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Toll-Like Receptor 3 Mediates a More Potent Antiviral Response Than Toll-Like Receptor 4[1]

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We have recently described an IFN regulatory factor 3-mediated antiviral gene program that is induced by both Toll-like receptor (TLR)3 and TLR4 ligands. In our current study, we show that activation of IFN/viral response gene expression in primary macrophage cells is stronger and prolonged with TLR3 stimulation compared with that of TLR4. Our data also reveal that the cytoplasmic tails of both TLR3 and TLR4 can directly interact with myeloid differentiation factor 88 (MyD88). However, although Toll/IL-1 receptor homology domain-containing adaptor protein/MyD88 adaptor-like is able to associate with TLR4, we were unable to detect any interaction between Toll/IL-1 receptor homology domain-containing adaptor protein/MyD88 adaptor-like and TLR3. By using quantitative real-time PCR assays, we found that TLR3 expression is inducible by both TLR3 and TLR4 ligands, while TLR4 expression is not inducible by these same stimuli. Furthermore, using cells derived from mice deficient in the IFN-$\alpha\beta$R, we show that both TLR3 and TLR4 require IFN-β autocrine/paracrine feedback to induce TLR3 expression and activate/enhance genes required for antiviral activity. More specifically, a subset of antiviral genes is initially induced independent of IFN-β, yet the cytokine further enhances expression at later time points. This was in contrast to a second set of genes (including *TLR3*) that is induced only after IFN-β production. Taken together, our data argue that, despite both TLR3 and TLR4 being able to use IFN-β to activate/enhance antiviral gene expression, TLR3 uses multiple mechanisms to enhance and sustain the antiviral response more strongly than TLR4. ***The Journal of Immunology*****, 2003, 170: 3565–3571.**

Mammalian Toll-like receptors (TLRs)[4] recognize conserved pathogen-associated molecular patterns (PAMPs) and are thought to be prominent among the initial triggers that allow the infected host to develop an immune response. Thus far, there have been 10 TLRs identified in mice and humans, with each receptor recognizing a unique set of PAMPs (1). Upon ligand binding, TLRs have been shown to activate a variety of signaling pathways, including phosphoinositide 3-kinase, Jun N-terminal kinase (JNK), p38, NF-κB, extracellular signal-related kinase, and IFN regulatory factor (IRF)3, leading to the induction of numerous target genes involved in inflammation, cellular differentiation, and direct antimicrobial activity (2–4). In addition to mediating innate immunity, TLRs play an important role in activating and directing adaptive immune responses (5–7). To better understand how TLRs regulate these events, a detailed understanding of the signaling mechanisms used by TLRs is required.

A well-known adaptor molecule used by TLRs is myeloid differentiation factor 88 (MyD88). MyD88 activates both the serine-threonine kinases IL-1R-associated kinase-1 and IL-1R-associated kinase-4, and TNFR-associated factor 6. Activation of these adaptor molecules leads to the subsequent induction of mitogen-activated protein kinases (such as p38, extracellular signal-related kinase, and JNK) as well as NF-κB. Signaling downstream from certain TLRs, such as TLR9, appears to be completely dependent on MyD88 (8, 9). However, NF-κB and mitogen-activated protein kinases can still be activated by TLR3 or TLR4 in cells derived from mice deficient in MyD88, supporting the existence of an alternative, MyD88-independent pathway (3, 8, 10). Recently, an adaptor molecule called Toll/IL-1 receptor homology domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like (MAL) has been identified (11, 12). TIRAP/MAL has been shown to bind directly to TLR4 and mediate NF-κB and JNK activation. Additionally, cells deficient in TIRAP/MAL are defective in their ability to induce expression of proinflammatory cytokine production following stimulation with TLR4 or TLR2 ligands (13, 14).

We have recently described a TLR3/4-specific antiviral gene program involving the activation of IRF3 (15). Once activated, IRF3 mediates the transactivation of a set of primary genes (as determined by resistance to cycloheximide treatment) including IFN-β. Secreted IFN-β then instigates an autocrine/paracrine loop leading to the production of a set of secondary genes, such as IFN-inducible 204 (*IFI-204*), many of which are thought to be involved in antiviral and antimicrobial responses. The unique ability of TLR3/4 to induce the IRF3-mediated antiviral program suggests that these receptors have diverged evolutionarily from other TLRs. Because the adaptor MyD88 is expendable for TLR3/4

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[4] Abbreviations used in this paper: TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; JNK, Jun N-terminal kinase; IRF, IFN regulatory factor; MyD88, myeloid differentiation factor 88; TIR, Toll/IL-1 receptor homology domain; TIRAP, TIR-containing adaptor protein; MAL, MyD88 adaptor-like; IFI-204, IFN-inducible 204; IFNAR, IFN-$\alpha\beta$R; BMM, bone marrow-derived macrophage; IP10, IFN-inducible protein 10; MHV68, murine gammaherpesvirus 68.

