

Nuclear Factor κ B Subunits RelB and cRel Negatively Regulate Toll-like Receptor 3-mediated β -Interferon Production via Induction of Transcriptional Repressor Protein YY1*

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Background: The expression of IFN- β is under tight regulatory control.

Results: NF- κ B induces YY1 that negatively regulates TLR3-mediated expression of IFN- β .

Conclusion: NF- κ B, via YY1, causes post-induction repression of IFN- β expression in response to TLR3.

Significance: This is a novel regulatory mechanism for controlling IFN- β expression.

The induction of β -interferon (IFN- β) is a key anti-viral response to infection by RNA viruses. Virus-induced expression of IFN- β requires the co-operative action of the transcription factors IRF-3/7, NF- κ B, and ATF-2/c-Jun on the IFN- β promoter leading to the orderly recruitment of chromatin remodeling complexes. Although viruses strongly activate NF- κ B and promote its binding to the IFN- β promoter, recent studies have indicated that NF- κ B is not essential for virus-induced expression of IFN- β . Herein, we examined the role of NF- κ B in regulating IFN- β expression in response to the viral-sensing Toll-like receptor 3 (TLR3). Intriguingly pharmacological inhibition of the NF- κ B pathway augments late phase expression of IFN- β expression in response to TLR3 stimulation. We show that the negative effect of NF- κ B on IFN- β expression is dependent on the induction of the transcriptional repressor protein YinYang1. We demonstrate that the TLR3 ligand polyribonucleic acid (poly(I:C)) induces expression and nuclear translocation of YinYang1 where it interacts with the IFN- β promoter and inhibits the binding of IRF7 to the latter. Evidence is also presented showing that the NF- κ B subunits c-Rel and RelB are the likely key drivers of these negative effects on IFN- β expression. These findings thus highlight for the first time a novel self-regulatory mechanism that is employed by TLR3 to limit the level and duration of IFN- β expression.

Toll-like receptors (TLRs)³ are primary sensors of pathogen-associated molecules that initiate innate immune reactions in

response to microbial challenge by activating transcription factors such as NF- κ B and inducing the expression of interferons (IFNs) and proinflammatory cytokines (1). With the exception of TLR3, all other mammalian TLRs employ the adaptor myeloid differentiation factor 88 (MyD88) as a key receptor proximal signaling molecule to stimulate downstream activation of NF- κ B (2). In contrast, TLR3, which is liganded by dsRNA from viral particles and the artificial ligand poly(I:C), interacts with a different adaptor protein termed Toll-interleukin-1 receptor domain-containing adaptor-inducing IFN- β (TRIF) and triggers differentiation factor 88-independent activation of NF- κ B and the interferon regulatory factor (IRF) family members IRF3 and IRF7 (3). TRIF induces phosphorylation of IRF3/7 via two kinases, TNF receptor-associated factor (TRAF) family member-associated NF- κ B activator (TANK)-binding kinase 1 (TBK1) and inducible I κ B kinase (also known as I κ B kinase ϵ) leading to nuclear translocation of IRF3/7 and their induction of type I IFNs (1, 3). The latter are critical anti-viral molecules that block viral replication (4, 5), and much effort has focused on the mechanisms underlying virus-induced expression of type I IFNs. The transcriptional regulation of the IFN- β gene by viruses has served as a paradigm in this regard (6, 7).

The enhancer region of the IFN- β gene contains four positive regulatory domains (PRDs). The coordinated binding of IRF3/7 to PRDI and -III, NF- κ B to PRDII, and ATF-2/c-Jun to PRDIV leads to recruitment of co-activator proteins, resulting in nucleosome acetylation and chromatin remodeling, ultimately facilitating access of the transcriptional machinery to the IFN- β promoter (6, 8). Although IRF3 and IRF7 have been demonstrated to play critical roles in enhancing transcription of IFN- β (9–11), the role of NF- κ B is less clear. Five subunits comprise the NF- κ B family and are termed RelA (p65), RelB, c-Rel, NF- κ B1 (p50 and its precursor p105), and NF- κ B2 (p52 and its precursor p100) (12, 13). These subunits interact in various dimeric combinations, with p50/p65 being most common. Although p50 and p52 lack a transactivation domain, RelA, RelB, and c-Rel each contain such a domain, and complexes containing these latter subunits are capable of promoting gene

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³ The abbreviations used are: TLR, Toll-like receptor; BMDM, bone marrow-derived macrophage; PRD, positive regulatory domain; TBK1, TRAF family member-associated NF- κ B activator (TANK)-binding kinase 1; TRAM, TRIF-related adaptor molecule; TRIF, Toll-interleukin-1 receptor domain-containing adaptor-inducing IFN- β ; YY1, YinYang1; IRF, interferon regulatory factor.

transcription. In resting cells NF- κ B is sequestered in the cytoplasm via its association with members of the I κ B family, and activation of the former requires prior phosphorylation of I κ Bs by I κ B kinases (14). Such phosphorylation facilitates subsequent polyubiquitination of I κ Bs leading to their degradation by the 26 S proteasome and allowing for nuclear translocation of NF- κ B (15). It is well known that viruses can strongly activate this NF- κ B pathway, with early studies demonstrating that viral challenge can promote the binding of the NF- κ B subunits RelA and p50 to the IFN- β promoter leading to its activation (16, 17). However, a more recent study using mice deficient in the genes encoding p50, RelA, or c-Rel demonstrated a nonessential role for NF- κ B in mediating induction of IFN- β in response to Sendai virus or Newcastle disease virus infection (18). Instead, the primary role for the NF- κ B subunits RelA appears to be in maintaining low basal expression of IFN- β before infection and facilitating low level autocrine IFN- β signaling in uninfected cells (19, 20). In response to viral infection, NF- κ B is important for early phase induction of IFN- β when IRF activation is low, but it fails to make a significant contribution to late phase peak levels of IFN- β when IRF proteins become the major drivers of expression. Indeed, contrary to stimulating late phase expression of IFN- β the recent studies, using NF- κ B-deficient mice provide clues that NF- κ B may negatively regulate late phase expression of IFN- β . Thus dendritic cells from c-Rel/p50-deficient mice produce higher levels of IFN- β mRNA than wild type cells in response to Sendai virus and Newcastle disease virus (18), and RelA-deficient murine embryonic fibroblasts (MEFs) show greater late phase expression of IFN- β than wild-type MEFs in response to Newcastle virus (19). This prompted the present study in which we speculated that NF- κ B may play a key role in post-induction repression of IFN- β .

Although the IFN- β promoter has served as a leading model for defining our understanding of how transcription factors can work together in a coordinated manner to promote eukaryotic gene transcription, the mechanism underlying gene repression at this promoter is less well understood. The protein Yin Yang 1 (YY1) is a transcription factor that, depending on cell type and promoter context, can act as an activator or repressor of transcription (21–23). YY1 binds to two sites on the murine IFN- β promoter and is capable of showing activation and repression of this promoter (24). The occupancy of both the –90 and –122 sites of the IFN- β promoter by YY1 leads to recruitment of the histone acetyltransferase CBP (cAMP-response element-binding protein (CREB)-binding protein) and activation of the IFN- β promoter (25). In contrast, the repressor role of YY1 at the IFN- β promoter is mediated by its binding to the –90 site and subsequent recruitment of a histone deacetylase that represses the IFN- β promoter (24). Given that NF- κ B positively regulates the expression of YY1 (26), we hypothesized that NF- κ B may effect post-induction repression of IFN- β via YY1. We show that NF- κ B negatively regulates late phase expression of IFN- β in response to TLR3 stimulation and confirm a key role for YY1 in mediating this inhibitory effect. The mechanistic basis to this process is also presented. We show that TLR3 stimulation can promote increased expression of YY1 in a NF- κ B-dependent manner most likely mediated by the c-Rel and RelB subunit. TLR3 also promotes nuclear localization of

YY1 where it interacts with IFN- β promoter and inhibits the binding of IRF7 to the promoter. These findings thus define a novel mechanism by which the viral-sensing receptor TLR3 can employ NF- κ B to self-regulate the magnitude and duration of IFN- β expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293 cells, stably expressing TLR3, were from InvivoGen. Human U373 astrocytoma cells stably transfected with CD14 (U373-CD14) and bone marrow-derived macrophages (BMDMs) from wild type and TRIF-deficient mice were gifts from Dr. Katherine Fitzgerald (University of Massachusetts Medical School). The HEK293 and THP-1 cell lines were from the Health Protection Agency Culture Collection and were grown in DMEM and RPMI 1640, respectively, with GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 μ g/ml), streptomycin (100 μ g/ml), and Normocin (InvivoGen) (100 μ g/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. G418 (200 μ g/ml) was used to select for the stably transfected TLR3 cell line and maintenance of CD14 expression. Highly pure protein-free LPS derived from *Escherichia coli* strain 011:B4 was from Alexis. Naked poly(I:C), a TLR3 activator, was from Invivogen. JSH-23 was from Calbiochem. The rabbit anti-RelA (p65) antibody and mouse anti-c-Rel and anti-YY1 antibodies were from Santa Cruz Biotech. The rabbit anti-RelB antibody was from NCI Biological Resources Branch. The rabbit and anti-mouse Alexa Fluor 568 and anti-mouse Alexa Fluor 633 secondary antibodies were from Invitrogen.

Expression Vectors/Recombinant Plasmids—The IFN- β promoter-, PRDI-III, PRDII-, and PRDIV-regulated firefly luciferase constructs were as described (3). pCMV YY1-HA plasmid was a generous gift from Yang Shi (Harvard Medical School, Boston, MA). The pcDNA expression constructs encoding c-Rel-FLAG and RelB-FLAG were generous gifts from Gioacchino Natoli (European Institute of Oncology, Milan, Italy). The pcDNA3.1 expression constructs encoding RelA-HA, IRF3-FLAG, and IRF7-FLAG were cloned by standard procedures in our laboratory. Murine YY1 lentiviral shRNA plasmids were generous gifts from Mary E. Donohoe (Weill Cornell Medical College) (27). Murine IRF7 shRNA lentiviral plasmids were from Sigma (NM_016850).

First-strand cDNA Synthesis—BMDMs, U373, and THP-1 cells were seeded (3.5×10^5 cells/ml; 2 ml) in 6-well plates and grown for 24 h. Cells were then stimulated with various ligands for different times as indicated in the figures. Total RNA was isolated from cells using the TRIzol® reagent according to the manufacturer's instructions (Invitrogen). Isolated RNA (1 μ g) was incubated with random hexamer primers (1 μ l; 500 μ g/ml) at 70 °C for 5 min. Thereafter, the other reaction components were added in the following order: 5 μ l of 5 \times RT buffer (Promega), 1.3 μ l of 10 mM dNTP, 0.7 μ l RNasin (Promega), 1 μ l of Moloney murine leukemia virus reverse transcriptase (Promega), and nuclease-free water to a total volume of 25 μ l. Reactions were incubated at 37 °C for 40 min followed by 42 °C for 40 min and heating to 80 °C for 5 min. The first strand cDNA was stored at –20 °C for up to 1 month.

