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Role for MyD88-Independent, TRIF Pathway in Lipid A/TLR4-Induced Endotoxin Tolerance[1]

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Repeated exposure to low doses of endotoxin results in progressive hyporesponsiveness to subsequent endotoxin challenge, a phenomenon known as endotoxin tolerance. In spite of its clinical significance in sepsis and characterization of the TLR4 signaling pathway as the principal endotoxin detection mechanism, the molecular determinants that induce tolerance remain obscure. We investigated the role of the TRIF/IFN-β pathway in TLR4-induced endotoxin tolerance. Lipid A-induced homotolerance was characterized by the down-regulation of MyD88-dependent proinflammatory cytokines TNF-α and CCL3, but up-regulation of TRIF-dependent cytokine IFN-β. This correlated with a molecular phenotype of defective NF-κB activation but a functional TRIF-dependent STAT1 signaling. Tolerance-induced suppression of TNF-α and CCL3 expression was significantly relieved by TRIF and IFN regulatory factor 3 deficiency, suggesting the involvement of the TRIF pathway in tolerance. Alternatively, selective activation of TRIF by poly(I:C)-induced tolerance to lipid A. Furthermore, pretreatment with rIFN-β also induced tolerance, whereas addition of IFN-β-neutralizing Ab during the tolerization partially alleviated tolerance to lipid A but not TLR2-induced endotoxin homo- or heterotolerance. Furthermore, IFNAR1$^{-/-}$ murine embryonal fibroblast and bone-marrow derived macrophages failed to induce tolerance. Together, these observations constitute evidence for a role of the TRIF/IFN-β pathway in the regulation of lipid A/TLR4-mediated endotoxin homotolerance. *The Journal of Immunology,* **2007, 179: 4083–4092.**

Endotoxin tolerance is a protective phenomenon in which prolonged exposure of hosts (and their immune cells) to suboptimal levels of endotoxin causes their progressive inability to respond to subsequent endotoxin challenge. This was first reported in humans, where it was observed that repeated administration of typhoid vaccine caused progressive loss of efficacy as a pyrogen and an escalation of the dose was required to achieve therapeutic effect (1). Subsequently, this phenomenon was reproduced in animal models and attributed to the monocytes/macrophage lineage of the host immune system. Tolerance to a particular endotoxin induced by prior exposure to the same endotoxin is referred to as "homotolerance," Conversely, tolerance to an endotoxin induced by prior exposure to a different endotoxin is referred to as "heterotolerance" (2). Cells from monocytes/macrophage lineage when exposed to suboptimal endotoxin (e.g., LPS, LPS, or lipid A (LPA)[3]) concentrations for 3–24 h were rendered "tolerant" to subsequent endotoxin challenge, characterized functionally by a marked inhibition of inflammatory cytokine (e.g., TNF, IL-1, and IL-6) production, upon rechallenge with endotoxin (3, 4). These observations hold true for endotoxin-tolerized leukocytes from both mice and humans. Leukocytes from patients with sepsis as well as nonsystemic inflammatory response syndrome (e.g., surgery, trauma, resuscitation) have many characteristics of endotoxin tolerance (1, 5, 6). It has been argued that the fatality of sepsis patients is partly attributed to their inability to respond to subsequent microbial challenge, which renders them highly susceptible to uncontrolled infection. Despite the high mortality in sepsis cases worldwide, the molecular mechanisms that regulate the induction of endotoxin tolerance have surprisingly not been sufficiently investigated and consequently the underlying mechanisms remain obscure (7). This is reflective in the limited therapies available to contain this phenomenon.

The discovery of TLR and subsequent analysis of signaling pathways downstream of TLR activation have vastly improved our understanding of this signaling paradigm over the past few years (8–10). The recognition that TLR4 signaling mediates response to endotoxin challenge (11–14), has evoked interest in studying its contribution in the molecular basis of endotoxin tolerance (7). TLR4 signaling can be segregated into two distinct pathways: one leading to activation of the MyD88-dependent arm and the other leading to the MyD88-independent (TRIF/TRAM-mediated) arm (10). Both of these cascades lead to distinct outcomes: the former giving rise to expression of proinflammatory genes such as TNF, IL-1, and cyclooxygenase 2 through activation of NF-κB (15),

