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Absence of MyD88 Results in Enhanced TLR3-Dependent Phosphorylation of IRF3 and Increased IFN-β and RANTES Production

Jakub Siednienko,* Thusitha Gajanayake,* Katherine A. Fitzgerald,[†] Paul Moynagh,* and Sinéad M. Miggin*

Toll-like receptors are a group of pattern-recognition receptors that play a crucial role in "danger" recognition and induction of the innate immune response against bacterial and viral infections. TLR3 has emerged as a key sensor of viral dsRNA, resulting in the induction of the anti-viral molecule, IFN-β. Thus, a clearer understanding of the biological processes that modulate TLR3 signaling is essential. Previous studies have shown that the TLR adaptor, Mal/TIRAP, an activator of TLR4, inhibits TLR3-mediated IFN-β induction through a mechanism involving IRF7. In this study, we sought to investigate whether the TLR adaptor, MyD88, an activator of all TLRs except TLR3, has the ability to modulate TLR3 signaling. Although MyD88 does not significantly affect TLR3 ligand-induced TNF-α induction, MyD88 negatively regulates TLR3-, but not TLR4-, mediated IFN-β and RANTES production; this process is mechanistically distinct from that employed by Mal/TIRAP. We show that MyD88 inhibits IKKε-, but not TBK1-, induced activation of IRF3. In doing so, MyD88 curtails TLR3 ligand-induced IFN-β induction. The present study shows that while MyD88 activates all TLRs except TLR3, MyD88 also functions as a negative regulator of TLR3. Thus, MyD88 is essential in restricting TLR3 signaling, thereby protecting the host from unwanted immunopathologies associated with the excessive production of IFN-β. Our study offers a new role for MyD88 in restricting TLR3 signaling through a hitherto unknown mechanism whereby MyD88 specifically impairs IKKε-mediated induction of IRF3 and concomitant IFN-β and RANTES production. *The Journal of Immunology*, 2011, 186: 2514–2522.

I nnate immunity is the first line of defense against microbial pathogens, eliciting the production of inflammatory cytokines and type I IFNs. A number of classes of pathogen recognition receptors have evolved to detect pathogens, including the TLRs (1), the RIG-I–like receptor (RLR) RNA sensors (2), and the recently described cytosolic DNA receptors (3–5). Of the 10 human TLRs that have been described, endosomally localized anti-viral TLRs, namely TLR3, TLR7/8, and TLR9, have evolved to detect dsRNA, ssRNA, and ssDNA, respectively. Upon activation of the TLRs, cytosolic TIR domain-containing adaptor proteins are recruited (1). To date, four activating adaptor proteins have been identifie: MyD88, MyD88 adaptor-like (Mal)/Toll-IL-1R domain-containing adaptor protein (TIRAP), Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). Upon initiation of the TLR signaling cascade through adaptor recruitment via TIR domain interactions, a series of signaling cascades are elicited that ultimately result in the production of proinflammatory mediators, including TNF-α and IL-6 and the production of type I IFN, α and β. Despite similarities between the TLRs in terms of signal transduction pathways, there is specificity with regard to their adaptor usage (6). MyD88 is the common downstream adaptor that is recruited by all TLRs except TLR3 (7) and leads activation of the transcription factor NF-κB (6). Mal is required for signaling by the LPS receptor TLR4 and the bacterial lipoprotein receptor TLR2 (8). TRIF mediates TLR3 and TLR4 signaling and is involved in the activation of the transcription factors IRF3 and IRF7 and consequential expression of type I IFNs (9). Regarding TRIF, it is the sole adaptor required for TLR3 signaling (10). Finally, TRAM mediates TLR4 signaling exclusively (9), acting as a bridging adaptor to recruit TRIF to the TLR4 complex.

Transcriptional activation of the IFN-β gene requires the assembly of a multiprotein complex encompassing the transcription factors ATF-2/c-Jun, IRF3, IRF7, and NF-κB to form the IFN-β "enhanceosome." The enhanceosome elements bind to a nucleosome-free region within the IFN-β promoter upstream of the transcription start site (11). The enhancer itself is divided into four positive regulatory domains (PRDs) whereby NF-κB binds to the PRDII element within the IFN-β enhancer region, ATF-2/c-Jun binds to the PRDIV element and is activated by JNK, and IRF3 and IRF7 bind to the PRDI–III element. Upon activation of TLR3 and TLR4, the IKK-related kinases TNFR-associated factor (TRAF) family member-associated NF-κB activator binding kinase 1 (TBK1) and IKKε lead to the C-terminal phosphorylation of IRF3 and IRF7, thereby facilitating their homo- and heterodimerization, nuclear localization, and transcriptional activation of IFN-β (12).

Recently, research has been focused toward understanding the mechanisms that cells employ to curtail TLR signaling and concomitant proinflammatory and type I IFN production with a view to limiting host damage as a consequence of excessive cytokine production. To this end, a number of molecules that curtail TLR-mediated IFN-β induction have been identified, including TAG, Ro52/TRIM21, and SHIP-1 (13–15). Regarding the TLR adaptor molecules themselves, until quite recently, the physiological role of these adaptors was considered to be facilitation of TLR activation and concomitant perpetuation of the signaling cascade. Recently, studies have been focused toward understanding the role of the TLR adaptors in noncognate TLR signaling pathways. For example, it has been shown that the TLR4 adaptor molecule, Mal, has the ability to limit TLR3-mediated JNK phosphorylation (16). More recently, we have shown that Mal inhibits TLR3-dependent IFN-β production through a mechanism involving the inhibition of IRF7 phosphorylation and nuclear translocation (17). Regarding the adaptor MyD88, it has been shown that MyD88 negatively regulates TLR3/TRIF-induced corneal inflammation though a mechanism involving JNK phosphorylation, but not p38, IRF3, or NF-κB (18). MyD88 has also been shown to inhibit TLR3-dependent IL-6 induction, but not IκB degradation or p38 activation in murine macrophages (16).

To date, the exact mechanisms used by MyD88 to curtail TLR3 signaling and concomitant IFN-β production remain unidentified. The current study demonstrates that MyD88 negatively regulates IFN-β cytokine and chemokine production downstream of TLR3, but not TLR4. We show that MyD88 coimmunoprecipitates with IRF3 in a TLR ligand-dependent manner. We also show that MyD88 inhibits IKKε-mediated phosphorylation of IRF3, and so our study identifies MyD88 as a novel negative regulator of TLR3-mediated IFN-β and CCL5 gene induction.

Materials and Methods

Cell culture and reagents

HEK293 and BEAS-2B cell lines were purchased from European Cell Culture Collection (Porton Down, U.K.). The HEK293-TLR3 and HEK293-TLR4 cells were from InvivoGen. HEK293-TLR7 cells were a gift from Professor Stefan Bauer, University of Marburg. The cells were grown in DMEM with GlutaMAX (Life Technologies-BRL) supplemented with 10% FCS, penicillin-streptomycin, and noromycin and maintained at 37°C in a humidified atmosphere of 5% CO_2. G418 (200 μg/ml) was added to maintain HEK293-TLR3, HEK293-TLR4, and HEK293-TLR7 cells. Highly purified protein-free LPS derived from *Escherichia coli* strain 011:B4 was used in all treatments. Naked polyinosinic-polycytidylic acid [poly (I:C)] and R848 were from InvivoGen. Control and MyD88 inhibitory peptides were purchased from Imgenex (catalogue no. IMG-2005-1). MyD88 endoribonuclease-prepared siRNAs (esiRNAs) were purchased from Sigma-Aldrich. Rhinovirus (RV)1b and RV16 were from Dr. Marc Hershenson, University of Michigan, and Professor Steve Goodbourn, University of London, respectively.