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IRF3 activation, the recent discovery of TIRAP/MAL has provided a possible link between TLR3/4 and IRF3 (10). However, a number of reports have presented conflicting data on the role of TIRAP/MAL-mediated activation of this pathway. Recent data obtained using TIRAP/MAL knockout mice has shown that IRF3 can still be activated in TIRAP/MAL-deficient cells following TLR4 stimulation and that TLR3 has no apparent defect in inducing its target genes (14). This is in contrast to other studies that have demonstrated that TLR4 induction of both IRF3- and IFN-β-specific reporter constructs was inhibited by dominant-negative TIRAP/MAL (3, 10). In addition, TLR4-mediated gene expression of IFN-β in RAW 264.7 cells was blocked by a TIRAP/MAL inhibitory peptide that has been previously described (10). Finally, work by Fujita and colleagues (3) showed that overexpression of TIRAP/MAL can lead to the activation of IRF3 while overexpression of dominant negative TIRAP/MAL is unable to block IRF3 reporter gene activity following stimulation with the TLR3 agonist poly(I:C). Despite being unable to clearly identify which adaptor molecule(s) are responsible for activating the IRF3 pathway, these data suggest that TLR3 and TLR4 may use distinct adaptor molecule-containing complexes to initiate signaling. To date, TLR4 has been shown to directly interact with MyD88 and TIRAP/MAL. However, the ability of TLR3 to directly interact with these adaptor molecules has not been analyzed (11, 12, 16).

Blocking Ab experiments strongly suggest that IFN-β is required for TLR3/4-mediated activation of the antiviral response; however, genetic data has not been presented to support this model (10, 15). Although induced by both TLR3 and TLR4, the intensity and duration of antiviral gene expression has not been determined on a comparative level between these receptors. In addition, the contribution of IFN-β to TLR3- and TLR4-inducible genes classified as both primary and secondary according to cycloheximide sensitivity has not been clearly shown.

In this study, we sought to clarify the differences between TLR3 and TLR4 induction of the antiviral gene program. Gene expression studies reveal that TLR3 can induce antiviral genes with greater intensity and duration than TLR4. Our data suggest that two mechanisms may contribute to this outcome. First, TLR3 and TLR4 appear to use distinct receptor-proximal signaling complexes. Second, while TLR4 gene expression is not inducible by TLR3 and TLR4 agonists, TLR3 is induced to high levels by the same stimuli. We also show, using IFN-$\alpha\beta$R (IFNAR)-1-deficient (IFNAR$^{-/-}$) cells, that both TLR3 and TLR4 use IFN-β to enhance the expression of certain antiviral genes, whereas the inducible expression of other genes, including TLR3, is completely dependent on IFN-β feedback. Finally, we find that IFNAR is absolutely required for TLR3- and TLR4-mediated antiviral activity.

Materials and Methods

Cell culture and reagents

Murine bone marrow-derived macrophages (BMMs) were differentiated from marrow as previously described (15). A129 (IFNAR-1$^{-/-}$) (17) and B6129SF2/J wild-type control mice were obtained from B&K Universal (Grimston, Aldbrough Hull, U.K.) and The Jackson Laboratory (Bar Harbor, ME), respectively. C57/B6 mice were used for all experiments not involving the A129 mice (The Jackson Laboratory). Specific TLR activation was achieved using F-583 (Rd mutant) *Escherichia coli* lipid A for TLR4 (Sigma-Aldrich, St. Louis, MO), CpG oligonucleotides for TLR9 (Invitrogen, San Diego, CA), and poly(I:C) for TLR3 (Pharmacia, Peapack, NJ). For experiments using the TIRAP/MAL inhibitory peptide (CN Biosciences, San Diego, CA), cells were pretreated for 1 h with 20 μM peptide or DMSO alone. Cells were then stimulated with PAMPs in the presence of the inhibitory peptide. For experiments using murine rIFN-β (R&D Systems, Minneapolis, MN), wild-type macrophage cells were stimulated with 10, 100, or 1000 U. Viral infection and harvest were performed using MHV68 at a multiplicity of infection of five, as previously described (15).

mRNA quantification

RNA was isolated by standard guanidium isothiocyanate methods. cDNA template for quantitative real-time PCR analysis was then synthesized, and PCR was performed using the iCycler thermocycler (Bio-Rad, Hercules, CA) as previously described (15). IFN-β, IFN-inducible protein 10 (IP10), IκBα, and IFI-204 primers were the same as those previously described (15). For other genes, the following primers were used: IL-6, forward, CACAGAGGATACCACTCCCAACA, and reverse, TCCACGATTTC CCAGAGAACA; TLR3, forward, TCTGGAAACGCGCAAACC, and reverse, GCCGTTGGACTCTAAATTCAAGAT; TLR4, forward, AGAAA TTCCTGCAGTGGGTCA, and reverse, TCTCTACAGGTGTTGCACAT GTCA; TIRAP, forward, CAGGCAGGCTCTGTTGAAGAA, and reverse, TGTGTGGCTGTCTGTGAACCA; MyD88, forward, CATGGTGGTGG TTGTTTCTGAC, and reverse, TGGAGACAGGCTGAGTGCAA; ICAM1, forward, TGTCAGCCACTGCCTTGGTA, and reverse, CAGGATCTGGTC CGCTAGCT; and L32, forward, AAGCGAAACTGGCGGAAAC, and reverse, TAACCGATGTTGGGCATCAG.