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[3] Abbreviations used in this paper: LPA, lipid A; BMDM, bone-marrow derived macrophage; IFNAR1, IFN (α and β) receptor 1; IRF3, IFN regulatory factor 3; IRAK, IL-1 receptor-associated kinase; MEF, mouse embryonal fibroblast; TRIF, TIR domain-containing adapter-inducing IFN-β; TRAM, TRIF-related adapter molecule; WT, wild type; ChIP, chromatin immunoprecipitation.

while the latter giving rise to IFN regulatory factor 3 (IRF3)-mediated expression of type I IFNs (IFN-α and IFN-β) and IFN-inducible chemokines like CXCLl0, CCL5, and CCL2 (10, 16). The predominant MyD88-dependent pathway is activated by virtually all of the TLRs (except TLR3) and, therefore, responsible for defense against a wide variety of pathogens (Gram-negative bacteria, Gram-positive bacteria, fungi, virus, protozoans, etc.) (10). On the other hand, the MyD88-independent (TRIF/TRAM) pathway is mainly activated by TLR3 and is functionally responsible for defense against viral infections through activation of type I IFN and other IFN-inducible genes (16). However, TLR4 can signal using both MyD88 and TRIF pathways in response to Gram-negative endotoxins. The reason why TLR4 signaling uses both of these pathways has not yet been understood.

Careful dissection of TLR signaling has shed light on multiple alterations in the normal MyD88-dependent signaling which may play roles in mediating endotoxin tolerance. These include changes in cell surface receptors, intracellular signaling components, expression of proinflammatory and anti-inflammatory cytokines, and induction of negative regulators of this signaling (17, 18). At the receptor level, the effect seems to be species specific. Although suppression of TLR4 expression was documented in mouse macrophages (19), up-regulation of TLR4 mRNA (as well as surface expression) and MD-2 mRNA was noted in endotoxin-tolerized human patients or in vitro-tolerized human monocytes (18, 20). Decreased TLR4-MyD88 complex formation, impairment of IL-1R-associated kinase (IRAK) 1 activity, MAPKs, and downstream transcription factors like NF-κB and AP-1 have also been observed and proposed to be the effectors in tolerized murine macrophages (2, 21, 22). The involvement of NF-κB and AP-1 in regulating LPS tolerance has been reported in the human monocytic cell line THP-1 (23). It is believed that tolerized monocytes/macrophages overexpress anti-inflammatory cytokines like IL-10 and TGF-β, which can contribute to the suppression of proinflammatory cytokine expression in these cells (24, 25). Finally, changes in the levels of negative regulators of the TLR pathway like MKP1, FLN29, ST2, and IRAK-M are also thought to contribute toward tolerance (26–29), although the mechanism of their induction and their regulation are still poorly defined.

Studies by Yoza et al. (30) on the transcriptional regulation of endotoxin tolerance in human monocytes and THP-1 cells support a mechanism of endotoxin tolerance that appears to be associated with the inability of DNA-bound transcription factors to activate transcription, perhaps due to the activity of an associated repressor (30). Overexpression of p50 and RelB containing NF-κB dimers has been reported in endotoxin-tolerant monocytes (31, 32). Recent data also demonstrate disruption of chromatin remodeling and persistent histone deacetylation at inflammatory gene promoters, which might be yet another mechanism that plays a role in regulating endotoxin tolerance (30, 33).

Although substantial evidence implicates the MyD88-dependent pathway in mediating tolerance (17, 21, 22), the role of the TRIF pathway in endotoxin tolerance has not been studied (34). We hypothesized the involvement of TRIF signaling in endotoxin tolerance, based on several observations. The discoveries that TNF-α (35) and IFN-β (36) mediate much of the toxicity of LPS have given relevant end points in assessing LPS tolerance both in vivo and in vitro. Although IFN-β is a prototypic end point for the TRIF pathway and TNF-α for the MyD88-dependent pathway, recent studies from the Baltimore laboratory using mouse embryonic fibroblasts (MEF) deficient for MyD88 and TRIF have elegantly shown the role of TRIF/IRF3 in mediating sustained, late-phase TNF-α expression during TLR4 activation (37). Furthermore, the TRIF-specific TLR3 ligand poly(I:C) has been reported to induce heterotolerance to LPS in liver cells and to macrophage-activating lipopeptide 2 (MALP2) in macrophages (38, 39). Furthermore, microarray analysis of LPS transcriptome has demonstrated that the majority of LPS-inducible genes are regulated in a MyD88-independent fashion, suggesting a crucial role for the TRIF pathway in inflammatory responses (40). In addition, characterization of IFN-β-deficient mice has demonstrated their resistance to endotoxin shock and a crucial requirement of IFN-β in mediating expression of proinflammatory cytokines at late time points (41, 42). These observations make it tempting to speculate that the MyD88-independent TRIF pathway downstream of TLR4 might play a nontrivial role in generating endotoxin tolerance. In this article, we document that the TRIF/IRF3-mediated IFN-β pathway downstream of TLR4 is indeed a determinant in mediating endotoxin tolerance. Our observations constitute the first direct evidence for the regulatory role of a TRIF/IFN-β signaling circuit in mediating LPA/TLR4-induced endotoxin homotolerance.