NF- κ B Inhibits TLR3-induced IFN- β Expression via YY1

Real-time PCR—Total cDNA was subjected to RT-PCR and real-time quantitation with the DyNAmo[®]HS SYBR Green kit (Finnzymes) on a real-time PCR system (DNA Engine OPTICON[®] system; MJ Research). The following primers were used to amplify the fragments of the indicated genes: *mIFN- β* , forward (GGAGATGACGGAGAAGATGC) and reverse, (CCCAGTGCTGGAGAAATTGT); *hIFN- β* forward, (AAGTGCAACCTTTCGAAGCC) and reverse (TGTCGCCTACTACCTGTTGTGC); *mTNF α* forward (CATCTTCTCAAAATTCGAGTGACAA) and reverse (TGGGAGTAGACAAAGGTACAACCC); *hIL6*, forward (AGCCACTCACCTTTCAGAACGAA) and reverse (CAGTGCCTCTTTGCTGCTTTCACA); *mYY1*, forward (TCACCATGTGGTCCCTCGGATGAAA) and reverse (TTCTGCCAGTTGCTTAGGGTC-TGA). Each mRNA quantification was normalized relative to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (*HPRT*) using the primers *mHPRT* forward (CCCTGAAGTACTCATTATAGTCAAGGGCAT) and reverse (GCTTGCTGGTGAAGGACCTCTCGAAG), *hHPRT* forward (AGCTTGCTGGTGAAGGAC) and reverse (TATAGTCAAGGGCATATCC). Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method as previously described (28).

Lentiviral Transduction—The plasmids encoding shRNA constructs targeting mYY1 (mYY1 U6 shRNA) or mIRF7 (pLKO.1-puro IRF7) or their respective control plasmids (p β 5 U6 control shRNA and pLKO.1-puro Control Vector) were integrated into lentiviral particles as previously described (29). BMDMs (1×10^5 cells/well; 6-well plate) were transduced with control shRNA, mYY, or mIRF7 shRNA lentiviral particles, and cells were subsequently grown for 1 week under puromycin (10 μ g/ml) selection. The efficiency of YY1 and IRF7 knockdown was assessed by RT-PCR using the following primers: *mYY1*, forward (TCACCATGTGGTCCCTCGGATGAAA) and reverse (TTCTGCCAGTTGCTTAGGGTCTGA); *mIRF7*, forward (TCAGTTTCTGCAGTACAGCCACAT) and reverse (ATGATGGTACATCCAGGAACCCA); *mHPRT*, forward (CCCTGAAGTACTCATTATAGTCAAGGGCAT) and reverse (GCTTGCTGGTGAAGGACCTCTCGAAG).

siRNA Transfection—HEK293 or U373 cells were seeded in 12-well (1×10^5 cells/well) or 96-well (2×10^4 cells/well) plates. Cells were allowed grow for 16 h and then transfected with control or YY1-specific siRNA (20 nM) using Lipofectamine 2000 as described by the manufacturer (Invitrogen). After 36 h, efficiency of YY1 knockdown was assessed in U373 cells by Western blotting using a YY1-specific antibody and in HEK293 cells by semiquantitative RT-PCR.

Reporter Assays—HEK293, HEK293-TLR3, and HEK293-TLR4 cells were seeded in 96-well plates (2×10^4 cells/well) and grown overnight. Cells were co-transfected with the indicated amounts of expression constructs encoding Trif, RelA-HA, RelB-FLAG, c-Rel-FLAG, or YY1-HA and firefly luciferase reporter constructs regulated by the IFN- β promoter or the PRDI/III, II, or IV domains (80 ng) using Lipofectamine as previously described (30). In all cases an expression construct encoding constitutively expressed TK Renilla luciferase (40 ng) was cotransfected to normalize for transfection efficiency. Cells were allowed to recover for 24 h after transfection

and then stimulated with poly(I:C) or LPS as indicated. Cell lysates were prepared, and reporter gene activity was measured using the Dual Luciferase Assay system (Promega) as described (30).

IRF7/RelA Nuclear Translocation Assay—U373 cells were stimulated with poly(I:C) (10 μ g/ml) or LPS (100 ng/ml) for 0–2 h. Cells were centrifuged, and cell pellets were resuspended and incubated for 2 min on ice in 10 mM HEPES, pH 8.0, containing 50 mM NaCl, 17% (w/v) sucrose, 1 mM EDTA, 0.4 mM spermidine, 0.2% (v/v) Triton X-100, 0.8 mM PMSF, 0.05% (v/v) β -mercaptoethanol, and 0.2 units/ml aprotinin. After centrifugation at $12,000 \times g$ for 1 min at 4 $^{\circ}$ C, the cell pellet was washed with 10 mM HEPES, pH 8.0, containing 50 mM NaCl, 25% (w/v) glycerol, 0.1 mM EDTA, 0.4 mM spermidine, 0.8 mM PMSF, 0.05% (v/v) β -mercaptoethanol, and 0.2 units/ml aprotinin. The supernatant was removed, and the nuclear pellet was resuspended in 3 times the packed nuclear volume of ice-cold 10 mM HEPES, pH 8.0, containing 340 mM NaCl, 25% (w/v) glycerol, 0.1 mM EDTA, 0.4 mM spermidine, 0.8 mM PMSF, 0.05% (v/v) β -mercaptoethanol, and 0.2 units/ml aprotinin. The samples were gently vortexed at 4 $^{\circ}$ C for 1 h and centrifuged at $12,000 \times g$ for 10 min at 4 $^{\circ}$ C, and the supernatants (the nuclear extracts) were saved. Nuclear extracts were normalized for protein concentration and subjected to Western blotting using anti-IRF7 and anti-RelA antibodies.

IFN- β Enhanceosome Pulldown Assay—HEK293-TLR3 cells were seeded in 90-mm dishes (2×10^6 cells) and transiently transfected using Lipofectamine 2000 (Invitrogen) with a pCMV2 construct encoding YY1-HA and a pcDNA3.1 construct encoding IRF7-FLAG (5 μ g). Corresponding empty vector constructs were used as controls. Cells were allowed to recover for 24 h and then stimulated with poly(I:C) for various times. Nuclear extracts were generated as previously described (31). Nuclear extracts (250 μ g) were then incubated for 1 h at 20 $^{\circ}$ C with 3 nmol of biotinylated double-stranded oligonucleotides corresponding to nucleotide sequences from the IFN- β enhanceosome. The sense strand has the sequence 5'-CTAA-AATGTAAATGACATAGGAAAAGTCAAAGGGGAGAAG-TGAAAGTGGGAAATTCCTCTGAATAGAGAGGACC-3'. Streptavidin-magnetic beads (Invitrogen Dynabeads M-280) were added to collect the biotinylated oligonucleotides and associated proteins, and incubation was continued with a rotation for 1 h at 4 $^{\circ}$ C. The beads were washed 3 times with washing buffer (5 mM Tris, pH 7.5, containing 0.5 mM EDTA, and 1 M NaCl) and then subjected to Western blot analysis using an anti-FLAG or anti-HA antibody.

Assay of IFN- β —U373 cells were seeded in 12-well plates (0.5×10^6 cells/well). Cells were then treated with or without JSH-23 (30 μ M) for 1 h before stimulation with poly(I:C) (10 μ g/ml) for 16 h. Cell supernatants were collected and cleared by centrifugation, and IFN- β was assayed by sandwich ELISA using rabbit anti-human IFN β (Santa Cruz) as a capture antibody and biotinylated mouse anti-human IFN- β (Peprotech) in conjunction with streptavidin peroxidase as a detection system.

Immunoblot Analysis—Cells were stimulated with poly(I:C) or LPS as indicated, and lysates were subjected to SDS-PAGE followed by Western blot analysis with an anti-phospho-(Thr¹⁸⁰/

Tyr¹⁸²) p38 MAPK, anti-phospho-JNK (Thr-183/Tyr-185, Cell Signaling), anti-I κ B α (Cell Signaling), anti- β -actin (Sigma), anti-FLAG (Sigma), or anti-HA (Cell Signaling) antibody.

Confocal Microscopy—U373 cells (1×10^6 cells per well/6-well plate) were stimulated with poly(I:C) (1 μ g/ml) for the indicated times. Cells were then rinsed with PBS, permeabilized with Triton X-100 (0.2% v/v), and fixed at room temperature for 5 min with 2% (v/v) paraformaldehyde. Cells were then incubated overnight at 4 °C with rabbit anti-RelA (p65), rabbit anti-RelB, mouse anti-c-Rel, or mouse anti-YY1 antibody (all at 1/50 dilution). Cells were rinsed with PBS and incubated with the anti-rabbit or anti-mouse Alexa Fluor 568 secondary antibody (1/200 dilution) or anti-mouse Alexa Fluor 633 secondary antibody (1/200 dilution) for 1 h in the dark with slow agitation. Cells were again washed with PBS and counterstained using DAPI nuclear stain (Sigma). Fluorescence was examined using an Olympus laser confocal microscope (Olympus Life and Material Science Europe, Hamburg, Germany). Acquired images were analyzed using the Olympus FV 1.6b imaging software. Negative control experiments were performed by replacing the primary antibody with relevant IgG isotype controls (Millipore) and using equal gain settings during acquisition and analysis.

Chromatin Immunoprecipitation Assay—U373 cells were grown to confluency in 6-well plates. Cells were pretreated for 30 min with or without JSH-23 (30 μ M) before stimulation for 4 h with poly(I:C) (10 μ g/ml). Chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described (32) with some minor modifications. Briefly cells were cross-linked with 1% (v/v) formaldehyde for 10 min at 37 °C. Isolated nuclei were subjected to three 15-s sonication pulses using a Hielscher UP 200S sonicator at 50% of the total power. This procedure yielded chromatin fragments of 200–1000 bp. Chromatin preparations were precleared with protein A/G Plus beads (Santa Cruz Biotechnology) and then incubated overnight at 4 °C with anti-YY1 or anti-IRF7 antibody (Santa Cruz Biotechnology) or rabbit IgG. The immunoprecipitates were then incubated with protein A/G-agarose beads (Santa Cruz Biotechnology), washed, and collected for subsequent reverse cross-linking by overnight incubation at 65 °C. DNA was then extracted using phenol-chloroform and precipitated in ethanol. Standard PCR was conducted with specific primers designed to amplify the enhancer region of the IFN- β promoter. The primers were forward (5'-CTA AAA TGT AAA TGA CAT AGG-3') and reverse (5'-GGT CCT CTC TAT TCA GAG-3'). PCR products were resolved by 2% (w/v) agarose gel electrophoresis and then analyzed using a multiimager Typhoon 8600 (Molecular Dynamics).