Plasmids and GST pull-down assays

A human TLR4 construct was generously provided by Dr. R. Modlin (University of California, Los Angeles). Expressed sequence tags containing the intracellular domain of human TLR3 and full-length human MyD88 were obtained from Research Genetics (Huntsville, AL). Each of the two constructs was used as a PCR template for amplification of the sequence corresponding to their respective intracellular domains. *Eco*RI and *Xho*I sites were engineered into the forward and reverse primer sequences, respectively, and used to ligate the PCR products into pGEX1λT. The recombinant constructs were then transformed into Topp10 cells by electroporation. Following isopropyl β-D-thiogalactoside-induced expression, the cells were lysed in a Sarkosyl buffer (1% Sarkosyl, 100 mM EDTA, and 1 mM DTT, in PBS) followed by sonication. The fusion proteins were then immobilized on glutathione beads (Sigma-Aldrich). The pCDNA3–2xFlag-mTIRAP/MAL construct was donated by T. Roni from the laboratory of Dr. S. Smale (University of California, Los Angeles). The TIRAP/MAL and MyD88 constructs were overexpressed in 293T cells and lysed in immunoprecipitation lysis buffer (1% Triton X-100, 400 μM EDTA, 150 mM NaCl, 20 mM HEPES (pH 7.2), 10 mM NaF, and a protease inhibitor mixture). The lysate was then incubated with the immobilized GST-TLR fusion proteins, and interactions were detected by immunoblotting with an anti-flag monoclonal or anti-MyD88 polyclonal Ab.

Immunoblotting

For STAT1 immunoblotting, cells were lysed in modified radioimmunoprecipitation assay buffer and 20 μg of protein were loaded per lane and separated by SDS-PAGE. Gels were transferred to nitrocellulose filters and immunoblotted using the Ab manufacturers' recommended instructions. Abs specific to the STAT1 or the phosphorylated forms of STAT1 were obtained from Cell Signaling Technologies (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The anti-MyD88 Ab was purchased from ProSci (Poway, CA). For detection of murine gammaherpesvirus 68 (MHV68), equal amounts were loaded in each lane and analyzed by Western blotting techniques using rabbit anti-M9. Blots were stripped and reprobed with anti-actin (Sigma-Aldrich) to verify equal loading.

Results

TLR3 is a more potent inducer of antiviral gene expression than TLR4

We have previously shown that both TLR3 and TLR4 can induce a number of antiviral/IFN-β-inducible genes (15). To compare the intensity and duration of expression of these genes following TLR3 vs TLR4 stimulation over an extended time course, we stimulated BMM cells for up to 12 h with either the TLR3 agonist poly(I:C) or the TLR4 agonist lipid A. Using quantitative real-time PCR technologies, we next assessed the expression levels of a number of antiviral genes throughout the time course. As seen in Fig. 1, IFN-β, IP10, and IFI-204 were all induced to higher levels and for extended periods of time by TLR3 compared with TLR4. Despite the increased induction of antiviral gene expression mediated by TLR3 relative to TLR4, both receptors induced IκBα

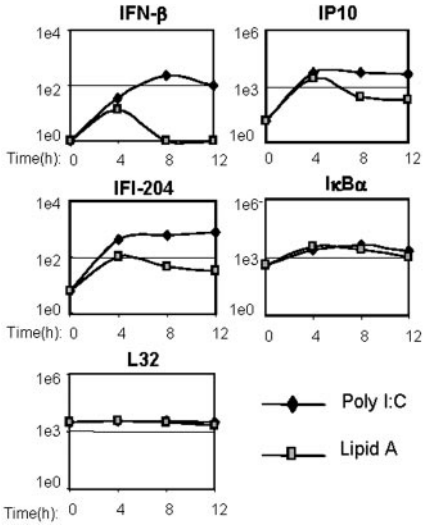

FIGURE 1. TLR3 is a more potent inducer of antiviral gene expression than TLR4. Murine BMMs were stimulated with poly(I:C) (10 μg/ml) or lipid A (1 ng/ml) for the indicated times. Total RNA was isolated and converted to cDNA for quantitative real-time PCR analysis using primers specific for IFN-β, IFI-204, IP10, IκBα, or L32. Experiments were repeated three times, and the data are presented in relative expression units on a log scale.