Materials and Methods

Cell lines, reagents, and cell culture

Wild-type (WT), MyD88$^{-/-}$, TRIF$^{-/-}$, and IFNAR1$^{-/-}$ MEF and bone marrow cells were obtained from the Akira laboratory (Department of Host Defense; Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). IRF3$^{-/-}$ MEF were provided by Dr. T. Tanaguchi (Department of Immunology, University of Tokyo, Tokyo, Japan).

All murine cells were cultured in DMEM (Invitrogen Life Technologies) containing 4500 mg/L D-glucose, L-glutamine without sodium pyruvate, and sodium bicarbonate. Medium was supplemented with 10% FBS (HyClone) and 100 U/ml penicillin-streptomycin. The following reagents were used for cell treatment: LPA, *Escherichia coli*, serotype R515(Re) (Alexis Biochemicals), poly(I:C) (InvivoGen), Pam_3Cys_4 (InvivoGen), recombinant mouse IFN-β (PBL Biomedical Lab), anti-IFNβ neutralizing Ab (PBL Biomedical Laboratory), and recombinant human IFN-β (PeproTech).

Preparation of bone marrow-derived macrophages

Briefly, 10×10^6 bone marrow cells from WT or IFNAR1$^{-/-}$ mice were plated in a 15-cm cell culture petri dish (Nuncleon) and incubated in DMEM (containing 100 IU/ml penicillin and 100 μg/ml streptomycin, i.e., incomplete medium) for 1 h at 37°C. Thereafter, nonadherent Bone marrow cells were retrieved. Nonadherent bone marrow cells (2×10^6 cells) were plated into 6-well plates (Nuncleon) in 2 ml of DMEM complemented with 10% FCS and 20% (v/v) L929-conditioned medium as a source of M-CSF and placed in the incubator at 37°C in a 5% CO_2 humidified atmosphere. One milliliter of medium was replaced from each well and replenished with fresh medium supplemented with 20% L929-conditioned medium every 2 days. At day 7, the cells formed an adherent monolayer that resembled macrophages in their morphology and showed 98% purity by F4/80 staining in FACS. At this stage, cells were washed with complete medium and ready for treatment.

Quantitative real-time PCR

Cells were lysed with TRIzol (Invitrogen Life Technologies) and total RNA was prepared according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed for 1 h at 42°C using an $oligo(dT)_{12–18}$ primer (Invitrogen Life Technologies) and the SuperScript II RT kit (Invitrogen Life Technologies). Quantitative real-time PCR was performed using the SYBR Green PCR core reagents mix (Applied Biosystems) containing 1× SYBR Green PCR buffer; 3 mM $MgCl_2$; 100 μM dATP, dCTP, and dGTP; 200 μM dUTP; 0.025 U/μl AmpliTaq Gold DNA polymerase; 0.01 U/μl AmpErase UNG; and 2 pmol/μl gene-specific forward and reverse primers designated using the Primer Express software (Applied Biosystems). The reaction conditions were as follows: 2 min at 50°C (one cycle), 10 min at 95°C (one cycle), 15 s at 95°C, and 1 min at 60°C (40 cycles). Gene-specific PCR products would be measured by means of an Applied Biosystems PRISM 7500 detection system (PerkinElmer). Samples were normalized using the housekeeping gene *β-actin* expression. Three replicates for each experimental point were performed and differences were assessed with the two-tailed Student *t* test. Results are expressed as the relative fold changes of the stimulated over the control group, which was used as a calibrator.