Data Analyses—Statistical analysis was carried out using the unpaired Student's *t* test using the SigmaPlot 2001 program. *p* values of less than or equal to 0.05 were considered to indicate a statistically significant difference where *, *p* < 0.05 and **, *p* < 0.01.

RESULTS

NF- κ B Negatively Regulates TLR3-induced Expression of IFN- β —To explore the potential of NF- κ B to mediate the repression of IFN- β after the activation of viral sensing TLRs, BMDMs were pretreated in the absence or presence of the

NF- κ B inhibitor JSH-23 (33) before stimulation with the TLR3 agonist poly(I:C) and assaying of IFN- β expression by quantitative PCR. Poly(I:C) promoted strong induction of IFN- β expression in wild-type BMDMs, and this was strongly augmented in cells pretreated with JSH-23 (Fig. 1A), suggesting that NF- κ B can negatively regulate poly(I:C)-induced IFN- β expression. Poly(I:C) and JSH-23 failed to affect expression of IFN- β in TRIF-deficient BMDMs, indicating that under the described experimental conditions both agents exert their effects by regulating the TLR3 pathway. The positive effect of JSH-23 on the TLR3 pathway shows specificity for IFN- β expression as JSH-23 failed to affect poly(I:C)-induced expression of TNF (Fig. 1B). Furthermore the augmentation of IFN- β mRNA expression by JSH-23 shows selectivity for TLR3 as LPS-induced expression of IFN- β and TNF was decreased by JSH-23 in BMDMs (Fig. 1, A and B). JSH-23 also selectively augments poly(I:C)-induced expression of IFN- β in the human monocytic cell line THP-1 while failing to affect poly(I:C) induction of TNF or LPS-induced expression of IFN- β and TNF (Fig. 1C). These findings indicate that the negative regulatory effect of NF- κ B on TLR3-induced expression of IFN- β mRNA expression is not restricted to murine BMDMs and instead is species- and cell-type independent. In addition, JSH-23 also augments TLR3-induced expression of IFN- β protein (Fig. 1D), further validating the above findings that were based on measuring levels of IFN- β mRNA. Furthermore, to confirm that these effects of JSH-23 on IFN- β expression are associated with inhibition of NF- κ B, cells were transfected with an expression construct encoding I κ B super repressor protein and assessed for effects on TLR3-induced expression of IFN- β . Like JSH-23, the I κ B super repressor protein augmented poly(I:C), but not LPS-induced expression of IFN- β (Fig. 1E), further validating the conclusion that activation of NF- κ B by TLR3 serves as a mechanism to self-regulate its induction of IFN- β .

YY1 Is Induced by NF- κ B and Negatively Regulates TLR3-induced Expression of IFN- β —To assess if the negative effect of NF- κ B on TLR3-induced expression of IFN- β is mediated by direct inhibition of the IFN- β promoter or indirectly by induction of a NF- κ B-induced repressor protein, BMDMs were treated with the protein synthesis inhibitor cycloheximide and examined for effects on poly(I:C)-induced expression of IFN- β mRNA. Like JSH-23, cycloheximide augmented the ability of poly(I:C) to induce expression of IFN- β mRNA (Fig. 2A), whereas it failed to potentiate poly(I:C)-induced expression of TNF mRNA (Fig. 2B) or LPS-induced expression of IFN- β (Fig. 2A) and TNF mRNA (Fig. 2B). Furthermore, no synergy or additive effects were observed when cells were co-treated with JSH-23 and cycloheximide (Fig. 2A), suggesting that JSH-23 manifests its potentiating effects by blocking the induction of a NF- κ B-regulated repressor protein. Given that YY1 is known to be regulated by NF- κ B and can act as a repressor at the IFN- β promoter, we next probed the role of YY1 in poly(I:C)-mediated IFN- β induction. First, both poly(I:C) and LPS were shown to induce the expression of YY1 in BMDMs, and this expression was reduced by pretreatment of cells with JSH-23 (Fig. 2C). The role of YY1 in regulating TLR3- and -4-induced expression of IFN- β was then directly addressed by infecting BMDMs with

NF- κ B Inhibits TLR3-induced IFN- β Expression via YY1

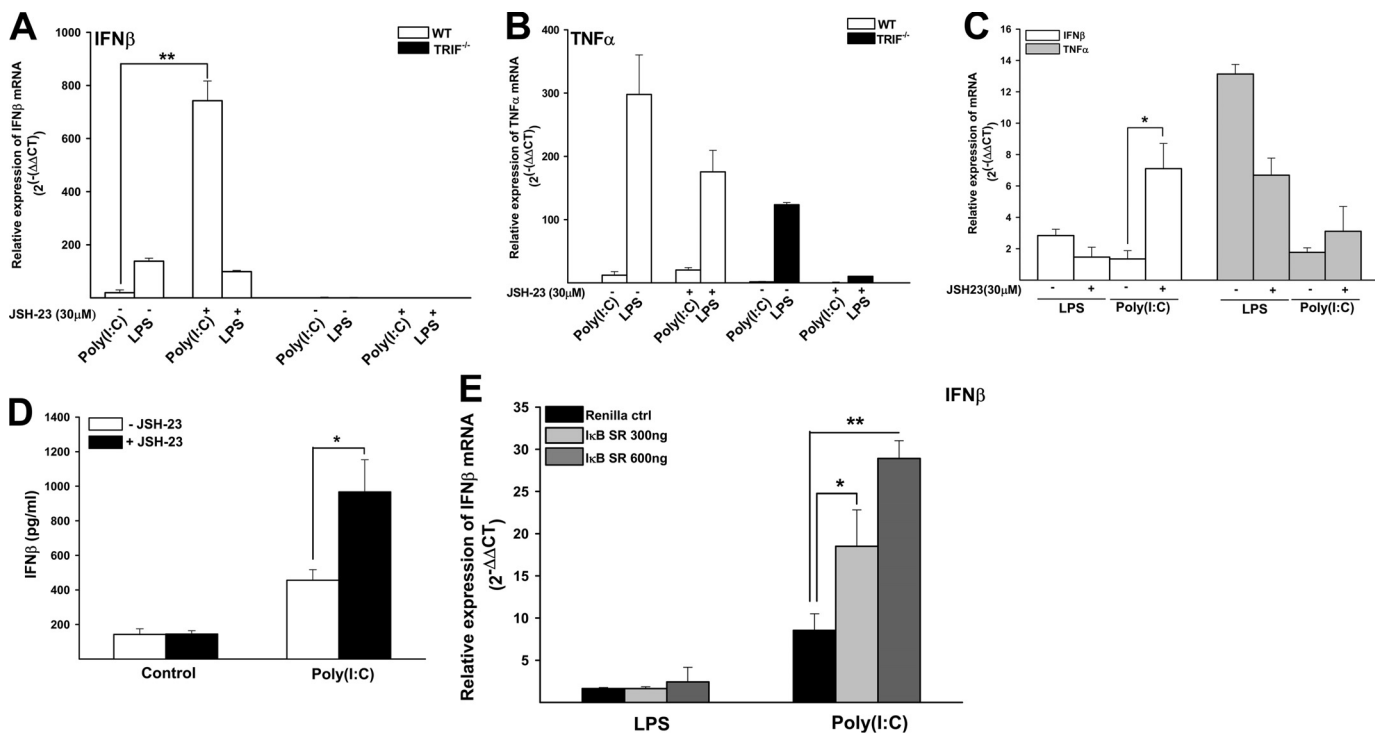


FIGURE 1. Inhibition of NF- κ B augments poly(I:C) induced expression of IFN- β . Immortalized BMDMs from wild type (WT) and TRIF^{-/-} mice (A and B) and M-CSF-differentiated THP-1 cells (C) were treated with or without the NF- κ B inhibitor JSH-23 (30 μ M) for 30 min followed by further stimulation in the absence or presence of poly(I:C) (1 μ g/ml) or LPS (10 ng/ml) for 2 h. E, U373 cells were previously transfected with an expression construct encoding the I κ B super repressor (SR) protein or Renilla luciferase protein as control. Total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR to assay the mRNA expression levels of (A, C, and E) IFN- β and (B and C) TNF α . The levels of the relevant mRNAs were normalized relative to the housekeeping gene HPRT and are expressed relative to normalized values from unstimulated cells. D, U373 cells were treated with or without JSH-23 (30 μ M) for 1 h before stimulation with poly(I:C) (10 μ g/ml) for 16 h. Cell supernatants were subsequently assayed for levels of IFN- β protein by sandwich ELISA. All data represent the mean \pm S.E. of three independent experiments and were subjected to unpaired Student's *t* test, **p* < 0.05; ***p* < 0.01.

lentiviral particles containing control or mYY1-specific shRNA. The latter was shown to selectively and specifically suppress endogenous expression of mYY1 as measured by semiquantitative RT-PCR (*inset*, Fig. 2D). Knockdown of mYY1 resulted in strong augmentation of poly(I:C), but not LPS-induced expression of IFN- β mRNA (Fig. 2D), indicating a specific repressor role for mYY1 in regulating TLR3-induced expression of IFN- β . We next assessed whether the specific effect of YY1 on IFN- β mRNA also applies to human cells and is cell type-independent. Thus human U373 cells were transfected with control or YY1-specific siRNA to specifically suppress YY1 expression (*inset*, Fig. 2E). Poly(I:C) showed greatly increased potential to induce IFN- β in YY1-knockdown cells relative to cells transfected with control siRNA (Fig. 2E), further confirming a negative role for YY1 in TLR3 signaling. To directly address whether YY1 mediates the negative effects of NF- κ B on TLR3-induced expression of IFN- β , JSH-23 was examined for its effects on IFN- β expression in control and YY1-knockdown U373 cells. As before, JSH-23 augmented poly(I:C)-induced expression of IFN- β mRNA in control cells, but notably potentiation was no longer evident in YY1 knockdown cells (Fig. 2E), strongly indicating that the positive effects of JSH-23 on IFN- β expression are mediated by negatively affecting the expression of NF- κ B-regulated YY1.