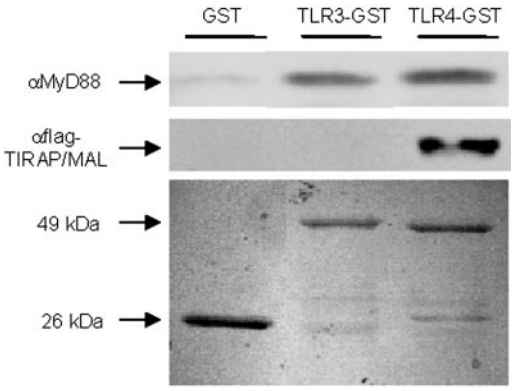

FIGURE 2. MyD88, but not TIRAP/MAL, directly interacts with TLR3. 293T lysate-containing MyD88 or flag-TIRAP/MAL was incubated with the intracellular domains of TLR3 and TLR4 fused to GST and immobilized on glutathione beads. TLR-MyD88 interaction was determined by Western blotting using a polyclonal anti-MyD88 Ab (*upper panel*). TLR-TIRAP/MAL interaction was determined by Western blotting using an anti-flag Ab to detect flag-TIRAP/MAL (*middle panel*). Equal amounts of beads containing GST-TLR3 or -TLR4 intracellular domains were boiled and the eluted proteins were size-fractionated by SDS-PAGE. Coomassie blue staining (*lower panel*) was used to ensure that comparable amounts of GST-TLR protein were loaded on the beads. The data represent three independent experiments.

mRNA to similar levels, albeit with slightly different kinetics (Fig. 1). The constitutively expressed ribosomal protein L32 was assayed to ensure equal cDNA loading. In Fig. 1, we used 10 μg/ml poly(I:C) for stimulations because it gave us comparable IκBα levels between both TLR3 and TLR4 stimulated cells. We also use 1 μg/ml poly(I:C), as shown in later figures, which still results in higher TLR3-mediated antiviral gene induction compared with TLR4 (see Fig. 5), because it is less toxic to the cells.

TLR3 can directly interact with MyD88 but not with TIRAP/MAL

The receptor-proximal signaling complexes used by TLR3 and TLR4 to activate the antiviral gene program are relatively uncharacterized. We hypothesized that these receptors interact with distinct adaptor molecule-containing complexes which may contribute to the differences in signaling output observed in Fig. 1. MyD88 and TIRAP/MAL are both TIR-domain containing adaptor molecules that have been shown to directly bind to the cytoplasmic tail of TLR4 (11, 12, 16). However, these same experiments have not been performed with TLR3. To see whether MyD88 or TIRAP/MAL is able to interact with TLR3, we performed GST pull-down assays. To conduct these experiments, we fused the complete intracellular domains of TLR3 and TLR4 to GST and immobilized the fusion proteins on glutathione agarose beads. Next, we attempted to pull down overexpressed MyD88 or flag-TIRAP/MAL with the GST-TLR3 or GST-TLR4 beads. As expected, MyD88 and TIRAP/MAL were both able to bind to TLR4. The TLR3 intracellular domain was also able to associate with MyD88. However, we found that TLR3 was incapable of interacting with TIRAP/MAL (Fig. 2). These data strongly suggest that the receptor-proximal signaling complex directly engaged by TLR3 differs compositionally from the complex engaged by TLR4.

The TIRAP/MAL inhibitory peptide is able to block TLR4 but not TLR3 signaling

Although knockout studies have suggested that both MyD88 and TIRAP/MAL are dispensable for induction of IFN-β by TLR3 and TLR4, dominant-negative TIRAP/MAL has been shown to prevent TLR4 but not TLR3 signaling through IRF3 (3, 8, 14, 16). In addition, a cell-permeable TIRAP/MAL-inhibitory peptide has been shown to block TLR4-mediated induction of an IFN-β-specific reporter construct in RAW 264.7 cells (10). However, this inhibitory peptide has not been used to study TLR4 signaling in primary macrophage cells, nor have its effects on TLR3 signaling been addressed.

Because knockout studies leave open the possibility for redundancy, we decided to assess whether the TIRAP/MAL peptide could block TLR3 or TLR4 induction of *IFN-β* gene expression and activation of STAT1 following primary macrophage treatment with either TLR3 or TLR4 ligands. Results from these experiments show that the TIRAP/MAL peptide abrogated TLR4-mediated expression of IFN-β and STAT1 activation in primary macrophage cells. These findings corroborate peptide studies using macrophage cell lines, but disagree with TIRAP/MAL knockout results (10, 13, 14) (Fig. 3, *A* and *B*). In contrast to its effect on TLR4 signaling, the peptide was completely unable to block TLR3-induced expression of IFN-β and IL-6, which is in complete agreement with all previous studies (Fig. 3*A*). Likewise, STAT1 was still activated in cells stimulated with poly(I:C) in the presence of the TIRAP/MAL peptide (Fig. 3*B*). Even at lower concentrations of poly(I:C) stimulation (Fig. 3*B*) or higher concentrations of the TIRAP/MAL peptide (data not shown), the inhibitor was still incapable of blocking STAT1 activation via TLR3 signaling. Poly(I:C) has been shown to weakly induce proinflammatory cytokine production in TLR3-deficient mice, while poly(I:C)-induced IFN-β induction appears to be TLR3 dependent (18). Although we cannot exclude the possibility that poly(I:C) may signal through alternative receptors other than TLR3, our studies show that the peptide is incapable of reducing poly(I:C)-mediated gene expression and that TIRAP/MAL cannot bind to the cytoplasmic tail of TLR3. These data strongly suggest that TLR3 does not use TIRAP/MAL for signaling.