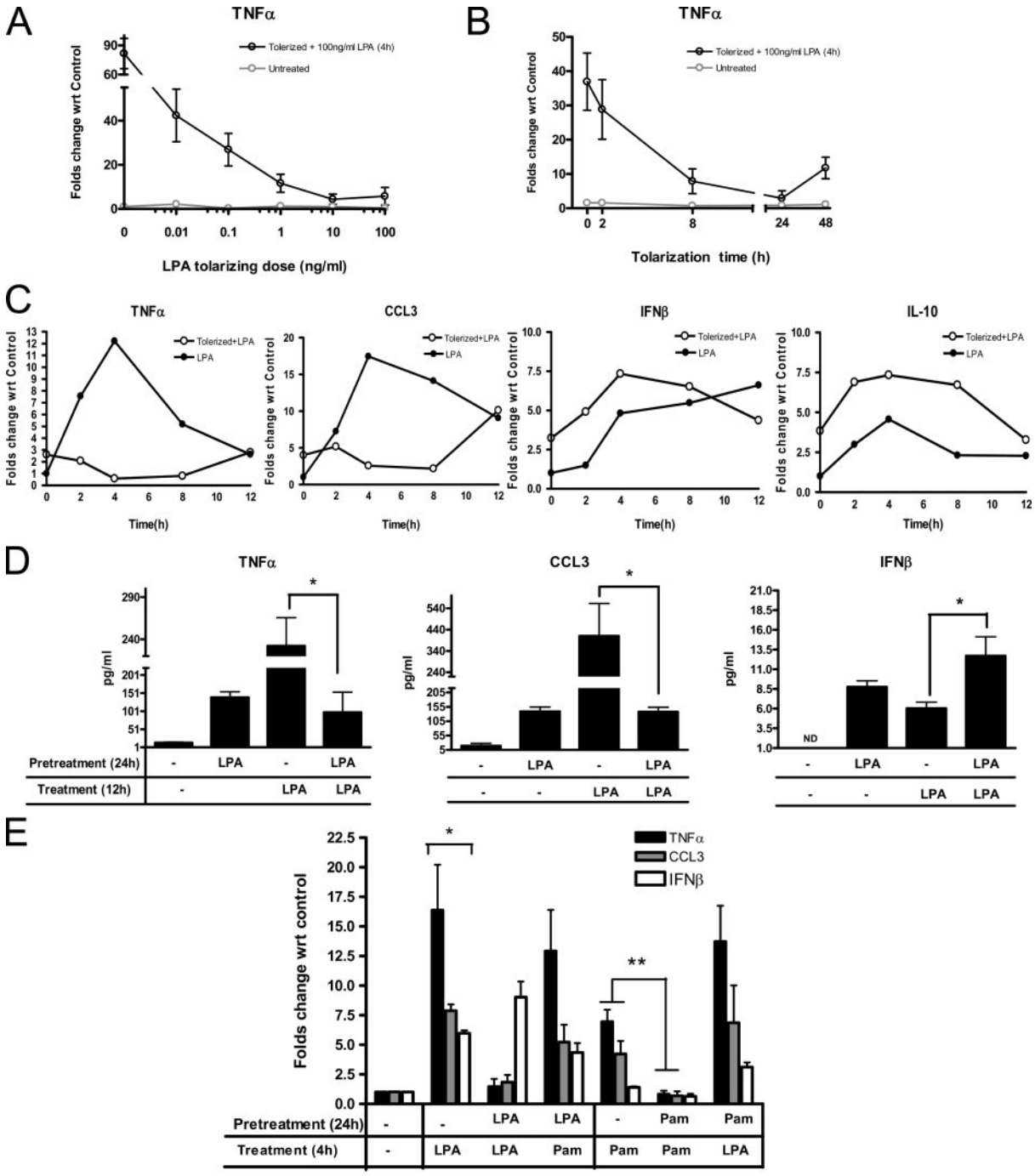

FIGURE 1. Differential regulation of MyD88/TRIF-dependent cytokine genes in endotoxin tolerance. WT-MEF were tolerized with 0–100 ng/ml LPA for 24 h (*A*) or 10 ng/ml LPA for the indicated time periods (*B*). Thereafter, cells were washed and restimulated with 100 ng/ml LPA for 4 h (labeled as Tolerized + 100 ng/ml LPA (4h)). Cells that did not receive any treatments, i.e., incubated in medium alone for the indicated dose or time points (labeled as Untreated) were used as control. Real-time-PCR expression of the TNF-α gene is indicated as fold changes with respect to control. All data were normalized to β-actin gene expression. *C,* Cells were tolerized with 10 ng/ml LPA for 24 h, washed, and restimulation with 100 ng/ml LPA for the indicated time periods (labeled as Tolerized + LPA). In parallel, nontolerant cells were stimulated with 100 ng/ml LPA for the indicated time periods (labeled as LPA). Nontolerant cells that did not receive any treatments, i.e., incubated in medium alone, for the respective time points were used as the control (data not shown). Real-time PCR for TNF-α, CCL3, IFN-β, and IL-10 gene expression are shown as fold change with respect to control. All data were normalized to β-actin gene expression. Genes analyzed are indicated in bold at the top of each graph. *D,* ELISA measurement for TNF-α, CCL3, and IFN-β secretion in nontolerant and tolerant cells upon stimulation with 100 ng/ml LPA for 12 h (see Treatment (12h) row). Tolerization was performed by pretreatment of cells with 10 ng/ml LPA for 24 h (see Pretreatment (24h) row). Levels of cytokines measured in the cell-free supernatants are quantitated in picograms per milliliter per 2×10^6 cells. Values are shown as mean $\pm$ SD. $*$, $p < 0.05$ LPA-treated nontolerized vs LPA-treated cells tolerized cells. *E,* Cells were either incubated in medium alone or pretreated with 10 ng/ml LPA or 10 ng/ml Pam_3Cys_4 (Pam) for 24 h as indicated in the Pretreatment (24h) row. Thereafter, cells were washed and again treated with 100 ng/ml LPA or 100 ng/ml Pam_3Cys_4 for 4 h as indicated in the Treatment (12h) row. Untreated cells incubated in medium alone were taken as control. Real-time PCR expression of TNF-α, CCL3, and IFN-β