TLR2 Signaling Depletes IRAK1 and Inhibits Induction of Type I IFN by TLR7/9

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Pathogens may signal through multiple TLRs with synergistic or antagonistic effects on the induction of cytokines, including type I IFN (IFN-I). IFN-I is typically induced by TLR9, but not TLR2. Moreover, we previously reported that TLR2 signaling by Mycobacterium tuberculosis or other TLR2 agonists inhibited TLR9 induction of IFN-I and IFN-I–dependent MHC-I Ag cross processing. The current studies revealed that lipopeptide-induced TLR2 signaling inhibited induction of first-wave IFN-α and IFN-β mRNA by TLR9, whereas induction of second-wave IFN-I mRNA was not inhibited. TLR2 also inhibited induction of IFN-I by TLR7, another MyD88–dependent IFN-I–inducing receptor, but did not inhibit IFN-I induction by TLR3 or TLR4 (both Toll/IL-1R domain-containing adapter-inducing IFN-β dependent, MyD88 independent). The inhibitory effect of TLR2 was not dependent on new protein synthesis or intercellular signaling. IL-1R–associated kinase 1 (IRAK1) was depleted rapidly (within 10 min) by TLR2 agonist, but not until later (e.g., 2 h) by TLR9 agonist. Because IRAK1 is required for TLR7/9-induced IFN-I production, we propose that TLR2 signaling induces rapid depletion of IRAK1, which impairs IFN-I induction by TLR7/9. This novel mechanism, whereby TLR2 inhibits IFN-I induction by TLR7/9, may shape immune responses to microbes that express ligands for both TLR2 and TLR7/TLR9, or responses to bacteria/virus coinfection. The Journal of Immunology, 2012, 188: 000–000.

The innate immune system recognizes and responds to bacterial exposure with a coordinated cytokine response that must launch host defense to eradicate the offending pathogen, yet avoid excessive inflammation that may damage host tissues. To this end, signaling by innate immune pattern recognition receptors (PRRs) controls the identity, quantity, and duration of cytokine responses. Many pathogens express agonists of more than one PRR, and the immune system must integrate signaling through multiple PRRs to specify the correct cytokine response. Despite the importance of this integration of signaling pathways to immune homeostasis, the molecular mechanisms of such integration remain unclear. For example, the induction of type I IFN (IFN-I [e.g., IFN-α and IFN-β]) is controlled by multiple PRRs that can have positive or negative effects on the expression of IFN-I. Too little IFN-I response may compromise IFN-I–induced immunity to pathogens (1, 2), whereas excessive or overly prolonged IFN-I may be associated with autoimmunity, for example, systemic lupus erythematosus (SLE) (3).

IFN-I is induced by agonists of certain TLRs, for example, by dsRNA via TLR3, by LPS via TLR4, by ssRNA and certain antiviral compounds via TLR7, and by DNA and oligonucleotides (ODNs) containing CpG motifs (e.g., CpG-ODNs) via TLR9. IFN-I induction by TLR3 and TLR4 is dependent on Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) signaling, whereas IFN-I induction by TLR7 and TLR9 is dependent on MyD88. Recruitment and activation of IL-1R–associated kinase (IRAK)1 are required for induction of IFN-I by TLR7 or TLR9, whereas IRAK1 is not required for induction of proinflammatory cytokines by these receptors (4). Initial signaling by PRRs leads to induction of a first wave of IFN-I composed of IFN-β and IFN-α. First-wave IFN-I induces autocrine or paracrine signaling through IFN-I receptor (IFN-IR) to induce a second wave of IFN-I, including all forms of IFN-α and IFN-β, amplifying the IFN-I response. IFN-I signaling results in the induction of a number of IFN-I–stimulated genes (ISGs).

Most pathogens express agonists of multiple PRRs, which may differ in their signaling and regulatory effects. TLR2 and TLR9 induce partially overlapping arrays of cytokines. In contrast to TLR9, TLR2 does not generally induce IFN-I, although signaling by some pathogens through TLR2 may induce IFN-I (5). Simultaneous activation of two or more TLRs, which may mimic physiological situation during host–pathogen interaction, can have synergistic, antagonistic, or additive effects on cytokine responses. However, very little is known about the effect of cross-talk of different TLRs on induction of IFN-I.