We found that the TIRAP/MAL peptide was able to inhibit IL-6 expression following TLR4 ligation, which is consistent with TIRAP/MAL knockout data (13, 14). One possible explanation for

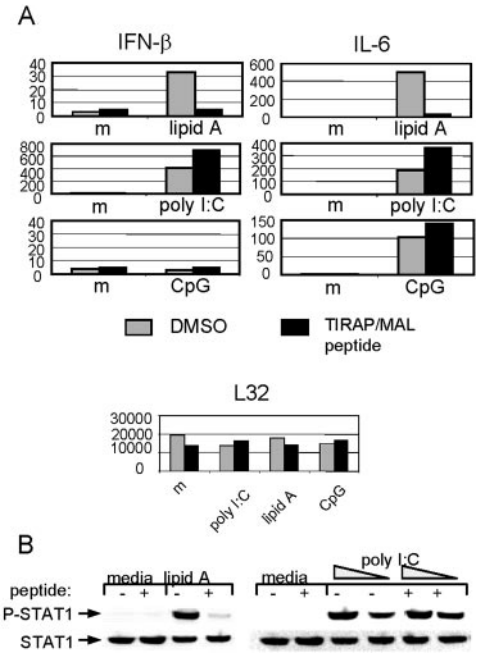

FIGURE 3. The TIRAP/MAL inhibitory peptide is able to block TLR4 but not TLR3 transactivation of IFN-β and IL-6, as well as IFN-β-mediated activation of STAT1. BMMs were pretreated with the TIRAP/MAL peptide (20 μM) or DMSO for 1 h and then stimulated with lipid A (1 ng/ml), poly I:C (1 μg/ml), or medium alone for 2 h. *A*, IFN-β, IL-6, and L32 mRNA levels were assayed by quantitative real-time PCR. All samples were run in duplicate or triplicate, and data are presented in relative expression units. *B*, STAT1 activation was determined by Western blotting analysis to detect phosphorylated STAT1. For STAT1 experiments, lipid A was used at 10 ng/ml, and poly I:C was administered at 100 and 10 ng/ml. Total STAT1 was also assayed to ensure equal loading. The data are representative of three independent experiments.

this would be that the TIRAP/MAL peptide not only affected TIRAP/MAL but also interfered with MyD88 function. TLR9 signaling has previously been shown to be completely dependent on MyD88 (18). Therefore, we used TLR9-mediated IL-6 activation as a readout to determine whether the TIRAP/MAL peptide could specifically interrupt MyD88 signaling. Consistent with previous reports using dendritic cells, we found that CpG-induced IL-6 expression in macrophages was not affected by the TIRAP/MAL peptide (Fig. 3*A*) (11). Thus, the TIRAP/MAL peptide does not specifically interfere with MyD88 signaling. These results show that the TIRAP/MAL inhibitory peptide can disrupt TLR4, but not TLR3 or TLR9, signaling in primary macrophage cells. Furthermore, because the peptide appears to inhibit both MyD88-dependent and -independent signaling events following TLR4 stimulation, the peptide may disrupt the entire TLR4-proximal signaling complex.

TLR3 and TLR4 ligands induce expression of TLR3, MyD88, and TIRAP/MAL

Thus far, our data suggest that TLR3 and TLR4 signal via unique adaptor molecule-containing complexes. We next wanted to see whether either TLR3 or TLR4 was capable of transcriptionally inducing molecules involved in eliciting early signaling events, which may explain why TLR3 can sustain and enhance antiviral gene expression to a greater degree than TLR4. To address this issue, we stimulated primary macrophage cells with poly(I:C) or lipid A for up to 12 h. By 4 h, treatment of macrophages with TLR3 or TLR4 agonists caused the induction of TLR3, but not TLR4, mRNA production (Fig. 4*A*). We also observed that TLR9 signaling, which does not induce IFN-β in primary macrophage cells, was incapable of inducing TLR3 expression (E. K. Chow and G. Cheng, unpublished data). In Fig. 4*A*, we show that both TLR3 and TLR4 agonists can induce the expression of MyD88 as well as TIRAP/MAL. Taken together, these data suggest that TLR3 is able to prolong and enhance its induction of antiviral genes by rapidly up-regulating the expression of additional TLR3.

TLR3 and TLR4 induce TLR3 expression through IFN-β

We have shown that both TLR3 and TLR4 use the IRF3 transcription factor to induce *IFN-β* gene expression (15). Once secreted from the cell, IFN-β is believed to act in an autocrine/paracrine manner leading to STAT1 activation and secondary antiviral gene induction (10, 15, 19). Due to the fact that both TLR3 and TLR4 can induce TLR3 expression, and because this induction takes place after IFN-β production has begun, we investigated whether TLR3 expression was induced by IFN-β. Using cells deficient in the IFNAR, we show in Fig. 4*B* that treatment of cells with TLR3 or TLR4 agonists does not cause the induction of TLR3 expression in the absence of IFNAR. Furthermore, stimulating primary macrophage cells with rIFN-β resulted in a dose-dependent up-regulation of TLR3 mRNA (Fig. 4*B*). TLR4 mRNA levels were relatively unaltered in the IFNAR-deficient cells or by induction of wild-type cells with rIFN-β (Fig. 4*B*). These data indicate that TLR3 and TLR4 can potently induce TLR3, but not TLR4, expression through IFN-β production.