genes are shown as fold changes with respect to the control. Analyzed genes are indicated in the legend shown on the graph. All data were normalized to β-actin gene expression and shown as mean $\pm$ SD. $*$, $p < 0.05$ LPA-treated nontolerized vs LPA-treated cells tolerized cells; $**$, $p < 0.05$ Pam_3Cys_4-treated nontolerized vs Pam_3Cys_4-homotolerized cells. All results (*A–E*) are representative of three independent experiments.

ELISA

Cell-free supernatants from untreated or LPA (100 ng/ml)-treated nontolerized or tolerized cells after overnight incubation were tested for the indicated cytokines/chemokines using ELISA kits according to the manufacturers' instructions: TNF-α and IL-10 (QuantiKine kits; R&D Systems) and IFN-β and CCL3 (RayBio Mouse ELISA kits; RayBiotech). Data are representative of three independent experiments, done in triplicate.

Immunoblotting

After the indicated treatments, the cells were lysed and processed for subsequent running on SDS-PAGE, transfer, and immunoblotting as described elsewhere (43). The following primary Abs were used: anti-phospho-STAT1 (Tyr^{701}) and anti-phospho-IκBα (Ser^{32}) (Cell Signaling Technologies) and rabbit polyclonal anti-STAT1 (Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP)

Tolerized or nontolerized MEF were stimulated with 100 ng/ml LPA for the indicated time periods. Thereafter, the cells were processed for the ChIP assay according to the protocol described by Saccani et al. (44, 45). The Ab used for the ChIP was NF-κB (p65) Ab obtained from Santa Cruz Biotechnology (catalog no. C-20, SC-372). The sequence for murine TNF-α gene promoter was follows: sense primer, 5′-TCC TGA GGC CTC AAG CCT GCC-3′ and antisense primer, 5′-CAT GAG CTC CAT CTG GAG GAA G-3′.

Flow cytometry

Cells were incubated in Fc block for 10 min and stained thereafter with fluorescence-labeled Abs in FACS buffer (i.e., PBS containing 1% FBS (Invitrogen Life Technologies) plus 0.01% sodium azide) for 30 min on ice. Staining was done with either isotype-matched anti-rabbit, PE-conjugated Ab or the corresponding anti-TLR4 PE-conjugated Ab (purchased from BD Biosciences). Cells were resuspended in 0.5 ml of FACS buffer and analyzed by flow cytometry on a FACSCalibur machine (BD Biosciences).

