenic bacteria. One could envision a scenario in which macrophages encounter TLR ligands (exogenous or endogenous) in the setting of tissue damage and platelet degranulation, where extracellular nucleotides would be abundant. $P2RX_7$ stimulation would then act as a costimulatory "danger signal" for macrophages that augments subsequent cytokine production. Our studies reveal that these two systems may be tightly linked. These findings may also have important physiological ramifications in the context of viral infections, where large amounts of type I IFNs are produced, and substantial ATP is released after lytic cell death [3, 67]. Interestingly, low $P2RX_7$ activity has been associated with an increased risk of viral-induced/associated asthma exacerbations [68]. Considering the established antiviral properties of type I IFNs and the observation that IFN-$\beta^{-/-}$ mice are more susceptible to viral infection [69], it is possible that decreased $P2RX_7$ activity would lead to lower IFN-β levels during a viral infection and consequently, less viral clearance. Our observation that $P2RX_7$ activation potentiates TLR3 agonist-induced IFN-β expression (Fig. 1F) supports a role for extracellular nucleotides in modulating host responses to viral exposure that trigger the activation of this TLR. Further work into the role of $P2RX_7$ in the context of antiviral signaling in macrophages is warranted.

The understanding of the role of extracellular nucleotides in amplifying the inflammatory response has broad implications for multiple diseases, including cancer, atherosclerosis, asthma, and autoimmune diseases. As $P2RX_7$ is involved in the synthesis and release of multiple inflammatory mediators, it has been hypothesized that the production of a pharmacological inhibitor against $P2RX_7$ will reduce the magnitude of an inflammatory response

under physiological conditions where an immune reaction is unfavorable. As such, small molecule inhibitors of P2RX$_7$ have been in clinical trials as treatment for rheumatoid arthritis [8–11, 28, 70]. IFN-β is present in the synovial fluid of arthritic patients [71] and based on our observations, may be, in part, a result of the activation of P2RX$_7$ in the inflammed joints.

In summary, these studies suggest that P2RX$_7$ signaling may act as a danger signal, by augmenting TLR4 agonist-induced IFN-β expression in macrophages. Our data also begin to elucidate the mechanisms by which purine nucleotides regulate the expression of this proinflammatory, pleiotropic cytokine. To our knowledge, this is the first investigation into the mechanism of how P2RX$_7$ signaling modulates type I IFN responses.

AUTHORSHIP

All authors contributed to the conception and design of the study, interpretation of the data, and revision of the manuscript and provided final approval of the submitted version. P.J.B., L.C.D., and J.A.S. were responsible for obtaining funding. M.L.G. drafted the manuscript. M.L.G., Y.P.L., L.Y.L., L.Z., A.G.G., and J.B.B. contributed to data acquisition. M.L.G. and J.A.S. completed data analysis and interpretation.

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