TLR3 and TLR4 induce both IFN-β-enhanced and IFN-β-dependent antiviral genes

We have previously characterized TLR3 and TLR4 antiviral gene induction as either primary or secondary based upon sensitivity to cycloheximide (15). Our previous data suggested that primary genes are induced in the absence of novel protein synthesis, whereas secondary genes require the initial expression of IFN-β. To further characterize these primary and secondary genes, we induced both wild-type and IFNAR-deficient cells with either poly(I:C) or lipid A and assessed antiviral gene expression at 1 and 4 h. As seen in Fig. 5, *IFN-β* and *IP10* (both primary genes) are induced by 1 h in both wild-type and IFNAR knockout macrophage cells, while *IFI-204* (a secondary gene) remained at basal levels. By 4 h, IP10 expression was significantly enhanced in the wild-type cells, but remained relatively unchanged in the IFNAR knockout cells. These data indicate that the primary expression of IP10 is enhanced by the IFN-β positive feedback loop. The secondary gene, *IFI-204*, was induced to high levels by 4 h, yet was not detectable in the IFNAR knockout cells at the same time point. Similar results were also obtained for the primary and secondary genes *RANTES* and *Mx1*, respectively. As an induction control, ICAM1 mRNA was elevated by 1 h and remained high by 4 h in both wild-type and knockout cells stimulated with poly(I:C) or lipid A. As shown in Fig. 4*B*, TLR3 is part of the secondary gene subset.

The IFNAR is required for both TLR3 and TLR4 activation of STAT1 and resistance to MHV68 infection

In mice deficient in the IFNAR, STAT1 activation has been shown to be blocked in macrophage cells stimulated with LPS (19). Although blocking Ab studies have suggested that IFN-β is also essential for poly(I:C)-induced STAT1 activation, these studies have not been conducted to date in primary macrophage cells deficient in IFNAR (15). To address this issue, we stimulated IFNAR$^{-/-}$

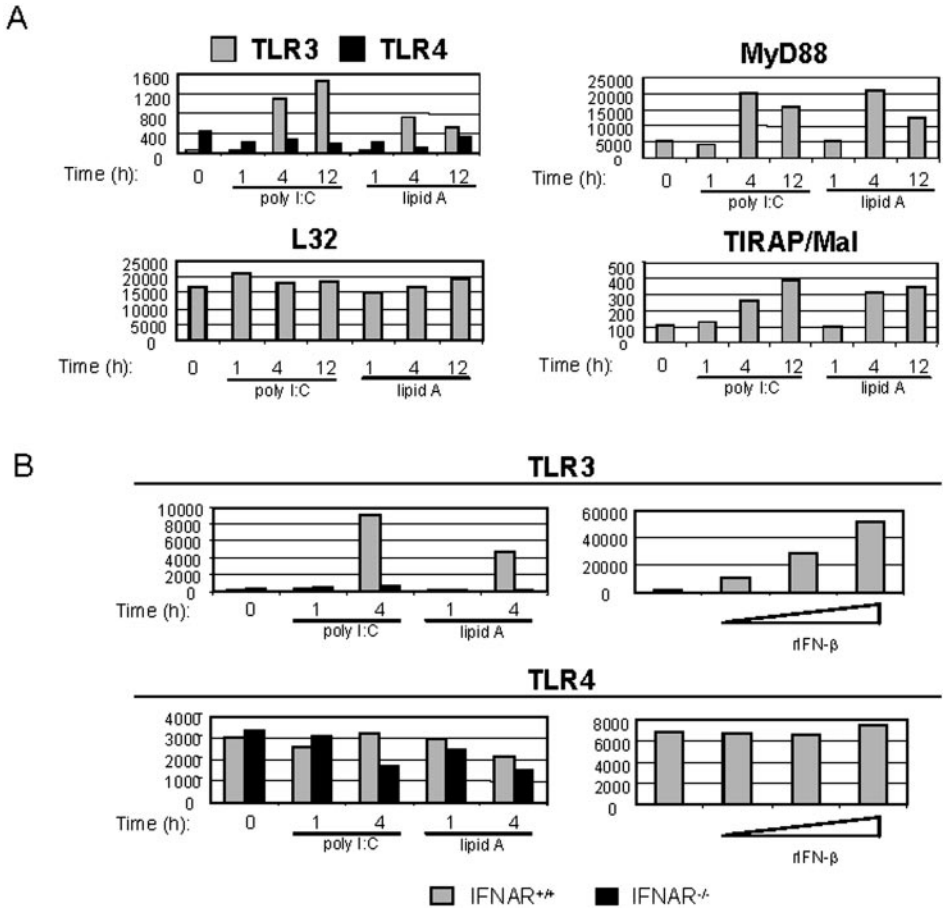

FIGURE 4. Both TLR3 and TLR4 ligands can induce expression of TLR3 through IFN-β. Primary macrophage cells derived from bone marrow cells were stimulated with poly I:C (1 μg/ml) or lipid A (1 ng/ml) for the indicated times. *A*, Quantitative real-time PCR was used to assay the expression levels of TLR3, TLR4, MyD88, and TIRAP/MAL. *B*, TLR3 and TLR4 mRNA levels were also assessed in cells deficient in IFNAR, and cells stimulated with rIFN-β (10, 100, and 1000 U). Experiments were repeated at least two separate times, and data are presented in relative expression units. L32 was used to normalize all samples.