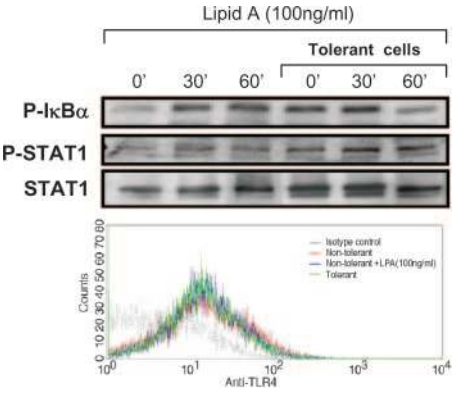

FIGURE 2. LPA-homotolerant cells show defective NF-κB but functional STAT1 signaling. *Upper panel,* Nontolerized and LPA-tolerized cells were stimulated with 100 ng/ml LPA for the indicated time intervals. Thereafter, the cell lysates were accessed by immunoblotting for the indicated Abs. STAT1 expression was used as equal loading control for both the immunoblots. *Lower panel,* FACS analysis of TLR4 surface expression was performed by staining cells with PE-conjugated TLR4 Ab or isotype-matched Ab, as described in *Materials and Methods*. Histograms for mean fluorescence intensity of TLR4 staining in untreated cells (Nontolerant), 4-h 100 ng/ml LPA-treated cells (Nontolerant + LPA (100 ng/ml)), and 24-h 10 ng/ml LPA-treated cells (Tolerant) are shown. Mean fluorescence intensity for untreated cells stained with isotype-matched control Ab (Isotype control) is also indicated. Refer to legend for color codes. All results are representative of three independent experiments. Data are represented as fold suppression in gene expression for 4-h LPA-treated tolerized cells as compared to that of 4-h LPA-treated nontolerant cells.

Results

Differential regulation of cytokines/chemokines is associated with tolerance

One of the important readouts of endotoxin tolerance is the inability of tolerized cells to induce a robust proinflammatory response when rechallenged with endotoxin. Using this assay, we studied the modulation of chemokines/cytokine expression during TLR4-induced endotoxin tolerance. Fig. 1, *A* and *B*, demonstrates the dose and time kinetic studies performed to standardize the in vitro tolerance-inducing protocol in WT-MEF using the TLR4-specific ligand LPA. LPA was preferred over LPS since it exclusively stimulates TLR4, whereas commercial LPS has contaminants which induce activation of TLR2 in addition to TLR4. Tolerance was induced by exposure of cells to various doses (0–100 ng/ml) of LPA for 24 h. Thereafter, the cells were washed, incubated in fresh medium for 1 h, and finally retreated with 100 ng/ml LPA for an additional 4 h. TNF-α expression was studied by real-time PCR. A dose-dependent decrease in TNF-α gene expression was observed in the tolerized cells upon restimulation with 100 ng/ml LPA for 4 h with minimal *TNF-α* expression observed at a tolerizing dose of 10 ng/ml LPA (Fig. 1*A*). Using this tolerizing dose, the optimal time interval for inducing tolerance was ascertained. Cells tolerized with 10 ng/ml LPA for 8–24 h showed minimal *TNF-α* expression upon restimulation with 100 ng/ml LPA for 4 h, suggesting that this might be the optimal time period for tolerization under the given conditions (Fig. 1*B*). Based on these results, we arrived at a dose of 10 ng/ml LPA for 24 h as the optimal tolerizing protocol for all of the subsequent studies. Furthermore, nontolerized cells stimulated with 100 ng/ml LPA were considered as positive controls for all of the subsequent experiments.

LPA signaling through TLR4 triggers both MyD88-dependent and TRIF-dependent pathways, each leading to distinct cytokine/chemokine expression (46). Therefore, we studied the expression of selected MyD88- and TRIF-dependent cytokine/chemokine genes in tolerized and nontolerized cells exposed to 100 ng/ml LPA. Fig. 1*C* shows that tolerized cells upon restimulation with LPA showed a significant time-dependent, down-regulation in the expression of MyD88-dependent proinflammatory TNF-α and CCL3 genes, as compared with LPA-treated nontolerized cells. In contrast, the expression of anti-inflammatory cytokine gene *IL10* and TRIF-dependent cytokine gene *IFN-β* were significantly up-regulated between the 2- and 8-h time points in the tolerized cells as compared with their nontolerized counterparts. The down-regulation of TNF-α and CCL3, but the up-regulation of IFN-β during LPA-induced endotoxin homotolerance was confirmed by ELISA for tolerant and nontolerant cells (Fig. 1*D*). Since our gene expression results from this and other experiments closely correlated with ELISA data, only gene expression data were presented for all of the subsequent experiments.