Expression vectors/recombinant plasmids

The NF-κB-luciferase reporter construct and Flag-TRIF were as described (9). The plasmid pcDNA3:MyD88-cmyc was a gift from Professor Luke O'Neill (Trinity College Dublin). The reporter gene contructs IFN-β luciferase, IFN-β PRDI–III-luciferase, and PRDIV-luciferase were as previously described (19). The CCL5 reporter gene construct, TBK1-Flag plasmid, IKKε-Flag plasmid, and IRF3-Flag plasmid were as described (19). The plasmids encoding the constitutively active forms of RIG-I and Mda5, namely the CARD domain regions of either RIG-I (RIG-IN) or Mda5 (Mda-5N) expression plasmids, were gifts from Professor Takashi Fujita (20).

Sources of macrophages

MyD88$^{-/-}$, TRIF$^{-/-}$, and mitochondrial antiviral signaling (MAVS)$^{-/-}$ mice were constructed as described (21–24). MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were on a C57BL/6 background. MAVS$^{-/-}$ mice were on a mixed C57BL/6 and 129 background. All mice were confirmed as being homozygous mutants by PCR genotyping of DNA. All animal protocols used in this study were approved by the Ethical Committee at the National University of Ireland, Maynooth, and in accordance with the Animals (Scientific Procedures) Act, 1986, U.K. Bone marrow-derived macrophages (BMDMs) were generated as previously described (16)

First-strand cDNA synthesis

Total RNA was isolated from all types of cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Thereafter, total RNA was converted to first-strand cDNA as described (25). Briefly, 1 μg RNA was incubated with 1 μl random hexamer primers (500 μg/ml) at 70°C for 5 min. Thereafter, the other reaction components were added in the following order: 5 μl 5xRT buffer, 1.3 μl 10 mM deoxyribonucleotide triphosphate, 0.7 μl RNasin (Promega), 1 μl Moloney murine leukemia virus reverse transcriptase (Promega), and nuclease-free water to a total volume of 25 μl. The tubes were incubated at 37°C for 40 min and at 42°C for 40 min followed by heating to 80°C for 5 min. The first-strand cDNA was stored at −20°C for up to 1 mo.

Real-time PCR

Total cDNA was used as starting material for real-time RT-PCR quantitation with DyNAmo HS SYBR Green kit (Finnzymes) on a real-time PCR system (DNA Engine Opticon system; MJ Research). For amplification of specific genes, the following primers were used; *mIFN-β*, forward, 5′-GGAGATGACGGAGAAGATGC-3′, and reverse, 5′-CCCAGTGCTGG-AGAAATTGT-3′; *hIFN-β*, forward, 5′-AACTGCAACCTTTCGAAGCC-3′, and reverse, 5′-TGTCGCCTACTACCTGTTGTGC-3′; *mTNF-α*, forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, and reverse, 5′-TGGGA-GTAGACAAGGTACAACCC-3′; *mCCL5*, forward, 5′-GGAGATGAGCT-AGGATAGAGGG-3′, and reverse, 5′-TGCCCATTTTCCCAGGACCG-3′. For each mRNA quantification, the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*) was used as a reference point using the following primers: *mHPRT* forward, 5′-CCCTGAAGTACTCATTATAGTC-AAGGGCAT-3′, and reverse, 5′-GCTTGCTGGTGAAAAGGACCTCTC-GAAG-3′; *hHPRT* forward, 5′-AGCTTGCTGGTGAAAAGGAC-3′, and reverse, 5′-TTATAGTCAAGGGCATATCC-3′. Real-time PCR data were analyzed using $2^{-\Delta\Delta CT}$ method as described (26).

esiRNA transfection

Human BEAS-2B cells were transfected with esiRNA to target the suppression of either MyD88 or control firefly luciferase (FLUC). Briefly, for each well in a 12-well plate, 200 ng esiRNA was tranfected using 1 μl N-TER nanoparticle siRNA transfection system (Sigma-Aldrich, catalogue no. N2913). After 36 h, efficiency of MyD88 knockdown was assessed by RT-PCR using the MyD88 forward (5′-TCCACAGTGATGCCTACT-GATGCT-3′) and reverse (5′-ATGCAGATGAGAGGTGGACCCATT-3′) primers and HPRT forward (5′-AGCTTGCTGGTGAAAAGGAC-3′) and reverse (5′-TTATAGTCAAGGGCATATCC-3′) primers.

Reporter assays

HEK293, HEK293-TLR3, HEK293-TLR4, and HEK293-TLR7 cells (2 × 10^4 cells/well; 96-well plate) were transfected with 80 ng/well luciferase reporter gene plasmid for NF-κB, CCL5, IFN-β, pRDI–III, or pRDIV as previously described (9) and cotransfected with the expression vector pcDNA3:MyD88, pcDNA3:TRIF, RIG-I, Mda-5, TBK1, or IKKε using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. In all cases, 40 ng/well phRL-TK reporter gene was cotransfected to normalize data for transfection efficiency. After 24 h, cells were stimulated with ligands as indicated. Thereafter, cell lysates were prepared and reporter gene activity was measured using the Dual-Luciferase Assay system (Promega) as described (27). Data were expressed as the mean fold induction ± SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Transfection and coimmunoprecipitation

HEK293-TLR3 and HEK293-TLR4 cells (2 × 10^6 cells/10-cm dish) were transfected using Lipofectamine 2000 (Invitrogen) with the indicated plasmids where the total amount of DNA (3 μg/dish) was kept constant. Twenty-four hours later, cells were stimulated and lysed as described (27). The indicated Abs (5 μg) were incubated with 40 μl 50% protein G slurry overnight at 4°C, washed, followed by the addition of the cell lysates for 2 h. The immune complexes were precipitated, washed, and eluted by the addition of sample buffer followed by SDS-PAGE and immunoblotting using the indicated Abs.

Cytokine analysis

BMDMs (5×10^5 cells/well/48-well plate) were stimulated with the following stimuli: ultrapure LPS, poly(I:C), or R848. After 16 h the cell supernatants were removed and analyzed for IFN-β release according to the manufacturer's recommendations (PBL). TNF-α and CCL5 cytokine release (PeproTech) were measured as indicated by the manufacturer.

Type I IFN bioassay

Detection of poly(I:C) and LPS-induced bioactive murine type I IFN was assessed using B16-Blue IFN-α/β cells (InvivoGen), essentially as described by the manufacturer.

IRF3/7 nuclear translocation assay

BMDMs were stimulated with poly(I:C) (1 μg/ml) for 0–1 h. Thereafter, nuclear proteins were isolated using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas) as described by the manufacturer. Thereafter, the nuclear fraction was subjected to immunoblot analysis using anti-IRF3 (Santa Cruz Biotechnology, sc-9082), anti-IRF7 (Santa Cruz Biotechnology, (F-1) sc-74471), and anti-nucleolin (Santa Cruz Biotechnology, C23 (H-6) sc-55486) Abs.

IRF3 immunoblot analysis

Cells were stimulated with ligand as described, and lysates were subjected to SDS-PAGE followed by immunoblot analysis with an anti-IRF3 (Santa Biotechnology, sc-9082) and anti–phospho-IRF3 Ab (Cell Signaling Technology, catalogue no. 4947).

Data analyses

Statistical analysis was carried out using the unpaired Student *t* test using SigmaPlot 2001 program. The *p* values ≤0.05 were considered to indicate a statistically significant difference (*p < 0.05; **p < 0.005).