We used Mycobacterium tuberculosis as a model, because this pathogen, like many bacteria, expresses agonists of both TLR2 (lipoproteins, glycolipids) and TLR9 (DNA-containing CpG motifs) (6–11), and both TLR2 and TLR9 contribute to host resistance to M. tuberculosis infection (12). Although the impact of IFN-I on tuberculosis pathogenesis remains unclear, M. tuberculosis induces
IFN-I and ISGs in peripheral blood neutrophils in human tuberculosis (13). *M. tuberculosis* induction of IFN-I is associated with decreased production of other cytokines, for example, IL-1β (14), TNF-α, and IL-12 (15). IFN-I increases growth of *M. tuberculosis* in macrophages and increases disease progression (16–18). We recently demonstrated that TLR2 signaling by *in macrophages and increases disease progression (16–18). We recently demonstrated that TLR2 signaling by *in macrophages and increases disease progression (16–18). We recently demonstrated that TLR2 signaling by

**M. tuberculosis** or other TLR2 agonists inhibited TLR9 induction of IFN-I and IFN-I–dependent MHC-I Ag cross processing (19). In the current study, we investigated the mechanisms by which TLR2 signaling inhibits induction of IFN-I. Our results show that TLR2 signaling inhibits MyD88–dependent induction of IFN-I through TLR9 or TLR7 by interfering with intracellular signaling through a novel mechanism that includes rapid degradation of IRAK1. This mechanism may shape the role of IFN-I in host–pathogen interactions when both TLR2 and TLR7/9 agonists are present. This mechanism may be exploited by pathogens to evade host defenses. Alternatively, it may be a mechanism for host protection against deleterious effects of IFN-I. Understanding this inhibitory pathway may allow its exploitation to inhibit deleterious effects of IFN-I in other disease settings, for example, autoimmune disease.

**Materials and Methods**

**Abs and reagents**

Triacylated LpqH lipopeptide containing 15 aa of the N-terminal sequence of *M. tuberculosis* LpqH (19-kDa lipoprotein) was purchased from EMC Microcollections (Tübingen, Germany). *M. tuberculosis* lipoprotein LpqG was purified, as described (20). CpG ODN-A2336 (5′-ggG GAC GAC GTC Gtg ggg ggG-3′) and CpG ODN-B1668 (5′-tcc atg aag ttc tct atg ct-3′) were synthesized by Eurofins MWG Operon (Huntsville, AL) or Sigma-Aldrich (St. Louis, MO); lowercase letters in ODN sequences refer to nucleotides for which the 3′ internucleotide linkage was phosphorothioate modified, and uppercase letters refer to standard phosphodiester-linked nucleotides. Poly(I:C), LPS (ultrapure Escherichia coli 0111:B4), ssRNA40, and synthetic lipopeptides Pam3CSK4 and FSL-1 were purchased from Cell Signaling Technology (Boston, MA). Anti–IRAK1 (4504), anti-IRAK4 (4363), and anti-MyD88 (4283) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–IL-12p40 or IL-10 (R&D Systems, Minneapolis, MN). For quantitative real-time RT-PCR (qRT-PCR), RNA was isolated from DCs using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA).

**Murine cell culture and media**

Standard medium was RPMI 1640 with 1-glutamine, glucose, 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 50 μM 2-ME, 1 mM sodium pyruvate, and penicillin-streptomycin. Dendritic cells (DCs) were prepared from femur and tibia bone marrow cells of C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME); TLR2−/−, TLR9−/−, or MyD88−/− mice (provided by S. Akira, Osaka University, on a C57BL/6 background); IFN-IR−/−. Murine IFN-β was from PBL InterferonSource (Piscataway, NJ). Cycloheximide (C7698) was from Sigma-Aldrich. Anti-IRAK1 (4504), anti-IRAK4 (4363), and anti-MyD88 (4283) Abs were purchased from Cell Signaling Technology (Boston, MA). Anti–β-actin and anti-TRAF6 (sc-7221) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Bacteria**