Next, we investigated whether the observed cytokine/chemokine profile was also true for homo- and heterotolerance induced through TLR2 activation, which also is known to mediate tolerance to Gram-negative endotoxin (2). Pretreatment with Pam_3Cys_4 (10 ng/ml) for 24 h caused homotolerance to subsequent Pam_3Cys_4 (100 ng/ml), but not to LPA (100 ng/ml) treatment, indicated by down-regulated TNF-α and CCL3 gene expression (Fig. 1*E*). The inability of the TLR2 ligand lipoteichoic acid to induce heterotolerance to LPS has been reported in human monocytic cells (using TNF-α and IL-1 as readouts) (47), which correlates well with our observations. In our hands, pretreatment of cells with LPA followed by Pam_3Cys_4 treatment also induced down-regulation of both of these cytokines, but the degree of suppression was not statistically significant (Fig. 1*E*). Up-regulation of IFN-β gene expression in tolerized cells was observable only for LPA homotolerance in these experiments.

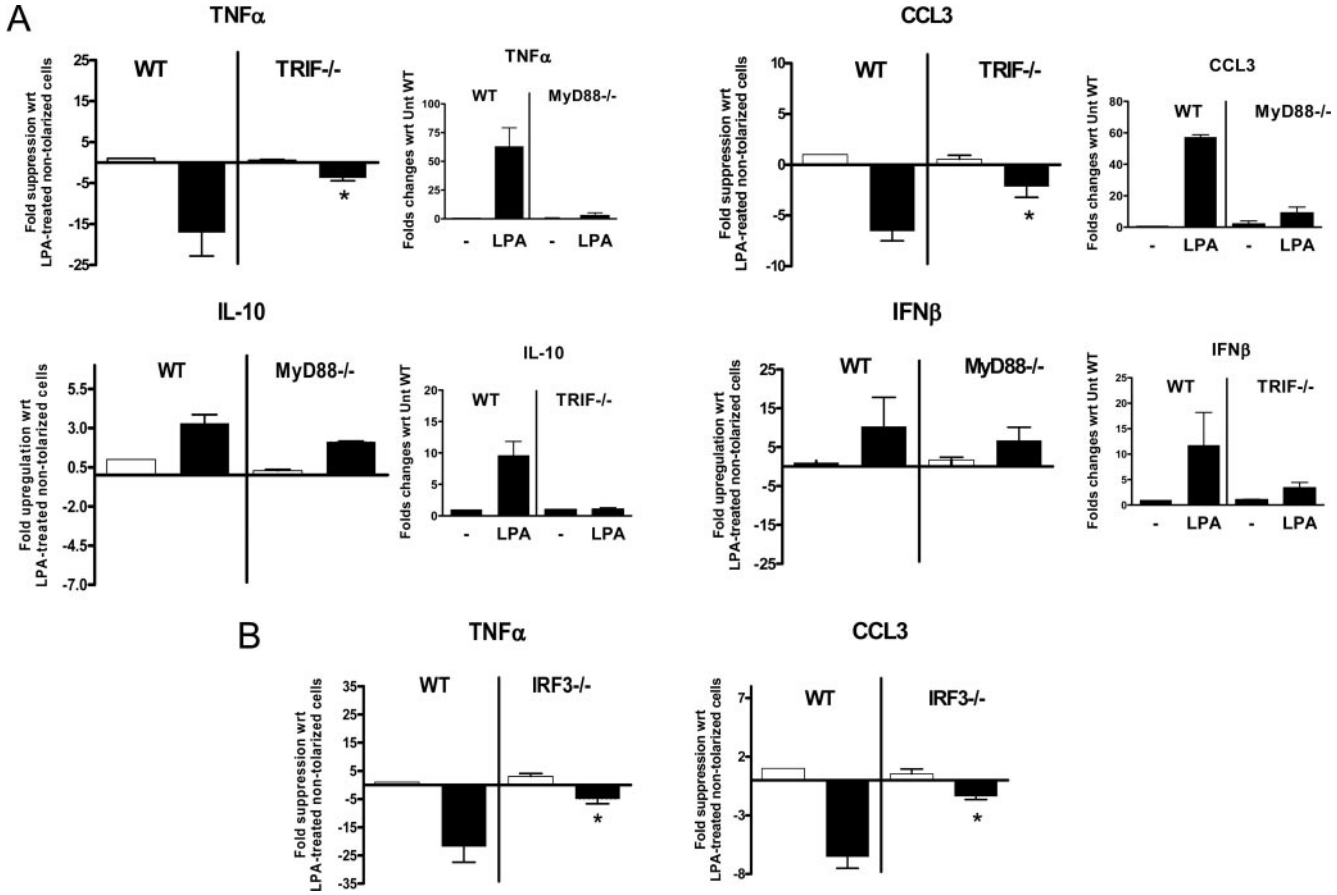

FIGURE 3. Effect of TRIF deficiency on endotoxin tolerance-induced cytokine/chemokine gene expression. Nontolerant and tolerant MEF from (*A*) WT, MyD88$^{-/-}$, TRIF$^{-/-}$ or (B) IRF3$^{-/-}$mice were stimulated with or without 100 ng/ml LPA for 4 h. Expression for the indicated cytokine genes (shown in bold above each graph) was determined by real-time-PCR. ■, Gene expression levels for LPA-treated tolerant cells represented as fold suppression (or up-regulation) with respect to LPA-treated nontolerized cells. □, Gene expression levels represented as fold suppression (or up-regulation) for tolerized cells treated with medium alone with respect to their nontolerized counterparts. Negative fold change signifies down-regulation, while positive fold change signifies up-regulation. *Insets,* Expression levels of LPA-induced MyD88-dependent genes in nontolerized MyD88$^{-/-}$ cells and TRIF-dependent genes in nontolerized TRIF $^{-/-}$ cells. Untreated cells incubated in medium alone are indicated as minus (−). Gene expression for all of the *insets* are represented as fold change with respect to WT-nontolerized cells incubated in medium alone (indicated as minus (−)). For all real-time PCRs, the analyzed gene is indicated in bold on the top of each graph. All values are representative of three independent experiments and shown as mean ± SD. ∗, $p < 0.05$ LPA-treated tolerant WT vs LPA-treated tolerant knockout cells.

Molecular phenotype of tolerized cells showed a defective NF-κB, but a functional TRIF/STAT1 pathway