Results

MyD88 negatively regulates IFN-β induction downstream of TLR3

Given that MyD88 has been shown to negatively regulate TLR3-mediated JNK activation (18) and that another noncanonical TLR3 adaptor, Mal, has been shown to negatively regulate IFN-β induction (17), we sought to investigate the ability of MyD88 to modulate TLR3-mediated cytokine production. To this end, we measured TLR3-mediated cytokine production, namely TNF-α, Rantes/CCL5, and IFN-β induction by ELISA and quantitative PCR. Following quantitative real-time RT-PCR measurements, we demonstrate that whereas stimulation of wild-type (WT) BMDMs with the TLR3 ligand poly(I:C) resulted in IFN-β gene induction, a significantly greater induction of IFN-β was evident in MyD88$^{-/-}$ BMDMs. In contrast, impaired IFN-β gene induction was evident in MyD88$^{-/-}$ BMDMs compared with WT controls following LPS stimulation (Fig. 1*A*). As expected, IFN-β gene induction was not evident in TRIF-deficient BMDMs following stimulation with either poly(I:C) or LPS (Fig. 1*A*). Similarly, increased induction of the IFN-β–dependent chemokine, CCL5, was evident in MyD88$^{-/-}$ BMDMs when compared with WT cells following stimulation with poly(I:C), but not with LPS (Fig. 1*B*). In contrast, whereas enhanced poly(I:C)-induced TNF-α induction was not evident in MyD88$^{-/-}$ cells compared with WT cells, a significant decrease in LPS-mediated TNF-α gene induction was evident (Fig. 1*C*).

Next, we sought to investigate the role of MyD88 in the translational regulation of IFN-β, Rantes, and TNF-α. Thus, MyD88$^{-/-}$, TRIF$^{-/-}$, and WT BMDMs were stimulated with the TLR3 ligand poly(I:C), LPS (TLR4), or R848 (TLR7/8), followed by cytokine measurement by ELISA. Consistent with the hypothesis that MyD88 is a negative regulator of TLR3 that mediates IFN-β, poly (I:C) treatment of MyD88$^{-/-}$ cells increased production of IFN-β when compared with WT cells (Fig. 1*D*). Correlating with real-time PCR data and previous reports (21, 28, 29), LPS- and poly

(I:C)-induced IFN-β production was significantly decreased in TRIF-deficient BMDMs when compared with WT BMDMs (Fig. 1*D*). In contrast, increased IFN-β and Rantes/CCL5 induction were evident in MyD88$^{-/-}$ BMDMs when compared with WT cells following stimulation with poly(I:C), but not with LPS or R848 (Fig. 1*D*, 1*E*). We also show that whereas TNF-α induction was not significantly different in MyD88$^{-/-}$ cells compared with WT cells following poly(I:C) stimulation, a significant decrease in LPS-mediated TNF-α gene induction was evident (Fig. 1*F*). Although poly(I:C) may be sensed by TLR3, it is also sensed by the RLR Mda-5, which utilizes the signaling adaptor MAVS (30). Hence, sensing of poly(I:C) by TLR3 and/or Mda-5 is determined by the relative expression of either molecule in a given cell, the subcellular distribution of TLR3, and ligand localization. Given these factors, it was imperative to assess the relative contribution of RLRs to poly(I:C)-mediated cytokine production in BMDMs and to determine the role of MyD88 therein. Correlating with previous findings (24), comparable induction of IFN-β, CCL5, and TNF-α was evident in MAVS$^{-/-}$ BMDMs compared with WT cells following treatment with a MyD88 inhibitory peptide (Fig. 1*G–I*), indicating that poly(I:C) mediates its effects through TLR3 rather than the RLRs in BMDMs. Although it has previously been shown that poly(I:C)-induced IFN-β induction is Mda5-dependent and TLR3/Trif-independent (31), our study conclusively shows that poly(I:C) mediates its effects in a TLR3-dependent mechanism. The apparent discrepancy between our study and that of Kato et al. (31) may be attributed to variations in the cell type chosen for study. Moreover, numerous studies showing that TRIF is essential for TLR3-mediated IFN-β gene induction (21, 29, 32) add credence to our finding that poly(I:C) mediates IFN-β gene induction in murine macrophages in a TRIF-dependent manner and so implicates TLR3 in the mediation of poly(I:C)-mediated IFN-β gene induction.