*M. tuberculosis* (strain H37Ra) was from the American Type Culture Collection (Manassas, VA). Bacteria were grown in 7H9 medium (Difco, Lawrence, KS), supplemented with 1% glycerol, 0.05% Tween 80 (Sigma-Aldrich), and 10% albumin/dextrose/catalase (BD Biosciences, Franklin Lakes, NJ). Bacteria were cultured with shaking at 37°C to midlog phase (∼1 wk) and harvested by centrifugation at 5000 × g for 30 min at 4°C. Bacteria were washed in RPMI 1640 medium, resuspended in RPMI 1640 medium supplemented with 10% FCS and 6% glycerol, and flash-frozen in a dry ice/ethanol bath. Prior to use in experiments, bacteria were thawed at 37°C for 30–60 min, declumped with 10 passes through a 23-gauge needle, and centrifuged at 200 × g for 2 min to pellet large clumps. The resulting bacteria in suspension were used for experiments. Bacterial CFU values were quantified by culture of serial dilutions of declumped bacteria. Gamma-irradiated *M. tuberculosis* H37Rv whole-cell lysate was prepared at Colorado State University, and was received as part of National Institutes of Health, National Institute of Allergy and Infectious Diseases Contract HHSN266200400091C, entitled “Tuberculosis Vaccine Testing and Research Materials.”

**ELISA and quantitative real-time PCR**

DCs were cultured at 2 × 106 cells/well in flat-bottom 96-well plates or 3–5 × 105 cells/well in 12-well tissue-culture-treated plates for 2–24 h with or without agonists. Plates were centrifuged to pellet cells, and supernatants were diluted and tested by ELISA for mouse IFN-α or IFN-β (PBL InterferonSource) or mouse IL-12p40 or IL-10 (R&D Systems, Minneapolis, MN). For quantitative real-time RT-PCR (qRT-PCR), RNA was isolated from DCs using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Total RNA was extracted following on-column DNase digestion using RNeasy Plus mini columns (Qiagen) and collected in RNase-free water. Yield was determined by OD. Oligo(dT)-primed reverse transcription of...
RNA into cDNA was performed with QuantiTect Reverse Transcription Kit (Qagen), and 4% of the product was used for each qRT-PCR sample using Bio-Rad iQ SYBR Green Supermix and the Bio-Rad CFX96 fluorescence detection system (Bio-Rad, Hercules, CA). All conditions were tested in triplicate. Primer pairs from murine gene sequences were for total IFN-α (sense, 5'-ATG GCT AGR CTC TGT GCT TTC CT-3'; antisense, 5'-AGG GCT CTC CAG AYT TCT GCT CTC-3'), IFN-β (sense, 5'-CAT CAA CTA TAA GCA GCT CCA-3'; antisense, 5'-TTC AAG AGG AGA GCA GGT GTT GAG-3'), and GAPDH (sense, 5'-AAC GAC CCC TCT ATT GAC-3'; antisense, 5'-TCC ACG ACA TAC TCA GGA C-3'), as described (22), or designed using Clone Manager Suite v7.11 and Primers Designers v5.11 (Scientific & Educational Software, Cary, NC). A BLAST search was performed to verify specificity.

Western blot analysis of whole-cell lysates

DCs were incubated with agonists in 12-well plates at 37°C. Subsequent steps were performed at 4°C, unless otherwise stated. Cells were washed in PBS with protease inhibitor mixture (P8340; 1:200; Sigma-Aldrich), 1 mM NaF, 1 mM PMSF, and 10 nM calyculin A. To prepare whole-cell lysates, cells were pelleted and resuspended in 160 μl RIPA lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% Na-deoxycholate, and 0.1% SDS [pH 7.4]) with protease inhibitors and mixed on a rotator for 1 h. Samples were centrifuged at 16,000 g for 10 min, and 150 μl supernatant was collected. Aliquots containing equal quantities of total protein were added to reducing sample buffer (Thermo Fisher Scientific; 39000), subjected to electrophoresis on 8% SDS polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. Membranes were washed in PBS with 0.1% Tween 20 (PBST), incubated for 1 h at room temperature in 5% nonfat milk in PBST, incubated overnight at 4°C with primary Ab in 3% BSA or 5% nonfat milk (PBST), incubated for 1 h at room temperature with HRP-labeled secondary Ab (Cell Signaling Technology), and developed with the ECL detection kit (Thermo Fisher Scientific; 32106).