BMMs with lipid A and poly(I:C) and assayed for STAT1 phosphorylation. In Fig. 6*A*, we show that, like that of TLR4, TLR3-mediated STAT1 activation was also abolished in IFNAR$^{-/-}$ macrophage cells. The TLR9 agonist CpG, which fails to induce IFN-β in primary macrophage cells, was used as a negative control.

Blocking Ab and conditioned medium experiments have suggested that TLR3- and TLR4-mediated viral resistance is IFN-β dependent (15). Using the MHV68 protein M9 as a readout for viral load, we show in Fig. 6*B* that the antiviral activity of macrophage cells infected with MHV68 was abolished in the absence

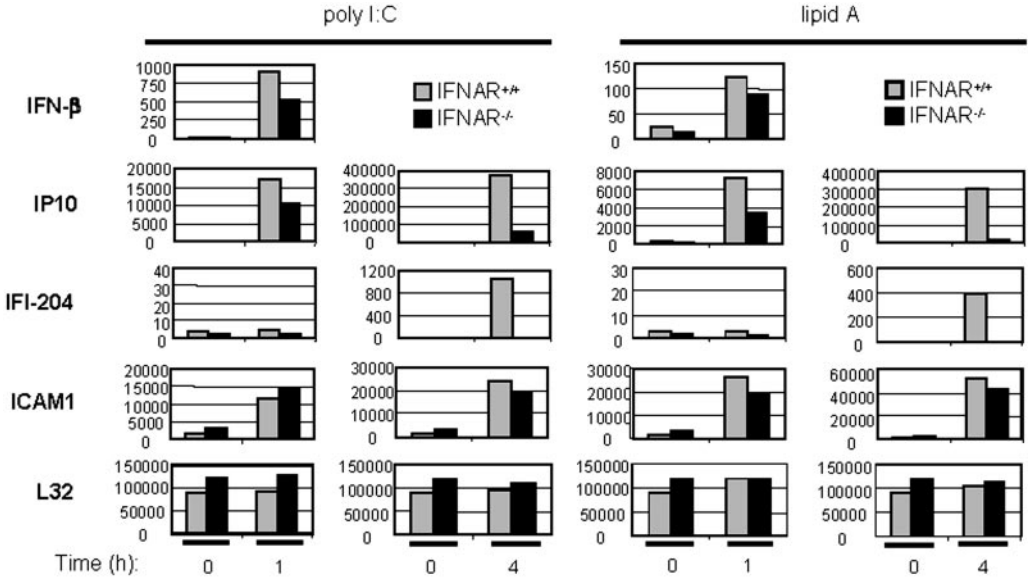

FIGURE 5. TLR3 and TLR4 induce both IFN-β-enhanced and IFN-β-dependent antiviral genes. Both wild-type cells and cells deficient in IFNAR were stimulated with poly I:C (1 μg/ml) or lipid A (1 ng/ml) for either 1 or 4 h. IFN-β, IP10, IFI-204, ICAM1, and L32 mRNA levels were assessed. Data are representative of three independent experiments and presented in relative expression units.

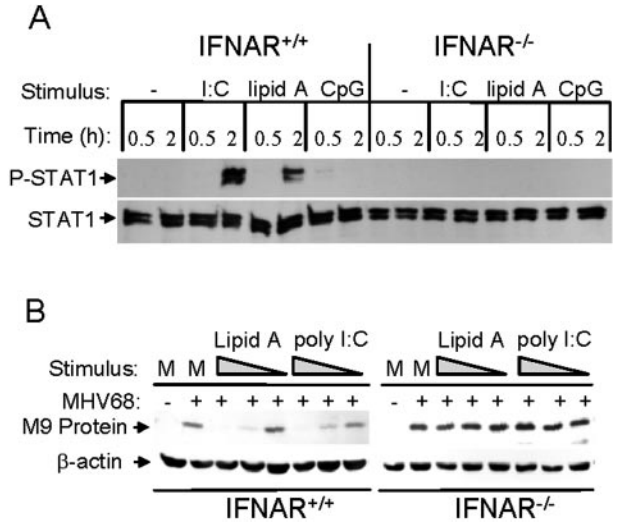

FIGURE 6. Both TLR3 and TLR4 fail to activate STAT1 and induce the antiviral gene program in IFNAR$^{-/-}$ primary macrophage cells. BMMs from both wild-type and IFNAR$^{-/-}$ mice were stimulated with lipid A (1 ng/ml), poly I:C (1 μg/ml), CpG (100 nM), or fresh medium (M) for the indicated time periods. *A*, Cell lysates were subjected to western blotting analyses to detect phosphorylated STAT1 (P-STAT1) and total STAT1. *B*, For viral replication assays, BMMs were simultaneously stimulated with PAMPs (10, 1, or 0.1 ng/ml lipid A, or l, 0.1, or 0.01 μg/ml poly(I:C)) and infected with MHV68 using a multiplicity of infection of five. Cell lysates were harvested at 48 h postinfection and subjected to Western blotting analysis using an Ab specific to the MHV68 protein M9. Actin levels were also assayed to ensure equal loading. The data represent two independent experiments.