C-Rel and RelB Negatively Regulate TLR3-induced Expression of IFN- β —Given that NF- κ B drives the expression of YY1 and negatively regulates IFN- β expression in response to TLR3 stimulation, we next characterized the relative contribution of each of the NF- κ B subunits containing a transactivation domain to this process. We initially assessed the ability of poly(I:C) to promote classical activation of NF- κ B in BMDMs, as judged by degradation of the inhibitory protein I κ B α . We observed only modest degradation of I κ B α in response to poly(I:C), whereas LPS caused classical total depletion of I κ B α followed by its rapid resynthesis (Fig. 3A). In contrast, poly(I:C) promoted strong activation of the p38 and JNK MAPK pathways, thus suggesting that even under conditions of strong TLR3 signaling, this receptor is a poor activator of the classical NF- κ B pathway. Using confocal microscopy, we next assessed the ability of poly(I:C) to regulate the expression and nuclear translocation of each of the transactivating NF- κ B subunits. In unstimulated cells, the p65 subunit showed a predominant cytoplasmic localization, and this was not affected by treatment of cells with poly(I:C) (Fig. 3B). In contrast, poly(I:C) promoted strong nuclear translocation of RelB (Fig. 3C) and c-Rel (Fig. 3D). The effects of each of these subunits on poly(I:C)-induced activation of the IFN- β promoter was next examined. Like YY1, expression of c-Rel or RelB strongly inhibited poly(I:C)-induced activation of the IFN- β promoter, whereas overexpression of p65 augmented this response (Fig. 4A). To validate the functional con-

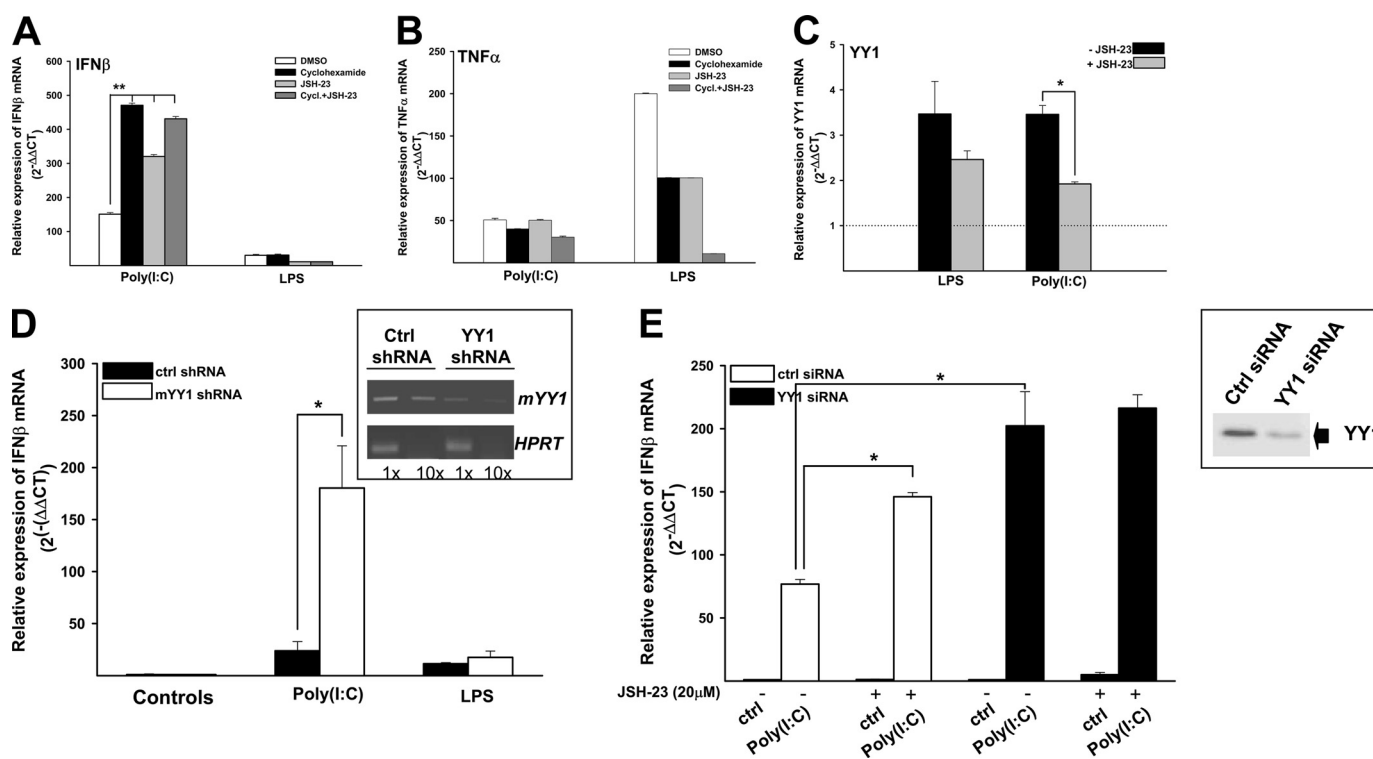


FIGURE 2. NF- κ B negatively regulates TLR3-induced expression of IFN- β via the induction of YY1. A–C, BMDMs were treated with or without the NF- κ B inhibitor JSH-23 (30 μ M) and/or cyclohexamide (Cycl., 10 μ g/ml) for 30 min followed by further stimulation in the absence or presence of poly(I:C) (1 μ g/ml) or LPS (10 ng/ml) for 2 h (A and B) or 90 min (C). Total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative RT-PCR to assay the mRNA expression levels of IFN- β (A and B) and YY1 (C). The levels of the relevant mRNAs were normalized relative to the housekeeping gene HPRT and are expressed relative to normalized values from unstimulated cells. D, BMDMs were transfected with lentivirus-encoding control or mYY1-specific shRNA. Cells were then stimulated with poly(I:C) (1 μ g/ml) or LPS (10 ng/ml) for 36 h. cDNA was generated and assayed for YY1 expression using semiquantitative PCR (inset) and for IFN- β expression by quantitative RT-PCR as described above. E, U373 cells were transfected with control (ctrl) or YY1-specific siRNA and allowed recover for 2 h. Cells were then treated with or without JSH-23 (30 μ M) for 30 min followed by further stimulation in the absence or presence of poly(I:C) (1 μ g/ml) for 2 h. cDNA was generated and assayed for IFN- β expression by quantitative RT-PCR. Cell lysates were probed by Western blotting using an anti-YY1 antibody (inset). All data represent the mean \pm S.E. of three independent experiments and were subjected to unpaired Student's *t* test. *, *p* < 0.05; **, *p* < 0.01.

sequences of such effects on the IFN- β promoter, the effects of each of the subunits on TLR3-induced expression of IFN- β protein was then assessed. In an analogous manner to the promoter studies, whereas p65 potentiated poly(I:C)-induced expression of IFN- β protein, c-Rel and RelB inhibited this process (Fig. 4B). The effects of the NF- κ B subunits on the expression levels of IFN- β protein were not as great in magnitude as those seen on the IFN- β promoter, but it is important to note that the effects on the former are limited by the degree of transfection efficiency. To further probe the mechanistic basis to the differential effects of the Rel subunits on the IFN- β promoter, the sensitivity of each of the PRD regions of the IFN- β promoter was then investigated. Both RelB and c-Rel inhibited activation of the PRD I-III domain (Fig. 4C) but failed to regulate PRDII (Fig. 4D) or PRDIV (Fig. 4E), whereas RelA showed a specific stimulatory effect on the NF- κ B-regulated domain PRDII (Fig. 4D). Taken together, these findings suggest a key role for the c-Rel and RelB subunits in the negative regulation of TLR3-induced activation of the PRD I-III domain of the IFN- β promoter and expression of IFN- β .

YY1 Negatively Regulates the PRDI/III Domains of IFN- β Promoter—We next focused on the potential mechanism(s) by which YY1 may be used by the TLR3 pathway to self-regulate IFN- β expression. Initial studies used confocal microscopy to characterize the ability of the TLR3 pathway to regulate cellular expression and subcellular localization of endogenous YY1.

Although unstimulated U373 cells showed basal cytoplasmic expression of YY1, poly(I:C) promoted a strong increase in the expression of YY1 with a predominant nuclear localization (Fig. 5A). Given the nuclear accumulation of YY1, we next addressed the direct effects of YY1 on expression of IFN- β . The expression of YY1 in U373 cells caused a strong inhibition of poly(I:C)-induced expression of IFN- β mRNA, whereas it failed to affect IL-6 expression (Fig. 5B), indicating that YY1 can directly and selectively regulate IFN- β expression. This effect of YY1 expression is also specific as overexpression of a control protein such as GFP fails to mimic the effects of YY1. To further probe its mechanism of action, the regulatory effects of YY1 on the IFN- β promoter and the various regulatory domains were then examined. Using the TLR3 adaptor Trif as a strong inducer of the transfected IFN- β promoter-regulated luciferase reporter gene, co-expression of YY1 in HEK293 cells was shown to strongly inhibit activation of the IFN- β promoter (Fig. 5C). The sensitivity of each of the PRD regions of the IFN- β promoter was also examined and, like RelB and c-Rel, YY1 strongly inhibited activation of the PRD I-III domain but failed to regulate PRDII or PRDIV. To further validate the finding that YY1 selectively targets the PRDI-III regions, endogenous expression of YY1 in HEK293 cells was suppressed by siRNA (inset, Fig. 5D), and this resulted in augmented Trif-induced expression of the luciferase reporter gene when the latter was regulated by the