Results

M. tuberculosis and TLR2 agonist lipopeptide inhibit TLR9-induced IFN-I production

To test the effect of TLR2 signaling on TLR9 induction of IFN-I, we incubated DCs with CpG ODN-A2336 (CpG-A ODN) in combination with M. tuberculosis strain H37Rv or synthetic LpqH lipopeptide (containing the N-terminal sequence of M. tuberculosis lipoprotein LpqH). CpG-A ODN induced IFN-α and IFN-β production in Flt3L DCs, but this induction was inhibited by M. tuberculosis or LpqH lipopeptide (Fig. 1A, 1B). The inhibition was dose dependent, and IFN-I induction was strongly inhibited with 400 nM LpqH lipopeptide or M. tuberculosis H37Rv at a multiplicity of infection of 1 (Fig. 1). In TLR2−/− DCs, however, CpG-A ODN induction of IFN-α and IFN-β was not significantly inhibited by M. tuberculosis or LpqH lipopeptide, indicating that the inhibitory effect was dependent on TLR2 (Fig. 1A, 1B). A lysate of M. tuberculosis H37Rv (see Materials and Methods) inhibited CpG-A ODN induction of IFN-β, similar to the inhibition achieved by LpqH lipopeptide, confirming that molecules expressed by M. tuberculosis recapitulate the inhibition of IFN-I observed with purified TLR2 agonist (Supplemental Fig. 1A). DCs also produce other cytokines in response to TLR signaling, including IL-12p40, IL-10, and others. CpG-A ODN induced higher production of these cytokines than LpqH lipopeptide in DCs (Fig. 1E, 1F). When DCs were incubated with both agonists, LpqH lipopeptide did not significantly inhibit CpG-A ODN-induced IL-12p40 (Fig. 1E) or IL-10 (Fig. 1F). We conclude that DCs incu-
bated with TLR2 agonists are viable and continue to produce IL-10 and IL-12p40, but show inhibition of TLR9-induced IFN-1.

**TLR2 agonist inhibits TLR9-induced IFN-1 mRNA expression**

IFN-1 production can be regulated at multiple levels, including mRNA expression. CpG-A ODN-induced expression of IFN-α and IFN-β mRNA in DCs was significantly inhibited by LpqH lipopeptide; the inhibition was greater for IFN-β than IFN-α (Fig. 2A, 2B). In addition to Flt3L-derived DCs, which represent our primary experimental system and comprise a mixture of mDCs and pDCs, the same effect was observed in GM-CSF-derived DCs (Fig. 2C), which are mDCs (21). Kinetic studies revealed significant TLR2-induced inhibition of mRNA for both IFN-α and IFN-β from 2 to 24 h (Fig. 2D–G). In studies of earlier time points (15–120 min), we found that TLR9 induced IFN-β mRNA at 1 h, and TLR2 inhibition of IFN-β mRNA occurred at this time point (data not shown), suggesting the inhibition occurs rapidly.

**TLR2 agonist inhibits first-wave IFN-1 induction**

We considered two stages of IFN-1 mRNA induction at which its expression could be regulated. TLR signaling directly induces first-wave IFN-1 (restricted to IFN-β and IFN-α4), and the expression of IFN-1 species can be substantially amplified by the autocrine/paracrine IFN-1 positive feedback loop via the IFN-IR, which induces second-wave IFN-1. Therefore, the TLR2–mediated inhibition of IFN-1 expression could involve inhibition of either first- or second-wave IFN-1.

We used two approaches to determine whether TLR2-mediated inhibition of IFN-1 expression involves inhibition of the first or second wave of IFN-1 induction. In the first approach, we treated DCs with exogenous mouse rIFN-β to induce second-wave IFN-1, which we assessed by mRNA expression. Addition of LpqH lipopeptide did not inhibit IFN-β–induced expression of IFN-β mRNA (Fig. 3A, 3B), suggesting that mechanisms for induction of second-wave IFN-1 were not inhibited. We considered that the design of our original experiments with simultaneous addition of LpqH lipopeptide and CpG-A ODN might activate TLR2 signaling hours prior to the time course of second-wave IFN-1 induction. Accordingly, in some experiments, we pretreated DCs with or without LpqH lipopeptide (400 nM) for 3 h before adding IFN-β (200 pg/ml) for 6 h to mimic this situation, but this protocol still did not produce inhibition of second-wave IFN-β (Fig. 3A). These results indicate that mechanisms intrinsic to the second-wave induction of IFN-1 are not inhibited by TLR2 signaling.