To negate the possibility of species-dependent differences in MyD88 functionality in the context of TLR3 signaling, the ability of MyD88 to impair TLR3 signaling in a human system was investigated. Thus, human lung bronchial epithelial BEAS-2B cells were chosen as a model, as they express TLR3 and respond well to poly(I:C), a TLR3 ligand (17). Following the suppression of MyD88 expression (Fig. 2*C*), we assessed IFN-β and CCL5 induction following stimulation with poly(I:C). As a control, we show that exposure of BEAS-2B cells to MyD88 and control esiRNAs alone does not significantly affect basal IFN-β/CCL5 mRNA expression levels when compared with vehicle-treated cells (Fig. 2*A*, 2*B*). We show that suppression of MyD88 significantly enhanced poly(I:C)-induced IFN-β and CCL5 gene induction in human cells (Fig. 2*A*, 2*B*). Next, we sought to investigate the physiological role of MyD88 in the modulation of virally induced IFN-β induction. To this end, lung epithelial BEAS-2B cells were treated with two ssRNA viruses that are known to be immunologically sensed by TLR3 (33), namely RV serotypes RV1b (a representative of the minor group of RV) and RV16 (a representative of the major group of RV), in the absence and presence of MyD88 suppressants followed by assessment of IFN-β, CCL5, and TNF-α gene induction. It was found that suppression of MyD88 using either a MyD88 inhibitory peptide or esiRNA technologies significantly enhanced RV1b- and RV16-induced IFN-β and CCL5 induction without significantly affecting virally induced TNF-α gene induction when compared with controls (Fig. 2*D–I*). Taken together, these data indicate that MyD88 inhibits TLR3-mediated and virally induced IFN-β and CCL5 gene induction and suggest that the effects of MyD88 are both TLR ligand-dependent and specific to the pathway that is triggered.
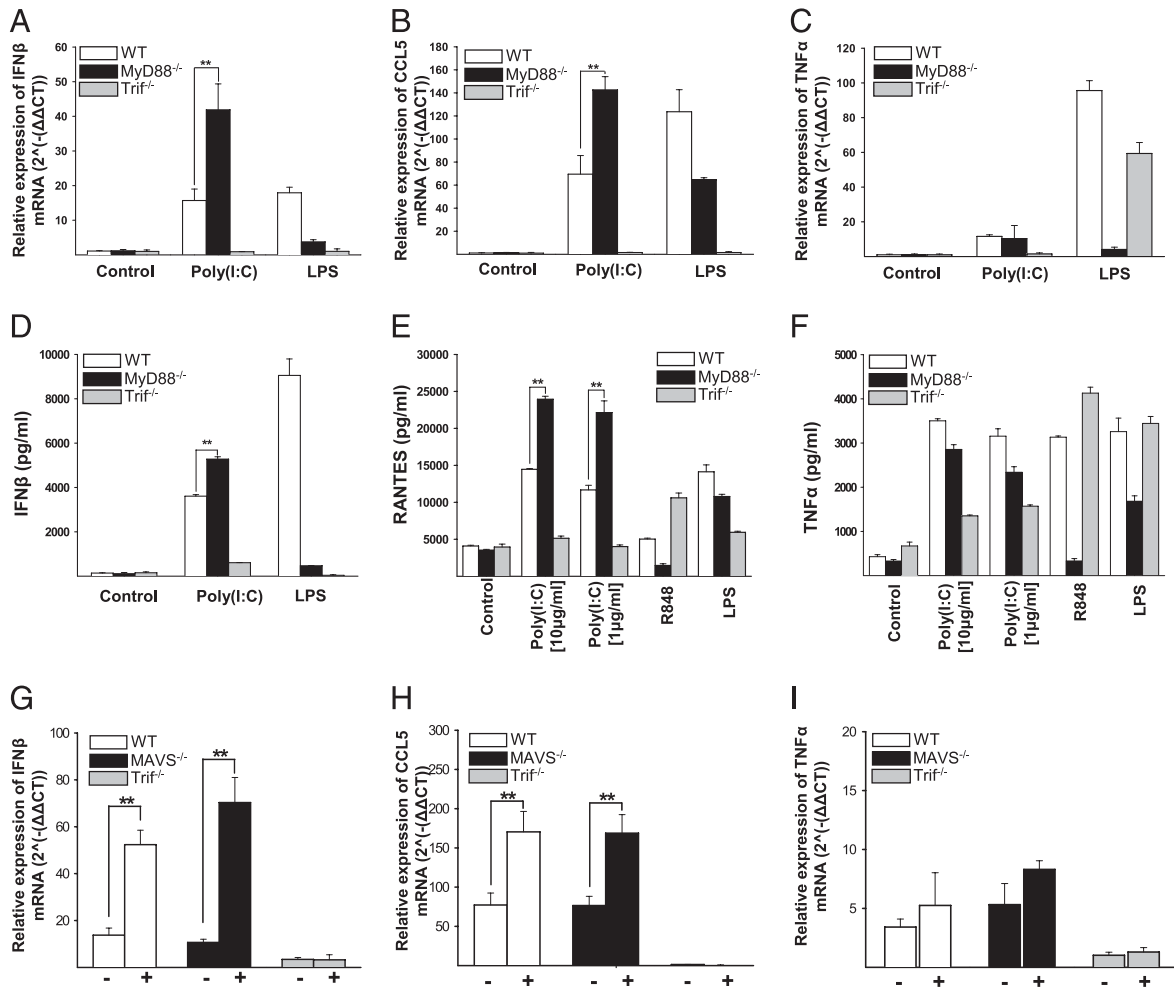
FIGURE 1. Poly(I:C)-induced IFN-β induction is exacerbated in MyD88$^{-/-}$ BMDMs. *A–C*, Immortalized BMDMs from WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice were treated with vehicle (control), poly(I:C) (1 μg/ml), or LPS (10 ng/ml) for 4 h. Thereafter, total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR as described in *Materials and Methods*. Quantitative real-time PCR was used to assay the expression levels of IFN-β (*A*), CCL5 (*B*), and TNF-α (*C*). Experiments were repeated at least three separate times and data are presented in relative expression units where HPRT was used to normalize all samples. *D–F*, Immortalized BMDMs from WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice were treated with poly(I:C) (1 or 10 μg/ml), LPS (10 ng/ml), or R848 (1 μM) for 16 h as indicated. Thereafter, IFN-β (*D*), RANTES (*E*), and TNF-α (*F*) were measured by ELISA as described in *Materials and Methods*. Results are representative of at least three independent experiments performed in triplicate (mean ± SE). *G–I*, Immortalized BMDMs from WT, MAVS$^{-/-}$, and TRIF$^{-/-}$ mice were pretreated with control peptide (−; 20 μM) or MyD88 inhibitory peptide (+; 20 μM) for 1 h. Next, cells were treated with poly(I:C) (1 μg/ml) for 4 h. Thereafter, total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR as described in *Materials and Methods*. Quantitative real-time PCR was used to assay the expression levels of IFN-β (*G*), CCL5 (*H*), and TNF-α (*I*). Experiments were repeated at least three separate times and data are presented in relative expression units where HPRT was used to normalize all samples and fold increases were determined relative to vehicle-treated cells.

MyD88 negatively regulates TLR3/TRIF-induced IFN-β and CCL5 promoter activity by inhibiting pathways regulated by IRF3/7, but not NF-κB or ATF2/c-jun

We next examined the ability of MyD88 to modulate TLR3-dependent transcription factor activation. To do this, HEK293 cells stably transfected with either TLR3 (HEK293-TLR3), TLR4 (HEK293-TLR4), or TLR7 (HEK293-TLR7) to render them poly (I:C), LPS, and R848 responsive, respectively, were transiently transfected with the IFN-β, NF-κB, PRDIV, PRDI–III, and CCL5 reporter gene constructs and increasing amounts of MyD88. Correlating with the analysis of IFN-β gene induction in MyD88$^{-/-}$ BMDMs, we found that although transfection of HEK293-TLR3 cells with MyD88 dose-dependently inhibited poly(I:C)-induced activation of the IFN-β, PRDI–III, and CCL5 reporter genes (Fig. 3*A*), MyD88 did not inhibit, but rather augmented, PRDIV and NF-κB reporter gene activity (Fig. 3*A*). In contrast, MyD88 did