NF- κ B Inhibits TLR3-induced IFN- β Expression via YY1

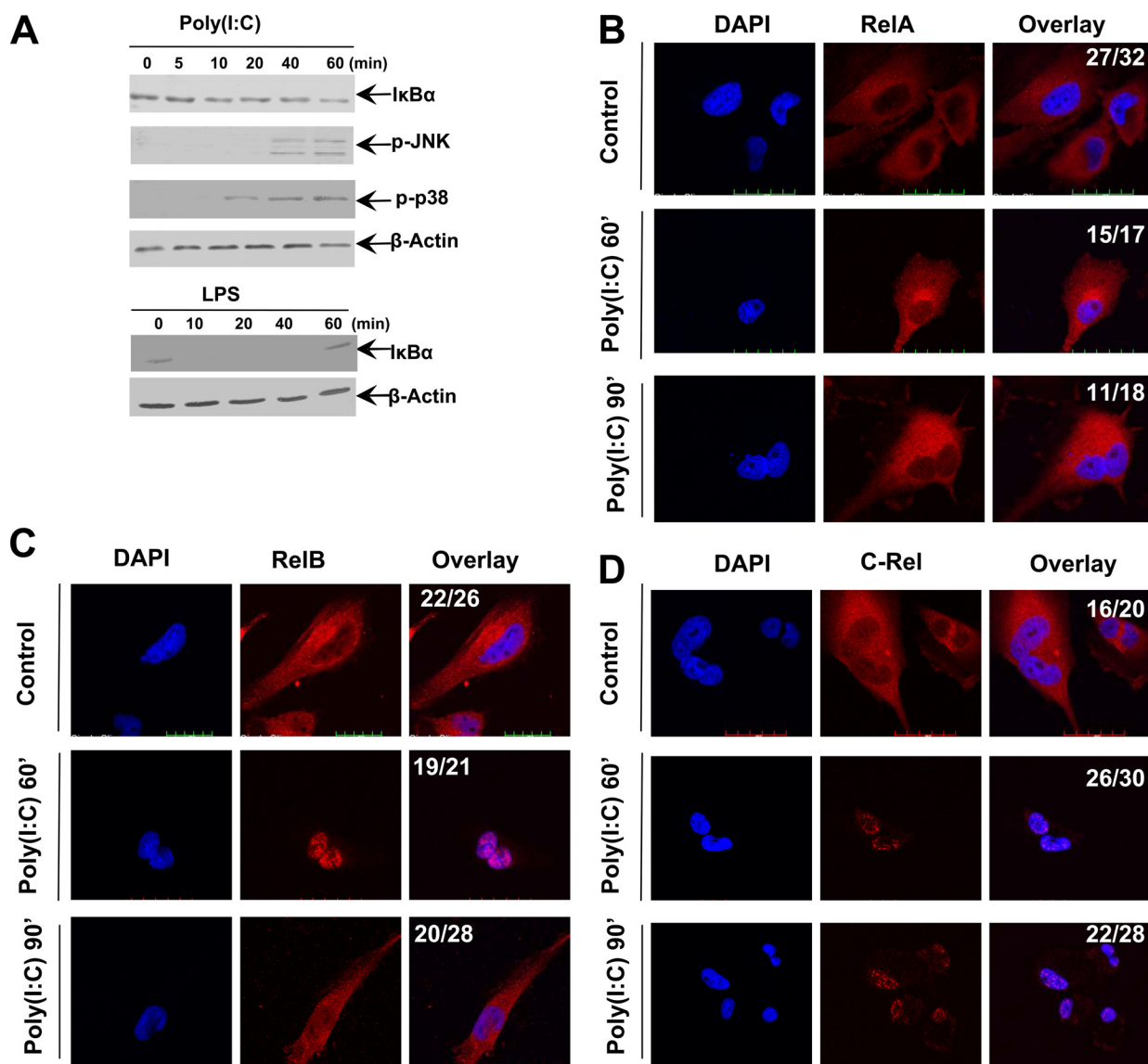


FIGURE 3. TLR3 signaling activates RelB and c-Rel but not RelA. *A*, BMDMs were treated with poly(I:C) (1 μ g/ml) or LPS (10 ng/ml) for the indicated times. Cell lysates were generated and assessed by Western blotting for levels of IkB α , phospho-JNK, phospho-p38 MAPK, and β -actin. *B–D*, U373 cells were grown on glass coverslips and treated with poly(I:C) (10 μ g/ml) for 60 and 90 min. Cells were fixed and probed for immunoreactivity with anti-RelA (*B*), anti-RelB (*C*), and anti-c-Rel (*D*) antibodies. Immunoreactivity was visualized by confocal microscopy using an Alexa Fluor 633-labeled secondary antibody. DAPI staining of nuclei is also included. Confocal images were captured using a Olympus FluoView FV1000 System laser scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the Olympus FV 1.6b imaging software. Individual cell images are shown for clarity, with the numbers in the overlay panels indicating the frequency of the observed cellular localization in a population of cells within larger fields.

IFN- β promoter or its PRDI-III region but not when controlled by the PRDII and PRDIV domains (Fig. 5*D*). This provides strong supporting evidence for YY1, like RelB and c-Rel, negatively regulating activation of the IFN- β promoter by targeting its PRDI-III domains.

YY1 Inhibits Binding and Activation of IRF7 at IFN- β Promoter—Given that the transcription factors IRF3 and IRF7 bind to the PRDI/III regions of the IFN- β promoter and promote its activation, YY1 was next assessed for its potential regulatory effects on IRF3 and IRF7. HEK293 cells were co-transfected with low amounts of expression constructs encoding Trif and IRF3 (Fig. 6*A*) and IRF7 (Fig. 6*B*), and these were shown capable of inducing luciferase when regulated by IFN- β promoter or the PRDI-III region but not when regulated by PRDII and PRDIV. Interestingly the co-expression of YY1 showed

only slight inhibition of the IFN- β promoter and its PRDI-III domains when driven by IRF3 (Fig. 6*A*), whereas it strongly inhibited activation of these promoters when regulated by IRF7 (Fig. 6*B*). These data suggested that YY1 may selectively target IRF7 at the IFN- β promoter. However co-immunoprecipitation analysis failed to show any interaction of YY1 with IRF3 or IRF7 (data not shown), thus excluding a direct association of YY1 with IRF7 as an inhibitory mechanism. YY1 was thus examined for its ability to directly bind to the IFN- β promoter in response to TLR3 stimulation and to regulate IRF7 binding to the promoter under these conditions. An oligonucleotide corresponding to the enhancer sequence from the IFN- β promoter was used in affinity precipitation of nuclear extracts from poly(I:C)-stimulated HEK293 cells previously transfected with expression constructs encoding YY1 and IRF7. The purity of the nuclear extracts was

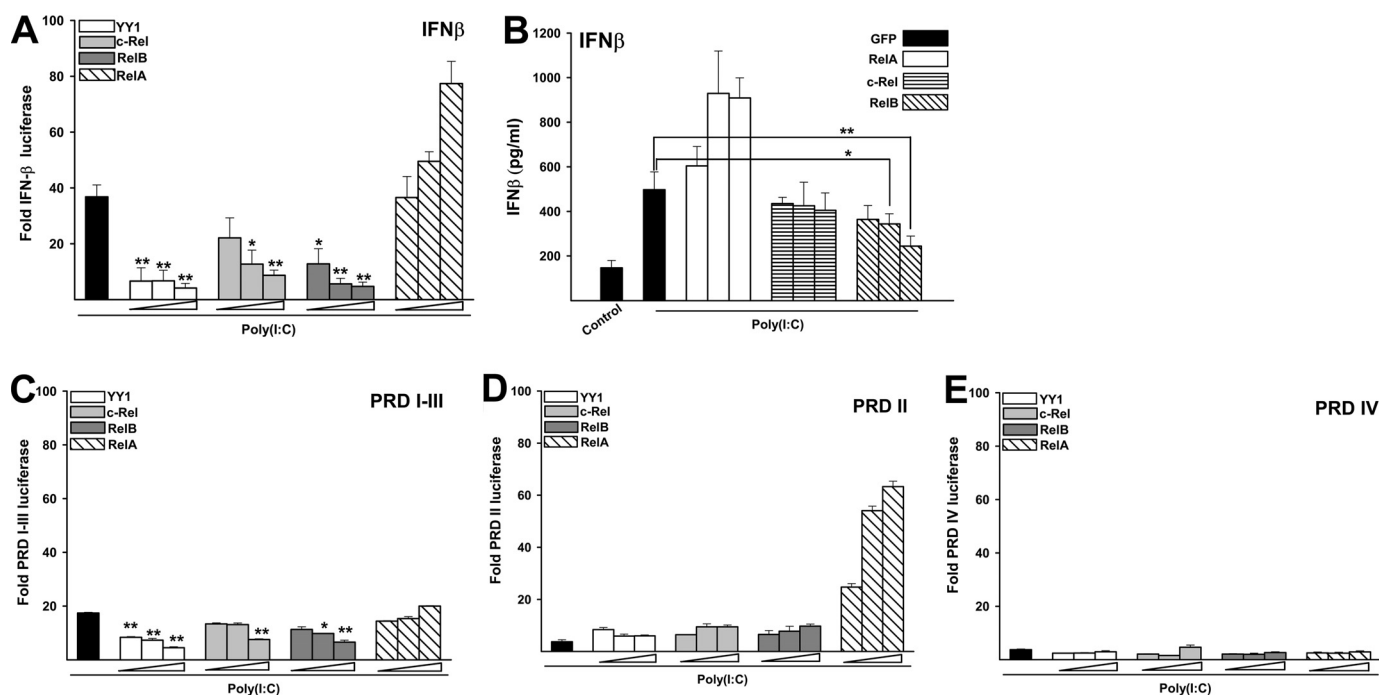


FIGURE 4. RelB and c-Rel negatively regulate TLR3-induced expression of IFN- β . A and C–E, HEK293-TLR3 cells were cotransfected with plasmid constructs encoding IFN- β promoter (A), PRDI-III (C), PRDII (D) or PRDIV (E)-regulated firefly luciferase (80 ng), constitutively expressed TK Renilla luciferase (20 ng), and varying amounts (1, 10, and 20 ng) of expression constructs encoding RelA, c-Rel, RelB, and YY1. Cells were allowed to recover for 6 h and then stimulated for 6 h with poly(I:C) (1 μ g/ml). Cell lysates were generated and assayed for firefly luciferase activity and normalized for transfection efficiency using Renilla luciferase activity. Data are presented as -fold stimulation of firefly luciferase expression relative to unstimulated cells. B, U373 cells were grown on 12-well plates and transfected with plasmid constructs encoding RelA, c-Rel, and RelB (300, 600, 900 ng). Cells were allowed to recover for 16 h and then stimulated for 16 h with poly(I:C) (1 μ g/ml). Cell supernatants were assayed for levels of IFN- β protein by sandwich ELISA. Data represent the mean \pm S.E. of triplicate determinations from three independent experiments and were subjected to unpaired Student's *t* test; *, *p* < 0.05; **, *p* < 0.01 relative to poly(I:C)-stimulated cells previously transfected in the absence of the Rel expression constructs.

demonstrated by the detection of high levels of the nuclear protein histone H3 and barely detectable levels of the cytosolic protein α -tubulin (Fig. 6, C and D). Confirming the above findings from confocal microscopy, poly(I:C) was again shown to promote strong nuclear localization of YY1 and YY1 precipitated with the enhancosome oligonucleotide (Fig. 6C), indicating that TLR3 stimulation causes nuclear translocation of YY1, thus allowing it to directly interact with the enhancosome sequence of the IFN- β promoter. Using this approach, poly(I:C) was shown to promote strong nuclear translocation of IRF7 and its binding to the enhancosome sequence (Fig. 6D). However the co-expression of YY1, although failing to affect the nuclear translocation of IRF7, reduced the TLR3-induced binding of IRF7 to the enhancosome sequence. These data thus strongly suggest that YY1 negatively regulates TLR3-induced expression of IFN- β by inhibiting the binding of IRF7 to the IFN- β enhancosome. These findings suggest a mechanism whereby the induction of YY1 by NF- κ B leads to the binding of YY1 to the PRDI-III domain of the IFN- β promoter and thus the reduced binding of IRF7 to the promoter. To further corroborate this hypothesis, U373 cells were treated with the NF- κ B inhibitor JSH-23, subsequently stimulated with poly(I:C), and then the *in vivo* binding of YY1 and IRF7 to the IFN- β promoter was assayed by chromatin immunoprecipitation. Poly(I:C) promoted strong binding of YY1 to the IFN- β promoter, and this was considerably reduced by pretreatment of cells with JSH-23 (Fig. 6E). In contrast, poly(I:C) caused modest binding of IRF7 to the IFN- β promoter, but this was considerably enhanced by pretreatment of cells with JSH-23. This adds further support to our

proposal in which YY1 is regulated by NF- κ B and mediates post-induction repression of IFN- β by inhibiting the binding of IRF7 to the IFN- β promoter.