The second approach eliminated second-wave IFN-1 by using IFN-IR−/− DCs, because the IFN-IR is required for second-wave IFN-1. Therefore, all IFN-1 production in IFN-IR−/− cells is by the first wave. In IFN-IR−/− DCs, LpqH lipopeptide inhibited induction of IFN-β mRNA by 3 h (Fig. 3C, 3D) and IFN-β protein by 4 h (Supplemental Fig. 1B) or 24 h (Fig. 3E, 3F) after stimulation by CpG-A ODN. These results indicate that the inhibitory mechanism blocks induction of first-wave IFN-1 by TLR9 signaling.

In these experiments, wild-type DCs produced higher amounts of IFN-β than IFN-IR−/− cells, which is expected when second-wave IFN-1 can supplement the first wave. For example, wild-type DCs produced 8- to 9-fold more IFN-β than IFN-IR−/− DCs after 24 h of stimulation with CpG-A ODN, a time point that allowed a substantial period for first wave to induce the second wave (compare Fig. 3E, 3F). After 3 h, when there was less time to induce the second wave, IFN-β mRNA was ∼3-fold higher in wild-type DCs compared with IFN-IR−/− DCs. We performed additional experiments to assess IFN-β expression by ELISA at early time points (1, 2, 4, and 6 h). IFN-β was not detected after 1–2 h of stimulation with CpG-A ODN (data not shown) and was first detected after 4 h of stimulation, at which time its production was already inhibited by LpqH lipopeptide (Supplemental Fig. 1).
TLR2-mediated inhibition of IFN-I induction is not dependent on intracellular signaling or new protein synthesis

TLR2 may inhibit induction of IFN-I either directly through intracellular signaling effects of TLR2 or indirectly through signaling by TLR2-induced cytokines. For example, cytokines such as IL-10 or TNF-α inhibit induction of IFN-I (23, 24). To examine this issue, we tested IFN-β mRNA expression at early time points in a mixed culture of TLR9−/− DCs and TLR2−/− DCs. TLR9−/− DCs cannot induce IFN-β mRNA in response to CpG-ODN, but can make TLR2-induced cytokines, whereas TLR2−/− DCs cannot respond directly to TLR2 agonists, but can respond to TLR2-induced cytokines from other cells. Therefore, this mixed culture would allow indirect inhibition (via TLR2-induced cytokines), but not direct TLR2 signaling inhibition of IFN-I induction by TLR9. LpqH lipopeptide inhibited CpG-A ODN-induced IFN-β mRNA in wild-type DCs alone, but not in TLR2−/− DCs alone (Fig. 4). When TLR2−/− and TLR9−/− DCs were mixed at a 1:1 ratio, LpqH lipopeptide did not inhibit CpG-A ODN-induced IFN-β mRNA (Fig. 4). IFN-I production in the mixed cell system was half of that seen in wild-type DCs alone, because half of the cells were unable to respond to CpG-A ODN. These results suggest that TLR2 inhibits IFN-I induction directly through intracellular signaling and not indirectly via intracellular signaling by TLR2-induced cytokines.

Regulation of IFN-I induction may involve de novo synthesis of negative regulators or the modification (e.g., phosphorylation, ubiquitination) and/or degradation of existing signaling components, which could occur rapidly and without synthesis of new proteins. Accordingly, we used cycloheximide, a protein synthesis inhibitor, to determine whether the inhibitory mechanism requires protein synthesis. Cycloheximide did not block the ability of LpqH lipopeptide to inhibit CpG-A ODN-induced IFN-β mRNA (Fig. 5). In contrast, the inhibition of IFN-β induction by CpG-B ODN [described previously (22)] was reversed with cycloheximide (and CpG-B ODN became an inducer of IFN-I, like CpG-A ODN, rather than an inhibitor), providing a positive control for cycloheximide function and establishing that TLR2-mediated inhibition occurs by a mechanism distinct from that previously reported for inhibition of IFN-I production by CpG-B ODN. These results indicate that inhibition of IFN-I induction occurs by direct TLR2 intracellular signaling mechanisms that affect the activity of pre-existing signaling components.