not inhibit IFN-β and PRDI–III reporter gene activity in control HEK293-TLR4 and HEK293-TLR7 cells (Fig. 3*B*, 3*C*). As the adaptor TRIF is critical for TLR3 functionality, we examined the ability of MyD88 to modulate TRIF signaling. We found that MyD88 inhibited TRIF-mediated activation of IFN-β, PRDI–III, and CCL5 reporter gene activity (Fig. 3*D–F*). As a control, we found that MyD88 did not inhibit, but rather augmented, IFN-β and CCL5 reporter gene activity that was driven by the RLRs RIG-I and Mda5 (Fig. 3*D–F*). We propose that this effect may be attributed to the synergistic effect of the RLRs and MyD88 on IFN-β reporter gene activity. Taken together, these data show that MyD88 exerts a ligand-dependent modulatory effect on TLR activity. Because MyD88 specifically inhibits the activity of the PRDI–III element of the IFN-β reporter gene and the CCL5 reporter gene, which contains an IRF3-dependent ISRE element, we propose that MyD88 mediates its inhibitory effects on TLR3 through a mechanism that involves IRF3.
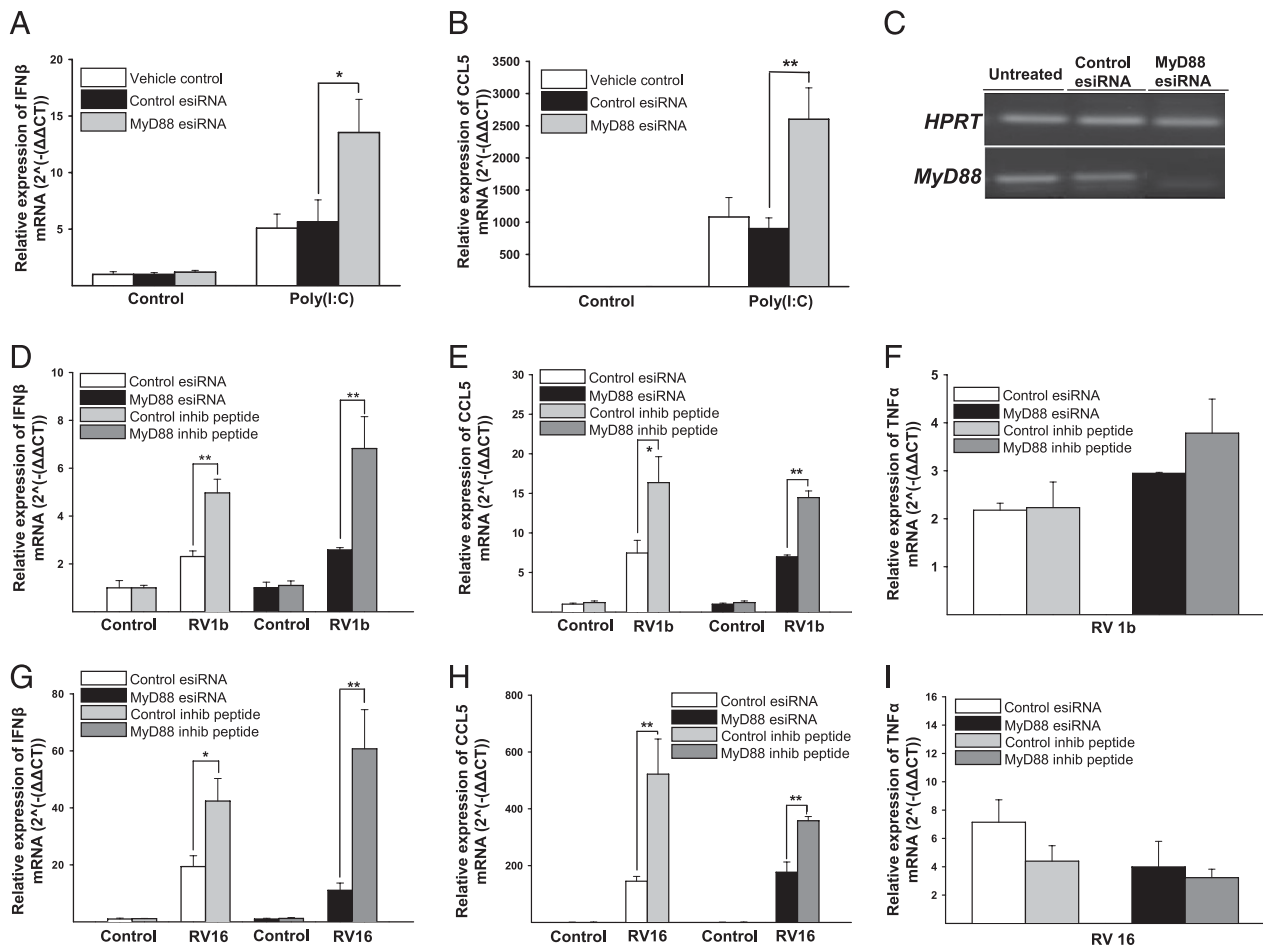
FIGURE 2. Suppression of MyD88 expression enhances IFN-β and CCL5 expression. *A–C*, BEAS-2B cells were pretreated with either control FLUC or MyD88 esiRNA to target the suppression of MyD88. After 36 h, cells were stimulated with vehicle (control) or poly(I:C) (10 µg/ml) for 4 h and total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR as described in *Materials and Methods* to assay the expression levels of IFN-β (*A*), CCL5 (*B*), or basal MyD88 expression in unstimulated cells (*C*). The data presented are representative of at least three independent experiments performed in triplicate (mean ± SE). *D–I*, BEAS-2B cells were pretreated for 24 h with either control FLUC or MyD88 esiRNA to target the suppression of MyD88 as described in *Materials and Methods*. Alternatively, cells were pretreated with control or MyD88 inhibitory peptide (20 µM) for 3 h. Next, cells were infected with RV1b or RV16 for 42 h at 33˚C, 5% CO_2 in a final concentration of 3% FCS with vehicle serving as a control. Next, total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR as described in *Materials and Methods* to assay the expression levels of IFN-β (*D*, *G*), CCL5 (*E*, *H*), and TNF-α (*F*, *I*). The data presented are representative of at least three independent experiments performed in triplicate (mean ± SE).

MyD88 inhibits IKKε-mediated induction of IRF3

To further explore the mechanism by which MyD88 regulates IRFs downstream of TLR3/TRIF, HEK293 cells were transfected with either TBK1 or IKKε and increasing amounts of MyD88. Given that both TBK1 and IKKε are weak activators of the IFN-β enhanceosome in HEK293 cells (data not shown), we opted to cotransfect the cells with TRIF (10 ng) to enhance the signal output and to aid in the engagement of auxiliary molecules necessary for reporter gene induction. Downstream activation of the IFN-β, PRDI–III, and CCL5 reporter genes was then examined. Whereas MyD88 inhibited IKKε-mediated IFN-β, PRDI–III, and CCL5 reporter gene activity, MyD88 did not inhibit, but rather enhanced, TBK1-driven reporter gene activity (Fig. 4*A–C*). Also, following poly(I:C) stimulation of HEK293-TLR3 cells, MyD88 dose-dependently inhibited IKKε- but not TBK1-driven IFN-β and PRDI–III reporter gene activity (Fig. 4*D*). In contrast, following LPS stimulation of HEK293-TLR4 cells, MyD88 dose-dependently enhanced IKKε and TBK1-driven IFN-β and PRDI–III reporter gene activity (Fig. 4*E*). Taken together, these data strongly suggest that the molecular target for MyD88 on the TLR3

pathway is IKKε and that MyD88 impairs IKKε-induced IRF3 activity and downstream induction of IFN-β.