Given such clear importance of IRF7 as a target for mediating the inhibitory effects of YY1, it was next addressed if this underlying mechanism could explain the differential inhibitory effects of YY1 on TLR3- and TLR4-induced expression of IFN- β . More specifically we evaluated the relative importance of IRF7 in TLR3 and TLR4 signaling. Poly(I:C) and LPS were initially compared for their abilities to activate IRF7. Poly(I:C) promoted strong nuclear translocation of IRF7, whereas LPS was ineffective even under conditions where the latter effected strong translocation of RelA to the nucleus (Fig. 7A). This suggested that poly(I:C) is a much stronger activator of IRF7 than LPS, and this was further corroborated by the greater efficacy of the former in inducing the expression of a luciferase reporter gene regulated by the IRF7-responsive PRDI-III region of the IFN- β promoter (Fig. 7B). Again, the poly(I:C)-induced activation of the PRDI-III domain was sensitive to strong inhibition by YY1, whereas the very modest activation by LPS was resistant to inhibition by YY1. This is hardly surprising given that LPS failed to promote the nuclear translocation of IRF7. These findings implied a very important role for IRF7 in the TLR3 but not TLR4 pathway. This was directly addressed by infecting BMDMs with lentiviral particles containing control or IRF7-specific shRNA. The latter was shown to selectively and specifically suppress endogenous expression of IRF7 as measured by semiquantitative RT-PCR (*inset*, Fig. 7C). Knockdown of IRF7

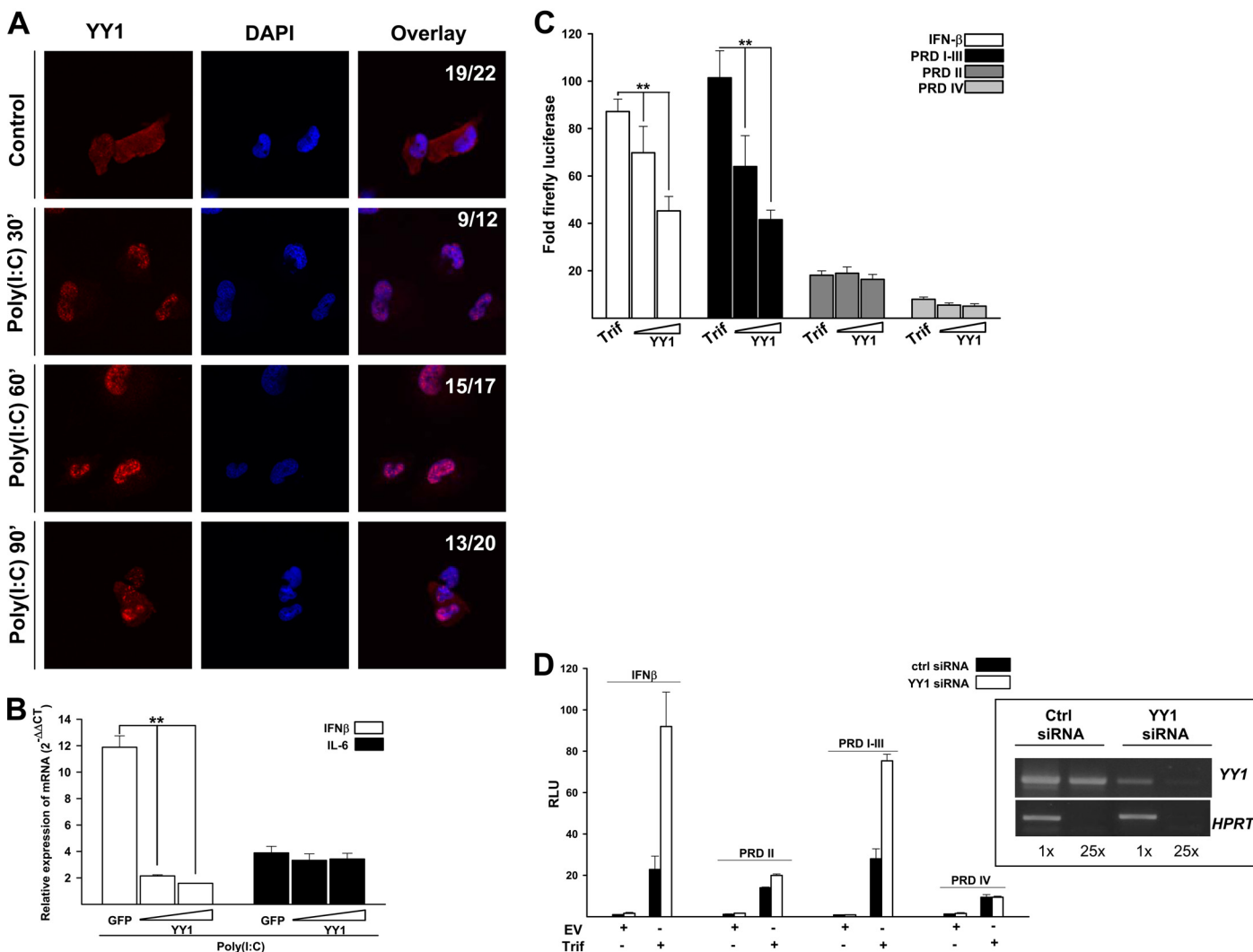


FIGURE 5. YY1 shows nuclear localization in response to TLR3 stimulation and negatively regulates the PRDI/III domains of the IFN- β promoter. *A*, U373 cells were grown on glass coverslips and treated with poly(I:C) (10 μ g/ml) for varying times. Cells were fixed and probed for immunoreactivity with an anti-YY1 antibody. Immunoreactivity was visualized by confocal microscopy using an Alexa Fluor 633-labeled secondary antibody. DAPI staining of nuclei is also included. Confocal images were captured using a Olympus FluoView FV1000 System laser-scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the Olympus FV 1.6b imaging software. Individual cell images are shown for clarity, with the numbers in the overlay panels indicating the frequency of the observed cellular localization in a population of cells within larger fields. *B*, U373 cells were transfected with varying amounts (300 or 600 ng) of expression plasmid encoding YY1 or GFP as a control. 24 h after transfection, cells were treated with poly(I:C) (10 μ g/ml) for 2 h. Total RNA was isolated, converted to first-strand cDNA, and used as a template for quantitative real-time RT-PCR to assay the mRNA expression levels of IFN- β and IL-6. The levels of the mRNAs were normalized relative to the housekeeping gene HPRT and are expressed relative to normalized values from unstimulated cells. *C* and *D*, HEK293-TLR3 cells were cotransfected with plasmid constructs encoding Trif (20 ng), YY1 (10, 20 ng), constitutively expressed TK Renilla luciferase (20 ng), and IFN- β promoter-, PRDI-III, PRDII-, or PRDIV-regulated firefly luciferase (80 ng). *D*, HEK293-TLR3 cells were also transfected with control and YY1-specific siRNA. Cells were allowed recover for 24 h, and cell lysates were then assayed for firefly luciferase activity and normalized for transfection efficiency using *Renilla* luciferase activity. Data are presented as -fold stimulation of firefly luciferase expression relative to cells transfected without Trif but with an empty vector (*EV*) construct. YY1 knockdown was confirmed by generating cDNA and assaying varying dilutions for YY1 and HPRT expression using semiquantitative PCR (*inset* in panel *D*). Data represent the mean \pm S.E. of triplicate determinations from three independent experiments and were subjected to unpaired Student's *t* test. *, *p* < 0.05; **, *p* < 0.01. RLU, relative luciferase units.

resulted in strong inhibition of poly(I:C)- but not LPS-induced expression of IFN- β mRNA (Fig. 7C). These findings clearly indicate that stimulation of the TLR3 pathway promotes prominent nuclear translocation of IRF7 where it strongly induces IFN- β expression, whereas LPS is relatively ineffective in activating IRF7, and thus IRF7 fails to play an important role in mediating TLR4-induced expression of IFN- β . Given that YY1 targets IRF7, this provides the likely mechanistic basis to the differential sensitivity of TLR3 and TLR4 signaling to YY1.

DISCUSSION

Viral-induced expression of type I IFNs is a key anti-viral response by the infected host. A signature of this response is rapid induction of the type I IFNs followed by further amplification of their expression through a feed-forward mechanism. Although this is advantageous in clearing viral infections, uncontrolled type I IFN expression can lead to autoimmune diseases (34), and thus it is vitally important that the body is equipped with mechanisms to repress IFN expression in the post-induction phase. Herein, we describe findings that indi-