TLR2 signaling inhibits induction of IFN-I by TLR7 or TLR9, but not TLR3 or TLR4

TLR9, TLR3, TLR4, and TLR7 can all induce IFN-I. Induction of IFN-I by TLR3 and TLR4 is dependent on TRIF, whereas induction of IFN-I by TLR7 and TLR9 is dependent on MyD88. To dissect the mechanism by which TLR2 signaling inhibits first-wave IFN-I

**FIGURE 4.** TLR2-mediated inhibition of IFN-I production occurs by an intracellular mechanism. Incubations included Flt3L-derived DCs (3–5 × 10⁶/well) from wild-type, TLR2−/−, or TLR9−/− mice, or a 1:1 mixture of TLR2−/− and TLR9−/− DCs (total of 3–5 × 10⁶ cells). DCs were cultured for 3 h in 12-well plates with medium, LpqH lipopeptide (400 nM), CpG-A ODN (300 nM), or both agonists. Expression of mRNA for IFN-β was determined by qRT-PCR with normalization to GAPDH and is expressed as fold-change relative to expression with medium alone. Data represent means and SDs of triplicate samples and are representative of three or more independent experiments. Student t test was performed to compare results with CpG-A ODN alone to results with CpG-A ODN plus LpqH lipopeptide (**p < 0.001, *results are plotted, but are too low to be visualized on this graph).
induction, we tested the ability of TLR2 signaling to inhibit IFN-I induction via MyD88-dependent versus TRIF-dependent pathways. LpqH lipopeptide inhibited IFN-β mRNA induction by CpG-A ODN (TLR9 agonist) and ssRNA40 (TLR7 agonist), but not poly(I:C) (TLR3 agonist) or LPS (TLR4 agonist) (Fig. 6). LpqH lipopeptide also inhibited TLR7 induction of IFN-β at the protein level (Fig. 6C). These data indicate that TLR2 signaling inhibits MyD88-dependent IFN-I induction, but not TRIF-dependent IFN-I induction.

**TLR2-mediated inhibition of IFN-I expression is correlated with rapid depletion of IRAK1**

We tested the hypothesis that TLR2 signaling disrupts the TLR9 signaling complex, focusing on IRAK1, because it has an important role in MyD88-dependent induction of IFN-I by TLR9 (4). Upon activation by CpG-A ODN, TLR9 recruits MyD88 and activates IRAK4, IRAK1, and TRAF6, which form an intermediate signaling complex. The complex then phosphorylates IFN regulatory factors, which translocate into the nucleus to transcribe IFN-I genes. In our studies, CpG-A ODN did not induce IFN-I in IRAK1−/− DCs, confirming that IRAK1 is required for induction of IFN-I (Fig. 7A). Interestingly, IRAK1 was rapidly and significantly degraded within 10 min of stimulation with LpqH lipopeptide (Fig. 7B). In contrast, TLR9 signaling did not cause significant degradation of IRAK1 until ∼2 h, and even then to a lesser degree than TLR2 signaling. When added simultaneously with CpG-A ODN, LpqH lipopeptide also induced IRAK1 degradation (Fig. 7C). IRAK1 degradation occurred with similar kinetics after stimulation with LpqH lipopeptide alone or LpqH lipopeptide plus CpG-A ODN (400 nM), or both. Cell lysates were prepared and analyzed by Western blot for IRAK1 and β-actin (loading control). Data in A represent the means and SDs of triplicate wells. Data are representative of three or more independent experiments. Student t test was performed to compare conditions as indicated (**p < 0.01, ***p < 0.001, #results are plotted, but are too low to be visualized on this graph).