To test the hypothesis that IKKε-driven IRF3 activity may be inhibited by MyD88, we tested whether the loss of MyD88 may affect IRF3 activity. As expected, stimulation of WT BMDMs with poly(I:C) and LPS enhanced IRF3 phosphorylation indicative of increased IRF3 activity (Fig. 5*A*). Furthermore, enhanced poly(I:C)-, but not LPS-, induced phosphorylation of IRF3 was detected in MyD88$^{-/-}$ cells compared with WT cells at similar time points (Fig. 5*A*). Additionally, whereas nuclear translocation of IRF3 and IRF7 were evident following stimulation of WT BMDMs with poly(I:C) for 1 h, enhanced nuclear translocation of IRF3, not IRF7, was detected in MyD88$^{-/-}$ cells compared with WT cells following poly(I:C) stimulation (Fig. 5*B*). Additionally, to show that enhanced nuclear translocation of IRF3 leads to concomitant increases in type I IFN in MyD88$^{-/-}$ BMDMs following poly(I:C) treatment, we opted to quantify bioactive type I IFN in MyD88$^{-/-}$ and WT BMDMs following stimulation with either poly(I:C) or LPS. Concomitantly, enhanced production of bioactive type I IFN was evident following stimulation of MyD88$^{-/-}$
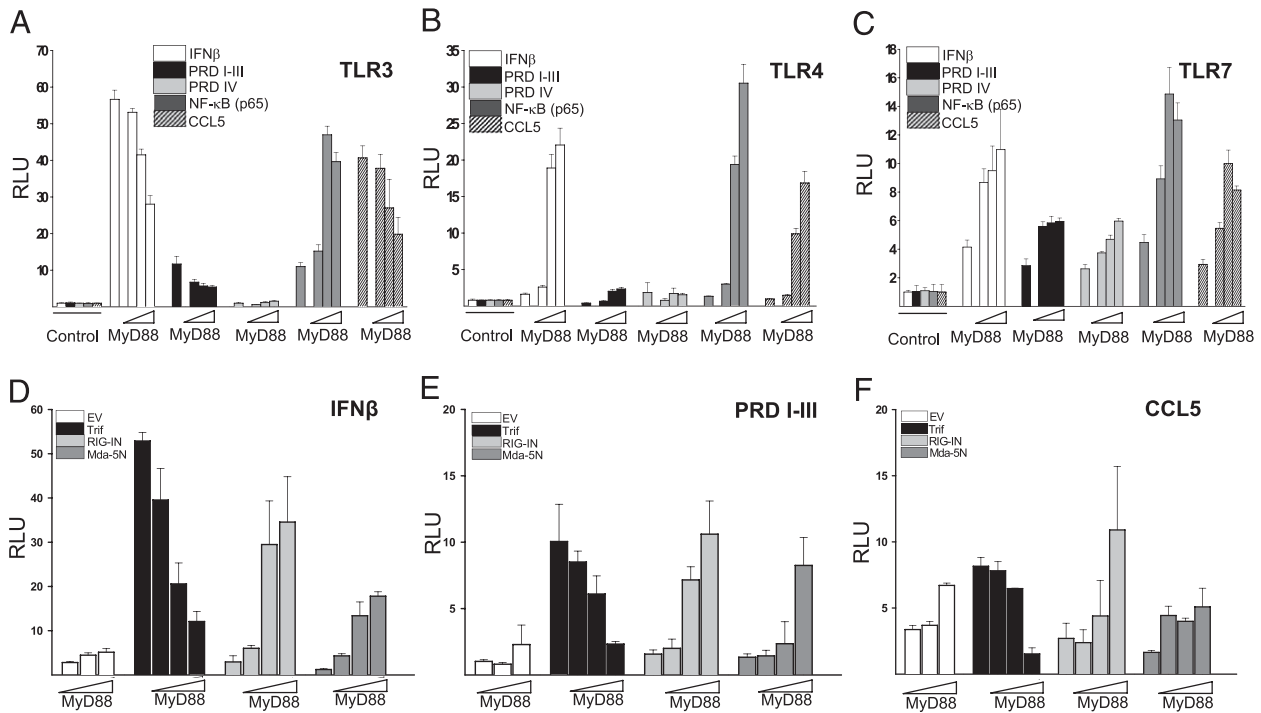
FIGURE 3. MyD88 negatively regulates TLR3:TRIF-induced activation of IFN-β gene and PRDI–III, but not NF-κB or PRDIV, gene induction. *A–C*, HEK293-TLR3, HEK293-TLR4, and HEK293-TLR7 cells were cotransfected with vectors encoding either a reporter gene for the IFN-β promoter, IFN-β PRDI–III, IFN-β PRDIV, NF-κB, or CCL5 and either empty vector (pcDNA3; 50 ng) or increasing amounts of an expression vector encoding MyD88 (10, 20, 50 ng) as indicated. After 24 h, cells were stimulated with poly(I:C) (25 μg/ml) (*A*), LPS (1 μg/ml) (*B*), or R848 (100 nM) (*C*) for 6 h followed by harvesting of the cell lysates and assessment of luciferase reporter gene activity. The results presented are representative of at least three independent experiments. *D–F*, HEK293 cells were cotransfected with vectors encoding a reporter gene for the full-length IFN-β promoter (*D*), IFN-β PRDI–III (*E*), or CCL5 promoter (*F*) and either TRIF (20 ng), RIG-I (20 ng), Mda-5 (20 ng), or empty vector (20 ng) and increasing amounts of the expression vector encoding MyD88 (1, 10, 20 ng) as indicated. After 24 h, cell lysates were harvested followed by assessment of luciferase reporter gene activity. The results presented are representative of at least three independent experiments.

BMDMs with poly(I:C) when compared with WT (Fig. 5*C*). Moreover, whereas coimmunoprecipitation studies have revealed a weak basal interaction between MyD88 and IRF3 (Fig. 5*D*, *left* and *right*, *lane* 3), treatment of HEK293-TLR3 cells with poly (I:C) enhanced the interaction of MyD88 with IRF3 (Fig. 5*D*, *left*, compare *lane 3* to *lane 4*). In contrast, treatment of HEK293-TLR4 cells with LPS did not enhance, but rather inhibited, the interaction of MyD88 with IRF3 (Fig. 5*D*, *right*, compare *lane 3* to *lane 4*). Taken together, these data suggest that the TLR3 ligand-dependent association of MyD88 with IRF3 may impair the activation and nuclear translocation of IRF3 and concomitant IFN-β and CCL5 gene induction.

To further test the hypothesis that curtailment of poly(I:C)-induced IRF3 activity by MyD88 is mediated through a mechanism involving IKKε, but not a TBK1, we transfected HEK293 cells with IRF3 and either TBK1 or IKKε in the absence and presence of MyD88. Thereafter, immunoblot analysis was performed using an anti-phosphorylated IRF3 Ab to assess IRF3 phosphorylation status, and increased phosphorylation indicates enhanced IRF3 activity. As expected, both expression of TBK1 and IKKε induced the phosphorylation of IRF3 (Fig. 5*E*, *lanes 3* and *5*). Notably, MyD88 suppressed IKKε-mediated phosphorylation of IRF3 (Fig. 5*E*, *lane 6*), whereas TBK1-induced phosphorylation remained unaffected in the presence of MyD88 (Fig. 5*E*, *lane 4*). Our observation that MyD88 suppresses IKKε-induced activation of IRF3 suggests that MyD88 specifically impairs TLR3:IKKε-induced phosphorylation and translocation of IRF3 in a ligand-dependent manner. Collectively, these findings strongly suggest that MyD88 targets IKKε-mediated activation of

IRF3 and that MyD88 inhibits TLR3 signaling by impairing the phosphorylation and translocation of IRF3, but not IRF7 through a process involving IKKε.

Discussion

The adaptor protein MyD88 plays a critical role in TLR signaling whereby it is required for the activation of all TLRs except TLR3 (1). Recently, studies have been directed toward understanding the role of the TLR adaptors in the negative regulation of noncognate TLRs. Johnson et al. (18) provided one of the first studies in the field to highlight a role for TLR adaptors in the negative regulation of noncognate TLRs whereby they showed that MyD88$^{-/-}$ mice display an exacerbated inflammatory response following poly(I:C) stimulation and suppression of MyD88 in human corneal endothelial cells exacerbated TLR3-driven RANTES through a mechanism involving JNK (18). Another study has shown that the TLR adaptor Mal is involved in the negative regulation of TLR3 ligand-induced JNK phosphorylation in macrophages (16). Importantly, we have recently shown that Mal plays a novel regulatory role in TLR3-mediated IFN-β gene induction whereby Mal inhibits TRIF-mediated activation of IRF7 (17). To date, no study on the role of MyD88 in the regulation of type I IFN production in response to TLR3 ligands has been carried out.