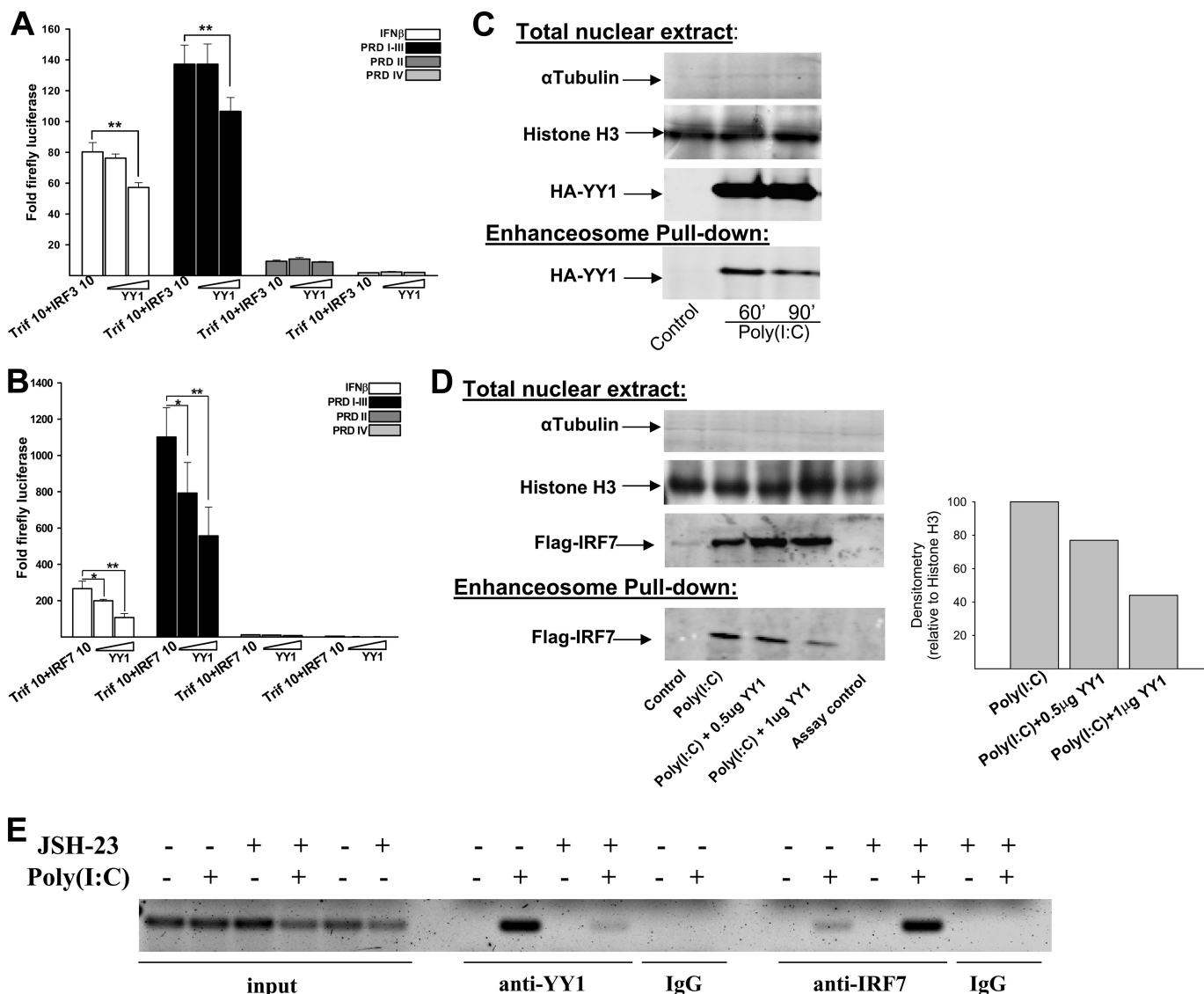


FIGURE 6. YY1 inhibits the binding and activation of IRF7 at the IFN- β promoter. *A* and *B*, HEK293-TLR3 cells were cotransfected with plasmid constructs encoding IRF3 (10 ng (*A*)) or IRF7 (10 ng (*B*)) and Trif (10 ng), YY1 (10, 20 ng), constitutively expressed TK Renilla luciferase (20 ng), and IFN- β promoter-, PRDI-III, PRDII-, or PRDIV-regulated firefly luciferase (80 ng). Cells were allowed to recover for 24 h, and cell lysates were then assayed for firefly luciferase activity and normalized for transfection efficiency using *Renilla* luciferase activity. Data are presented as -fold stimulation of firefly luciferase expression relative to cells transfected without Trif. Data represent the mean \pm S.E. of triplicate determinations from three independent experiments and were subjected to unpaired Student's *t* test. *, *p* < 0.05; **, *p* < 0.01. *C* and *D*, HEK293-TLR3 cells were transfected with expression plasmids encoding HA-tagged YY1 with and without FLAG-tagged IRF7. Cells were allowed to recover for 16 h and then stimulated with or without (control) poly(I:C) (10 μ g/ml) for the indicated times. Nuclear extracts were prepared and affinity-precipitated using streptavidin magnetic beads and a biotinylated oligonucleotide corresponding to the enhanceosome sequence from the IFN- β promoter. Precipitations with magnetic beads in the absence of the oligonucleotide served as a negative control for the assay. Nuclear extracts and enhanceosome precipitates were subjected to Western blotting using anti- α -tubulin, -histone H3, -HA, and -FLAG antibodies. The data are representative of two independent experiments. *D*, the levels of IRF7 in the enhanceosome precipitates were quantitated by densitometric analysis and are expressed relative to the levels of histone H3 in the corresponding nuclear extracts. *E*, U373 cells were pretreated with or without JSH-23 (30 μ M) for 30 min and then stimulated in the absence or presence of poly(I:C) (10 μ g/ml) for 4 h. Cells were fixed in formaldehyde followed by nuclei isolation and sonication. Sonicated nuclear lysates were immunoprecipitated with an anti-YY1 or anti-IRF7 or rabbit IgG control antibody. Input DNA (before immunoprecipitation) and immunoprecipitated chromatin were analyzed by 20 or 35 cycles of PCR, respectively, with primers designed to amplify a region of the IFN- β promoter.

cate a novel self-regulatory mechanism employed by TLR3 in controlling the levels of IFN- β that are expressed in response to TLR3 stimulation (Fig. 8). In this model a TLR3 ligand such as poly(I:C) can activate the TRIF/TBK1/inducible I κ B kinase pathway, leading to the binding of IRF7 to the IFN- β promoter and increased transcriptional activation of the IFN- β gene. Although TLR3 is a poor activator of the classical NF- κ B pathway and especially p65, it can strongly activate other Rel subunits such as RelB and c-Rel, leading to induction of YY1. The

latter translocates to the nucleus where it displaces IRF7 from the IFN- β promoter, culminating in post-induction repression of IFN- β .

To date NF- κ B has been generally accepted to play a crucial and indispensable role in driving strong IFN- β expression. However, more recent reports have questioned this dogma by using mice that are deficient in various NF- κ B subunits to show that NF- κ B is primarily involved in maintaining low basal expression of IFN- β and in the very early induction phase when

NF- κ B Inhibits TLR3-induced IFN- β Expression via YY1

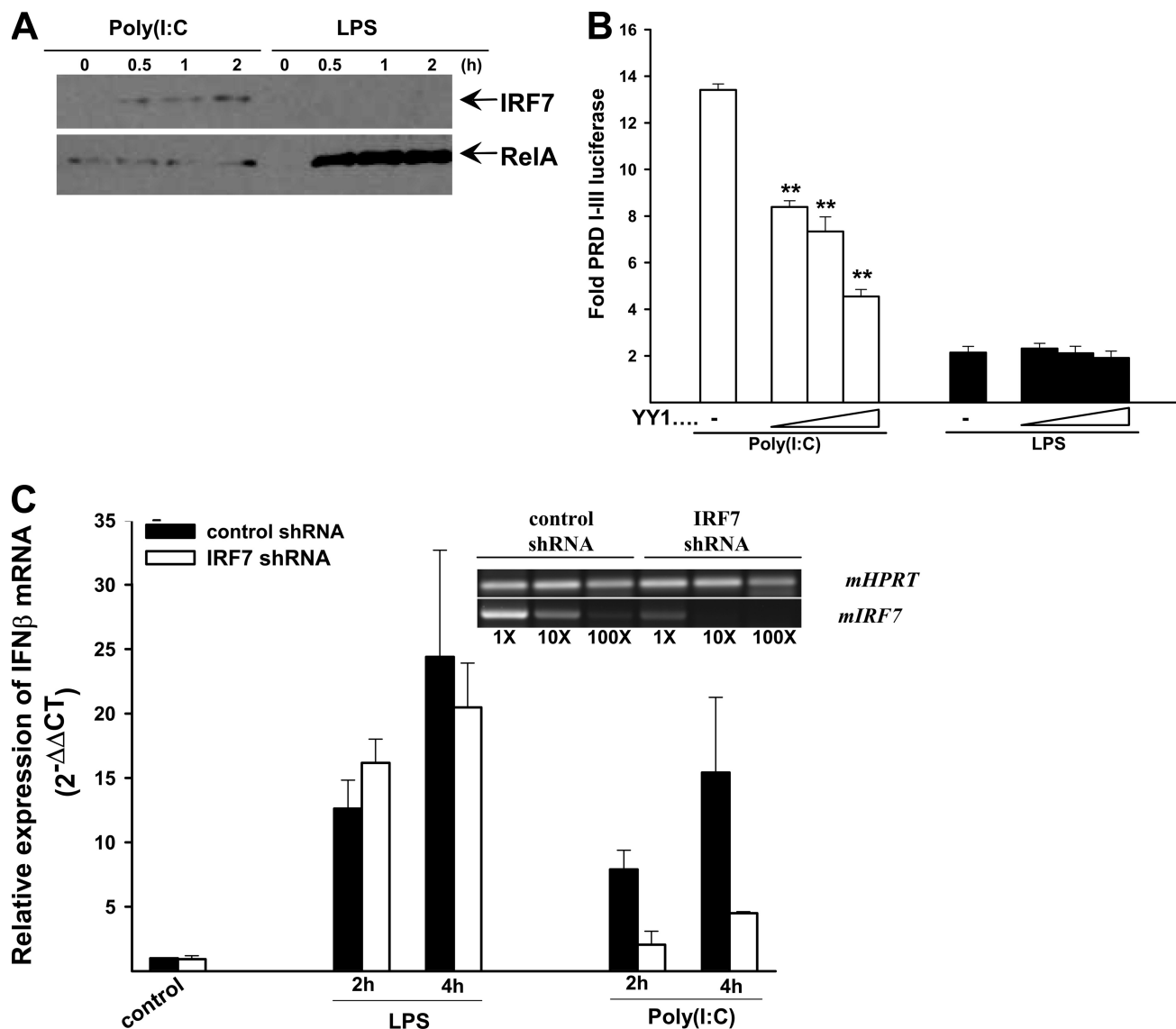


FIGURE 7. IRF7 mediates poly(I:C) but not LPS-induced expression of IFN- β . *A*, U373 cells were treated with poly(I:C) (10 μ g/ml) or LPS (100 ng/ml) for the indicated times. Nuclear extracts were generated and assessed by Western blotting for levels of IRF7 and RelA. *B*, HEK293-TLR3 and HEK293-TLR4 cells were cotransfected with plasmid constructs encoding PRD1-III-regulated firefly luciferase (80 ng), constitutively expressed TK Renilla luciferase (20 ng), and varying amounts (1, 10, 20 ng) of expression constructs encoding YY1. Transfected HEK293-TLR3 and HEK293-TLR4 cells were allowed recover for 16 h and then stimulated for 6 h with poly(I:C) (1 μ g/ml) or LPS (10 ng/ml), respectively. Cell lysates were generated and assayed for firefly luciferase activity and normalized for transfection efficiency using Renilla luciferase activity. Data are presented as -fold stimulation of firefly luciferase expression relative to unstimulated cells. *C*, BMDMs were transduced with lentivirus encoding control or IRF7-specific shRNA. Cells were then stimulated with poly(I:C) (1 μ g/ml) or LPS (10 ng/ml) for 2 h. cDNA was generated and assayed for IRF7 and HPRT expression using semiquantitative PCR (*inset*) and for IFN- β expression by quantitative RT-PCR. The levels of IFN- β expression are expressed relative to unstimulated (*control*) cells transduced with control shRNA.

IRF activation is still at a low level (18–20). In contrast to the prevailing view, NF- κ B knock-out mice showed no deficiency in overall expression levels of IFN- β in response to viral challenge, and indeed at later post-induction times levels of IFN- β were actually higher in the knock-out animals. Although not discussed in the detail in the original reports, these findings provided the first clues to us that NF- κ B may repress IFN- β expression. Herein, we employed a number of independent approaches, including a pharmacological inhibitor of NF- κ B and the I κ B super repressor protein to explore the concept that NF- κ B may act as a post-induction repressor of IFN- β expression. Such independent approaches provide very strong corroborative support for NF- κ B as an inhibitor of IFN- β expression, and in the proc-

ess we also define the key role of YY1 in mediating such repressor effects of NF- κ B.