of IFNAR despite cotreatment with either lipid A or poly(I:C). Thus, IFNAR-mediated up-regulation of secondary response genes, such as *IFI-204*, and enhancement of primary genes, such as *IP10*, is essential for antiviral activity. In fact, MHV68 protein synthesis was enhanced in IFNAR$^{-/-}$ vs wild-type cells under all conditions tested. These data provide genetic evidence that the IFN-β autocrine/paracrine loop is essential for induction of the TLR3- and TLR4-specific antiviral gene program.

Discussion

Our data suggest that, although both TLR3 and TLR4 induce antiviral gene expression, TLR3 is better suited than TLR4 to activate this program. We show that TLR3 is able to induce higher levels of IFN-β, which is most likely a result of using a different signaling complex that can more strongly activate IRF3 than TLR4. In addition, TLR3 is able to enhance its own expression (via an IFN-β-mediated positive feedback loop), thereby promoting an even stronger antiviral response. Viral infection or IFN-α stimulation of human macrophage cells has also been shown to induce TLR3 transcription (20). Collectively, the data argue that, while both TLR3 and TLR4 have been evolutionarily selected to induce antiviral gene expression, TLR3 seems to be even more specialized than TLR4 to initiate antiviral responses and is specifically up-regulated when a virus is detected.

Sequence analysis of the BB loop region (found in the Toll/IL-1 receptor homology domain (TIR)) reveals significant homology between the BB loop domains of TLR3, TLR4, MyD88, and TIRAP/MAL. The importance of this region in TLR signaling is exemplified by C3H/HeJ mice that contain a P712H mutation in the BB loop that renders these mice incapable of signaling via TLR4 (21). A P125H mutation in the homologous region of TIRAP/MAL prevents association with TLR4 (12). Interestingly, TLR3 naturally contains an alanine instead of a proline at this same BB loop position, which may explain why we do not detect TIRAP/MAL interacting with TLR3.

The fact that TLR4 can still activate IRF3 in primary macrophage cells deficient in TIRAP/MAL, but dominant-negative TIRAP/MAL and the TIRAP/MAL inhibitory peptide can block TLR4-mediated IFN-β expression presents a conflicting situation regarding the actual role of TIRAP/MAL in TLR4-mediated antiviral gene induction (3, 10, 14). As mentioned, the use of TIRAP/MAL-deficient cells leaves open the possibility that a redundant molecule may replace TIRAP/MAL in the TLR4-specific receptor-proximal signaling complex. In contrast, the inhibitory peptide and the dominant-negative form of TIRAP/MAL may nonspecifically interfere with other TIR-containing molecules. Thus, both experimental methods have possible defects that may lead to the conflicting results observed. Despite this, it is apparent that TIRAP/MAL can interact with TLR4 and is involved in certain aspects of TLR4 signaling. However, this does not appear to be the case for TLR3. The results presented in this report strongly suggest that TIRAP/MAL is unable to interact with TLR3 and is also not involved in TLR3-activated signal transduction.

It is apparent that both TLR3 and TLR4 can activate similar IFN-β-mediated antiviral gene programs, and that IFN-β is a key mediator of these responses. Our results clearly demonstrate that antiviral genes induced by TLR3 and TLR4 fall into two distinct categories: primary, which are insensitive to cycloheximide and are initially induced early (by 1 h) in the absence of IFN-β, yet are greatly enhanced by the IFN-β positive feedback loop; and secondary, which are not induced in the presence of cycloheximide or until IFN-β is produced and feeds back to signal through its receptor, IFNAR (by ~2–4 h). Our previous data have suggested that a key difference between primary and secondary gene induction is that primary genes appear to be transactivated directly by IRF3, in addition to NF-κB, following TLR3 or TLR4 ligand engagement (15).

It remains unresolved how IRF3 becomes activated following TLR3 or TLR4 receptor stimulation. It is also very likely that other TLRs may contain their own unique signaling pathways involving as-yet-unidentified signaling mediators. In addition to MyD88 and TIRAP/MAL, only a few other proteins have been shown to interact directly with the intracellular domains of TLRs, including the Rho GTPase Rac-1, phosphoinositide 3-kinase, and Toll-interacting protein (22, 23). The expressed sequence tag database currently contains a large number of TIR-containing sequences. It may be that one or more of these proteins plays a role in mediating the activation of IRF3 downstream of TLR3. By continuing to characterize these putative and established TLR-interacting adaptor molecules, the signaling and functional specificities between the different TLRs will surely become more clearly understood.

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