The current study aims to broaden our current understanding of TLR signaling and the role of TLR adaptors therein. We focused our attention on MyD88 and sought to explore its role in TLR3-driven IFN-β gene induction. Using macrophages derived from MyD88$^{-/-}$ mice, we demonstrate that IFN-β gene expression was significantly enhanced in the absence of MyD88. This correlates
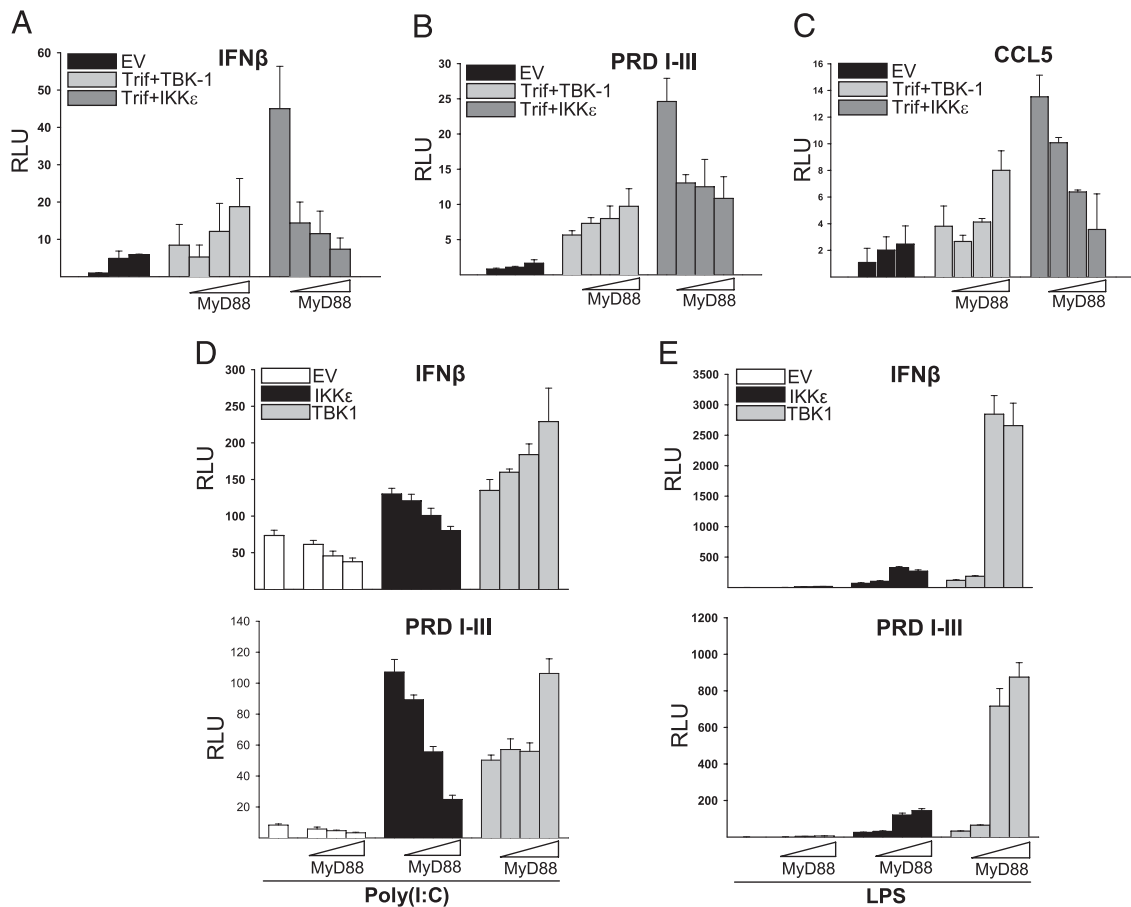
FIGURE 4. MyD88 negatively regulates IKKε-, but not TBK1-, mediated activation of IFN-β and CCL5 reporter genes. *A–C*, HEK293 cells were cotransfected with vectors encoding a reporter gene for the full-length IFN-β promoter (*A*), IFN-β PRDI–III (*B*), or CCL5 reporter gene (*C*) and cotransfected with TRIF (10 ng) plus TBK1 (10 ng) or TRIF (10 ng) plus IKKε (10 ng) and increasing amounts of the expression vector encoding MyD88 (1, 10, 20 ng) as indicated. After 24 h, cell lysates were harvested followed by assessment of luciferase reporter gene activity. The results presented are representative of at least three independent experiments. *D* and *E*, HEK293-TLR3 and HEK293-TLR4 cells, respectively, were cotransfected with vectors encoding either a reporter gene for the IFN-β promoter or IFN-β PRDI–III, as indicated, and either empty vector (20 ng), IKKε (20 ng), or TBK1 (20 ng) and increasing amounts of the expression vector encoding MyD88 (1, 10, 20 ng) as indicated. After 24 h, cells were stimulated with poly(I:C) (25 μg/ml) or LPS (1 μg/ml) for 6 h followed by harvesting of the cell lysates and assessment of luciferase reporter gene activity. The results presented are representative of at least three independent experiments.

with microarray data generated by von Bernuth et al. (34), who showed an increase in IFN-β mRNA in MyD88-defective individuals. Similarly, we show that suppression of MyD88 expression in epithelial cells enhanced TLR3-mediated IFN-β and CCL5 gene induction, adding further credence to our hypothesis that MyD88 impairs certain signaling pathways mediated through TLR3. Interestingly, our finding that IFN-β gene induction mediated by the ssRNA nonenveloped viruses RV1b and RV16 is enhanced following the suppression of MyD88 correlates with another study showing that MyD88^{−/−} mice exhibit enhanced IFN-β gene induction and enhanced dimeric IRF3 following exposure to the ssRNA nonenveloped coxsackievirus B3, also a member of the Picornaviridae family of viruses (35). In this study, we also demonstrate that the downstream marker of JNK activation, namely PRDIV reporter gene activity, was not affected by MyD88. We were also able to confirm that exacerbated RANTES (CCL5), an important modulator of antiviral defense, is observed in MyD88^{−/−} cells following poly(I:C) stimulation, as previously shown by others (18). In contrast to TLR3, abolition, rather than induction, of IFN-β was observed in response to the TLR4 ligand, LPS, indicating that the inhibitory role of MyD88 is TLR3-specific rather than a more generalized TRIF-dependent phenomenon.

To define the molecular mechanisms underlying MyD88-mediated inhibition of IFN-β and Rantes/CCL5, we examined the ability of MyD88 to modulate the PRD elements of the IFN-β gene. We demonstrate that MyD88 impaired poly(I:C)-induced activation of the PRDI–III element, but not the NF-κB or PRDIV elements of the IFN-β gene, and that CCL5 reporter gene activity was also impaired by MyD88 following poly(I:C) stimulation. These data suggested to us that MyD88 somehow modulated the IRF3/7 transcription factors. Further investigation revealed that MyD88 specifically impairs IRF3, not IRF7, functionality. Furthermore, this effect was mediated through IKKε, but not TBK1. Intriguingly, although Mal and MyD88 both have a negative impact on poly(I:C)-mediated IFN-β gene induction, MyD88, but not Mal, inhibited poly(I:C)-mediated Rantes production. Whereas Mal inhibits TLR3-mediated activation of IRF7, MyD88 exerts its inhibitory effects through a pathway involving IKKε:IRF3. Thus, it is clear that the molecular mechanism used by Mal and MyD88 to curtail innate immune responses emanating from TLR3 are quite distinct and that our study, which further defines the molecular role of MyD88 in TLR3 signaling, is clearly warranted.