Intriguingly, inhibition of the NF- κ B pathway augments poly(I:C)-induced expression of IFN- β while failing to affect LPS-induced expression of this gene. This suggests that the negative effects of NF- κ B on late phase expression of IFN- β show specificity for the TLR3 pathway. The underlying basis to such selectivity is not due to any difference between the TLR3 and TLR4 pathways in inducing YY1 as both poly(I:C) and LPS show comparable efficacy in promoting expression of YY1. Instead, the differential effects appear to be due to the contrasting roles of YY1 in TLR3 and TLR4 signaling. Thus, whereas knockdown of YY1 augmented poly(I:C)-induced expression of IFN- β , no such effect was apparent in LPS-induced expression

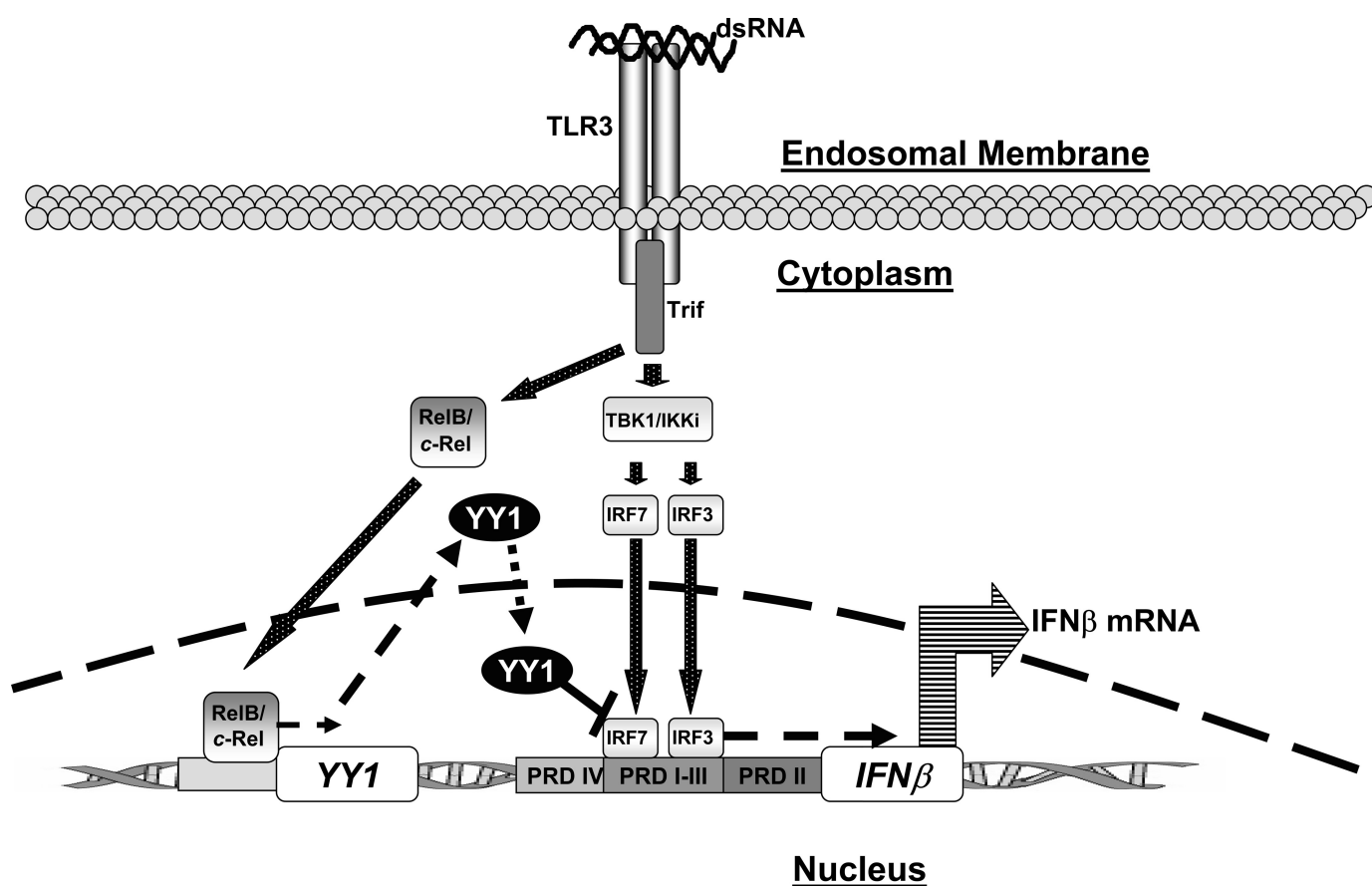


FIGURE 8. Schematic representation of post-induction repression of IFN- β expression by YY1 in TLR3 signaling pathway. TLR3 is engaged by a dsRNA ligand (e.g. poly(I:C)) promoting activation of the TRIF/TBK1/inducible I κ B kinase pathway leading to the binding of IRF3 and IRF7 to the PRD I/III domain of the IFN- β promoter. This results in increased transcriptional activation of the IFN- β gene. TLR3 is a poor activator of the classical NF- κ B pathway and especially p65 but can strongly stimulate the nuclear translocation of the Rel subunits RelB and c-Rel followed by induction of YY1. The latter translocates to the nucleus in response to TLR3 signaling, where it displaces IRF7 from the IFN- β promoter resulting in post-induction repression of IFN- β expression.

of IFN- β . It was interesting to speculate that TLR3 signaling may selectively regulate the ability of YY1 to act as a repressor by controlling its localization. However, in the present study we show that poly(I:C) promotes strong nuclear localization of newly synthesized YY1 (Fig. 4A), and the TLR4 ligand LPS can similarly induce nuclear localization of YY1 (data not shown). An alternative explanation for the differential effects of YY1 on TLR3 and TLR4 signaling lies in the relative importance of NF- κ B and IRFs in driving IFN- β expression. Whereas NF- κ B is a critical mediator of TLR4-induced IFN- β expression, our present findings in conjunction with data from other reports (18, 19, 35) clearly show that NF- κ B plays a relatively unimportant role in the TLR3 pathway, and instead IRFs are the major driving forces that mediate TLR3-induced expression of IFN- β . Indeed we show that IRF7 is strongly activated by TLR3 and plays an important mediatory role in driving expression of IFN- β . Furthermore, our data show that IRF7 lacks functional importance in mediating TLR4-induced expression of IFN- β . Given our findings that YY1 targets IRF7, it is not surprising that YY1 shows such prominent inhibitory effects on the TLR3 pathway without affecting TLR4 signaling.

Although the TLR3 and TLR4 signaling pathways share some downstream signaling components, the idea of certain mole-

cules differentially regulating the two pathways is not without precedent. Thus, we and others have previously shown that the adaptor molecule Mal negatively regulates TLR3 signaling while acting as a positive mediator in the TLR4 pathway (30, 36). In addition we have also shown that the synthetic cannabinoid *R*(+)-WIN55,212-2 can negatively regulate TLR4- but augment TLR3-induced expression of IFN- β (37). Furthermore, a previous report has shown that TLR4, but not TLR3, requires the NF- κ B subunit, p65, to activate the interferon-sensitive response element (35), and so it is not surprising that NF- κ B can differentially regulate IFN- β expression by TLR3 and TLR4.

It is interesting to note that although JSH-23 augments TLR3-mediated induction of IFN- β , this study shows that it fails to regulate TLR3-induced expression of TNF. Indeed given the role for NF- κ B in driving expression of TNF, it might have been predicted that a NF- κ B inhibitor would instead inhibit expression of TNF. Although our studies show that this certainly applies to TLR4-induced expression of TNF, the induction of the latter by TLR3 is refractory to inhibition by JSH-23. This questions the role for NF- κ B as a transcriptional activator of the TNF gene in the TLR3 signaling pathway, and this is consistent with a report showing that in macrophages TRIF can

NF- κ B Inhibits TLR3-induced IFN- β Expression via YY1

drive TNF induction by a mechanism independent of NF- κ B (38).

The proposal of YY1 as a post-induction repressor of TLR3-induced expression of IFN- β is consistent with its regulation by NF- κ B. In the present study we show that stimulation of TLR3 by poly(I:C) results in only very modest activation of the classical NF- κ B pathway, and instead the TLR3 pathway is associated with activation of NF- κ B subunits, like RelB, that are more associated with the non-classical pathway. Furthermore, the latter pathway is also more likely to play a key leading role in inducing YY1 and negatively regulating IFN- β expression as expression of RelB inhibits TLR3-induced activation of the IFN- β promoter and expression of the IFN- β gene, whereas overexpression of RelA, the subunit more closely associated with classical activation of NF- κ B, augments TLR3-induced expression of IFN- β . This is consistent with a very recent study showing that knockdown of RelB, but not RelA, in HT1080 cells results in greatly augmented TLR3-induced expression of IFN- β (39). Thus, a model arises where RelA may play an important role in the early phase induction of IFN- β by TLR3, and this is consistent with previous studies using RelA-deficient mice (19, 20), whereas the present study suggests that NF- κ B subunits like RelB play a more important role in mediating post-induction repression of IFN- β .

The post-induction repressor function of YY1 in the TLR3 pathway is also consistent with the ability of poly(I:C) to induce YY1 and strongly promote its nuclear localization. In this nuclear environment YY1 can exert its inhibitory effects on the IFN- β promoter. YY1 has been previously shown to suppress basal activation of the IFN- β promoter by recruiting histone deacetylases (24). We now add to the molecular understanding of how YY1 can repress the IFN- β promoter by showing that YY1 selectively targets the PRDI/III domain of the promoter by inhibiting the binding of IRF7 to this domain. Thus, we propose a model whereby stimulation of the TLR3 pathway can overcome the basal suppressive effects of YY1 on the IFN- β promoter thus allowing for increased *de novo* transcription from the promoter. This is facilitated by activation of transcription factors such as RelA and IRF3 and IRF7. However, TLR3-induced activation of the NF- κ B subunits c-Rel and RelB leads to subsequent induction and nuclear translocation of YY1, resulting in reduced binding of IRF7 to the PRDI/III domain and thus post-induction repression of IFN- β expression.

It will be of interest to explore the role of YY1 in autoimmune diseases that are associated with high expression levels of type I IFNs. Indeed the regulation of YY1 expression may serve as a valuable and novel therapeutic strategy in such cases. The undoubted value of targeting YY1 to control IFN- β expression is best illustrated by Rift Valley fever virus. The nonstructural protein NSs of this virus interacts with host proteins, including YY1, and blocks the transcriptional activation of the IFN- β promoter, thus acting as the major virulence factor of Rift Valley virus (40). The targeting of YY1 by a virus clearly highlights its regulatory importance with respect to IFN- β expression. We now show that the induction of YY1 by NF- κ B acts as a physiological braking system for TLR3-induced expression of IFN- β . This adds to our existing understanding of the complex regulatory network that controls activation of the IFN- β promoter

and promotes YY1 as a lead target to exploit in efforts to design new therapeutics to control autoimmune diseases associated with dysregulated IFN- β expression.

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