**FIGURE 6.** LpqH lipopeptide inhibits MyD88-dependent, but not TRIF-dependent induction of IFN-I expression. A and B, Flt3L-derived DCs were cultured for 3 h with medium, poly(I:C) (10 μg/ml), LPS (50 ng/ml), CpG-A ODN (300 nM), or ssRNA (1 μg/ml) with or without LpqH lipopeptide (400 nM). Expression of mRNA for IFN-β was determined by qRT-PCR with normalization to GAPDH and is expressed as fold-change relative to expression with medium alone. C, Flt3L-derived DCs were cultured for 24 h with medium, LpqH lipopeptide, ssRNA, or both agonists. Supernatants were assessed by ELISA for IFN-β. Data represent means and SDs for triplicate samples and are representative of three or more independent experiments. Student t test was performed to compare conditions as indicated (**p < 0.01, ***p < 0.001, #results are plotted, but are too low to be visualized on this graph; n.d., not detected).

**FIGURE 7.** LpqH lipopeptide induces rapid IRAK1 depletion in DCs. A, Flt3L-derived DCs from wild-type C57BL/6 or IRAK1−/− mice were cultured for 3 h with medium, LpqH lipopeptide (400 nM), CpG-A ODN (300 nM), or both agonists. Expression of mRNA for IFN-β was determined by qRT-PCR with normalization to GAPDH and is expressed as fold-change relative to expression with medium alone with wild-type DCs. B, Flt3L-derived DCs were left untreated or treated for various periods with CpG-A ODN (300 nM) or LpqH lipopeptide (400 nM). C, Flt3L-derived DCs were cultured for 30 or 120 min with medium, LpqH lipopeptide (400 nM), CpG-A ODN (300 nM), or both. Cell lysates were prepared and analyzed by Western blot for IRAK1 and β-actin (loading control). Data in A represent the means and SDs of triplicate wells. Data are representative of three or more independent experiments. Student t test was performed to compare conditions as indicated (**p < 0.001, #results are plotted, but are too low to be visualized on this graph).
also induced IRAK1 degradation (perhaps to a lesser degree than Pam3CSK4 or LpqH lipopeptide, but to a greater degree than CpG-A ODN) (Supplemental Fig. 1C). These data confirm that multiple TLR2 agonists can induce degradation of IRAK1, although different agonists may differ in potency. Thus, among the early TLR signaling components tested, a TLR2-dependent mechanism selectively targets IRAK1 for degradation.

**Discussion**

Although many TLR agonists drive IFN-I production, TLR2 agonists do not. Moreover, in our previous study, we showed that TLR2 signaling inhibited TLR9-induced IFN-I production and IFN-I–dependent MHC-I cross processing (19). In this study, we dissected the mechanisms by which TLR2 signaling inhibits induction of IFN-I. We demonstrate that TLR2 signaling rapidly inhibits MyD88-dependent induction of first-wave IFN-I through TLR9 or TLR7, as early as the initial time point when TLR9-induced IFN-I can be detected (~1 h for mRNA, and ~4 h for protein by ELISA). The inhibition involves a protein synthesis–independent intracellular signaling mechanism that affects MyD88-dependent, but not TRIF-dependent IFN-I induction. We considered a role for IRAK2, which has been implicated as a negative regulator of IFN-I in other systems (25), but the inhibitory mechanism was still observed in DCs from IRAK2 knockout mice (data not shown). Because IRAK1 is known to be required for MyD88-dependent induction of IFN-I by TLR9 (4), we considered the hypothesis that inhibition or depletion of IRAK1 might explain the inhibition of IFN-I induction. Interestingly, we observed that IRAK1 degradation was rapidly induced by TLR2 agonists, but not a TLR9 agonist (which caused lesser IRAK1 degradation only after longer periods). Because IRAK1 is essential for TLR9- or TLR7–induced production of IFN-I, but not other cytokines (e.g., IL-12p40) (Supplemental Fig. 1D, 1E) (4), we propose that rapid depletion of IRAK1 is a novel mechanism by which TLR2 signaling inhibits IFN-I induction by TLR9 or TLR7.