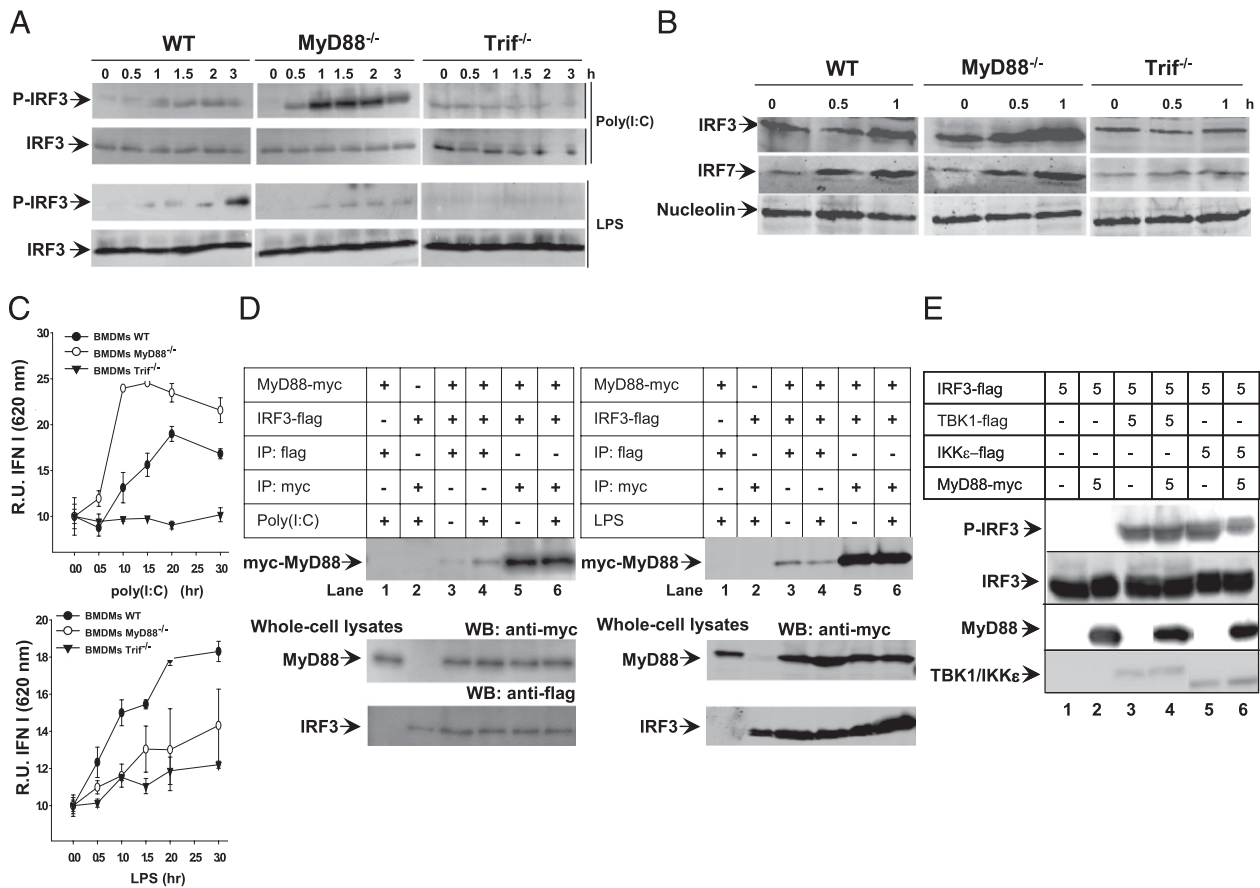The IKK-related kinases IKKε and TBK1 are 64% homologous, with both molecules containing a catalytic kinase domain,

FIGURE 5. MyD88 blocks IKKε-, but not TBK1-, induced activation of IRF3. *A*, WT, MyD88$^{-/-}$, and Trif$^{-/-}$ BMDMs were stimulated with poly(I:C) (1 μg/ml) or LPS (10 ng/ml) for 0–3 h. Thereafter, immunoblot analysis of whole cell lysates was performed using an anti-phosphorylated IRF3 Ab. Additionally, immunoblot analysis was performed to confirm the equal expression of IRF3 within each sample. The results presented are representative of at least three independent experiments. *B*, WT, MyD88$^{-/-}$, and Trif$^{-/-}$ BMDMs were stimulated with poly(I:C) (1 μg/ml) for 0, 0.5, or 1 h. Thereafter, isolation of the nuclear protein fraction was performed as described in *Materials and Methods*. Immunoblot analysis was then performed using an anti-IRF3 or an anti-IRF7 Ab. Equal protein loading was confirmed using an anti-nucleolin Ab. The data presented are representative of two independent experiments. *C*, WT, MyD88$^{-/-}$, and Trif$^{-/-}$ BMDMs were stimulated with poly(I:C) (1 μg/ml) or LPS (10 ng/ml) for 0–30 h. Thereafter, the cell-free supernatants were assessed for bioactive type I IFN as described in *Materials and Methods*. *D*, HEK293-TLR3 (*left*) and HEK293-TLR4 (*right*) cells were cotransfected with vectors encoding MyD88-cmyc, IRF3-Flag, or empty vector. After 24 h, cells were then stimulated with poly(I:C) (25 μg/ml) (*D*, *left*) or LPS (1 μg/ml) (*D*, *right*) for 90 min. Thereafter, immunoprecipitation (IP) of IRF3-Flag or MyD88-cmyc was performed as indicated. Immunoblot analysis was performed using anti-Myc Ab. Additionally, immunoblot analysis of whole cell lysates was performed to confirm the equal expression of the indicated proteins within each sample. The results presented are representative of at least three independent experiments. *E*, HEK293:TLR3 cells were transfected with IRF3-Flag (5 ng) and either TBK1-Flag (5 ng) or IKKε-Flag (5 ng) in the absence and presence of MyD88-cmyc (5 ng) as indicated. After 24 h, immunoblot analysis was performed to detect the presence of phosphor-IRF3 using an anti-phosphorylated IRF3 Ab, total IRF3 using an IRF3 Ab, MyD88-cmyc using an anti-myc Ab, TBK1 using an anti-Flag Ab, and IKKε using an anti-Flag Ab.

a leucine zipper domain, and a helix-loop-helix domain involved in protein–protein interactions (36). Whereas both TBK-1 and IKKε can directly phosphorylate IRF3 and IRF7 at key serine residues within their C-terminal signal-responsive domain (36), it is becoming increasingly apparent that each of these kinases exhibits functionally disparate responses to various ligands (36, 37). Regarding the role of these kinases in TLR3 signaling events, it has been reported that whereas normal IRF3 activation and IFN-β production was evident in poly(I:C)-treated IKK$^{-/-}$ embryonic fibroblasts, diminished IRF3 activation and IFN-β production was evident in TBK1$^{-/-}$ embryonic fibroblasts. In contrast, TBK1$^{-/-}$IKK$^{-/-}$ double-deficient embryonic fibroblasts exhibited complete abolition of IRF3 activation (38, 39), indicating that IKKε and TBK1 exhibit different roles in TLR signaling. Despite the apparent differential roles of TBK1 and IKKε in TLR signaling, minimal ligand-dependent functional analysis has been undertaken. In this study, we have broadened our understanding of TLR3 functionality and the differential role of

MyD88 in the modulation of TBK1 and IKKε. The obvious question is how and why MyD88 specifically blocks IKKε-mediated IRF3 activation, but not TBK1-mediated IRF3 activation, in the context of TLR3. Interestingly, using reporter gene assays, we have shown that dominant-negative TBK1 does not impair TRIF-mediated transactivation of IRF3; however, abolition of TRIF-mediated IRF7 transactivation was evident (data not shown). Thus, it is plausible to speculate that TBK1 may be redundant in the functioning of TLR3:TRIF:IRF3-mediated IFN-β and Rantes production. Studies are currently ongoing in our laboratory to address this question. Nonetheless, it is clear that MyD88 modulates IKKε-, but not TBK1-, mediated IFN-β and Rantes production following poly(I:C) stimulation.

In conclusion, by identifying MyD88 as a critical negative regulator of TLR3/IKKε-dependent IFN-β and CCL5 induction, the current study provides insight into the mechanism of TLR3-dependent signal transduction. MyD88 specifically inhibits TLR3-dependent type I IFN production, but not TLR4-mediated signal

transduction. We propose that upon TLR3 engagement, MyD88 is recruited and directly binds to IRF3, thereby inhibiting IKKε-mediated activation and translocation of IRF3 to the nucleus and concomitant IFN-β and RANTES production. These results demonstrate that MyD88 negatively regulates TLR3/IKKε-dependent IFN-β and RANTES production. In conclusion, MyD88 appears to play a novel major regulatory role in balancing the host inflammatory response to viral exposure. It is also clear that manipulation of the MyD88-regulated pathway to rebalance the host immune response may represent a novel therapeutic strategy.

Disclosures

The authors have no financial conflicts of interest.

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