We investigated the possibility that this inhibition may occur directly through intracellular signaling by TLR2 and TLR9 in the same cell or indirectly via intercellular communication, for example, through cytokines induced by TLR2 in one cell that act upon another cell. Cytokines such as IL-10 or TNF-α have been reported to be responsible for inhibiting IFN-I induction by TLR9 in some studies (23, 24). Accordingly, we investigated indirect inhibitory mechanisms by mixing TLR2/−/− DCs with TLR9/−/− DCs. Based on our discovery that the mechanism involves inhibition of first-wave IFN-I, we focused the mixed TLR knockout experiment on IFN-I mRNA expression at a relatively early time point (3 h). This experiment revealed that inhibition is not observed in mixed TLR2/−/− and TLR9/−/− DCs, indicating that the early rapid inhibition of IFN-I mRNA expression is dependent on direct intracellular signaling and did not occur by intercellular communication that was recapitulated by mixing TLR2/−/− with TLR9/−/− cells. Furthermore, TLR2-mediated inhibition was not dependent on new protein synthesis, on which the synthesis of potential inhibitory cytokines is dependent. In addition, we found the TLR9 agonist CpG-A ODN induced more IL-10 and TNF-α than the TLR2 agonist LpqH lipopeptide, yet CpG-A ODN induces high levels of IFN-I. These observations indicate that IL-10 and TNF-α are not the major drivers of the inhibition of IFN-I production in this system. However, indirect mechanisms could still be partly responsible for inhibition at later time points. In summary, these results indicate a rapid intracellular signaling mechanism by which TLR2 inhibits the induction of IFN-I by TLR9 or TLR7, consistent with the proposed mechanism involving IRAK1 degradation.

TLR2-mediated inhibition of MyD88-dependent IFN-I induction could shape host immune responses in response to stimuli that combine TLR2 agonists with TLR9 or TLR7 agonists. Pathogens that express agonists of both TLR2 as well as TLR9/7 include a wide range of bacteria (e.g., *M. tuberculosis*) and some viruses (e.g., HSV, CMV, EBV) (26–28). In addition, pathogens can induce host-derived TLR2 agonists, which may affect responses to pathogen-derived TLR9/7 agonists. Moreover, during bacteria/virus coinfection (e.g., *M. tuberculosis–HIV* coinfection), host cells may be stimulated by both viral TLR7/9 agonists and bacterial TLR2 agonists. In these situations, TLR2 agonists may inhibit TLR9/7–induced IFN-I production. Thus, TLR2–mediated inhibition of TLR9/7–induced IFN-I induction may be important in understanding immune responses to infections and vaccines when both TLR2 and TLR7/TLR9 agonists present, but additional study of specific models is required.

Mechanisms to control IFN-I production may play a role in autoimmune or inflammatory disorders. DCs and IFN-α play a central role in SLE (29), and neutralizing anti–IFN-α mAb therapies are in development or clinical trials (30). IRAK1 has been suggested to play a critical role in the pathogenesis of SLE. Jacob et al. (31) found five single-nucleotide polymorphisms spanning the *IRAK1* gene that showed disease association in both adult- and childhood-onset SLE. Moreover, IRAK1 deficiency abrogated all lupus-associated phenotypes in congenic mouse models bearing the *Sle1* or *Sle2* disease loci (31). Although more studies are needed to assess the role of these disease-associated single-nucleotide polymorphisms in IRAK1 expression and function, as well as IFN-α production or ISG signature in these patients, impaired IFN-α production caused by IRAK1 deficiency may be a protective factor in pathogenesis of certain IFN-I–driven autoimmune disorders, for example, SLE and Sjögren’s syndrome. In these scenarios, mechanisms to control IRAK1 expression or activity may have therapeutic potential.

In summary, our studies reveal rapid TLR2–mediated degrada- tion of IRAK1 as a mechanism to inhibit IFN-I induction by TLR9 or TLR7. This mechanism may shape the role of IFN-I in host–pathogen interactions when both TLR2 and TLR9/7 agonists are present. This mechanism may be exploited by pathogens to evade host defenses that depend on IFN-I. In contrast, we propose that negative regulation of IFN-I expression by TLR2 is part of a host regulatory program that allows IFN-I expression for host defense in viral infections, but inhibits IFN-I expression during infections with certain bacteria that trigger TLR2 (in which IFN-I may be harmful rather than helpful to host defense). Moreover, this may be a mechanism for host protection against deleterious effects of IFN-I in autoimmune disease, and understanding of this inhibitory pathway and mechanisms for IRAK1 degradation may reveal therapeutic approaches for these disorders.

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**Disclosures**

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**References**