Based on our results that show differential regulation of MyD88-dependent and TRIF-dependent cytokine/chemokine genes in LPA/TLR4-induced endotoxin homotolerance, we investigated the TLR4 signaling pathway in these tolerant cells using NF-κB activation as a readout of MyD88-dependent signaling, while using STAT1 activation as a readout for TRIF-dependent activation. Fig. 2 (*upper panel*) shows significant inhibition of IκBα phosphorylation (Ser^{32}) at 60 min after LPA treatment in the tolerized cells as compared with the corresponding nontolerized cells. Correlating with this, the ChIP assay for RelA binding to the TNF-α gene promoter also reflected significantly decreased binding in the tolerant cells at 60 min after LPA stimulation as compared with the LPA-stimulated nontolerant cells (see Fig. 5*B*). Both of these observations suggest that the activation of NF-κB signaling is defective during tolerance.

In contrast to NF-κB signaling, significant up-regulation of STAT1(Tyr^{701}) phosphorylation was observed in tolerized cells between 30 and 60 min after LPA treatment (as compared with their nontolerized counterparts that received the same LPA treatment for the same time periods) (Fig. 2, *upper panel*). Constitutive STAT1 phosphorylation (Tyr^{701}) was also noted in the tolerized cells under basal conditions (Fig. 2, *upper panel*). These observations correlate with and might provide a basis for the up-regulation of TRIF-dependent IFN-β expression during tolerance (Fig. 1, *C* and *D*).

To clarify whether down-regulation of the MyD88 pathway was not due to down-regulation of cell surface expression of TLR4, we also investigated TLR4 surface expression in normal and tolerized cells. As shown in Fig. 2 (*lower panel*), comparable levels of TLR4 surface expression were found in nontolerized cells treated with or without 100 ng/ml LPA (4 h) as well as cells tolerized with 10 ng/ml LPA for 24 h. This suggests a distinct mechanism of tolerance induction downstream of TLR4 in this model.

TRIF deficiency affects endotoxin tolerance-induced suppression of proinflammatory cytokines

To explain the opposite trends in the gene expression pattern for MyD88-dependent proinflammatory cytokines TNF-α and CCL3 and TRIF-dependent cytokine IFN-β in the LPA-tolerized MEF (Fig. 1, *C* and *D*), we investigated the relative roles of MyD88 and TRIF in the induction of tolerance. For this purpose, TNF-α and CCL3 gene expression was evaluated in normal vs tolerized MEF

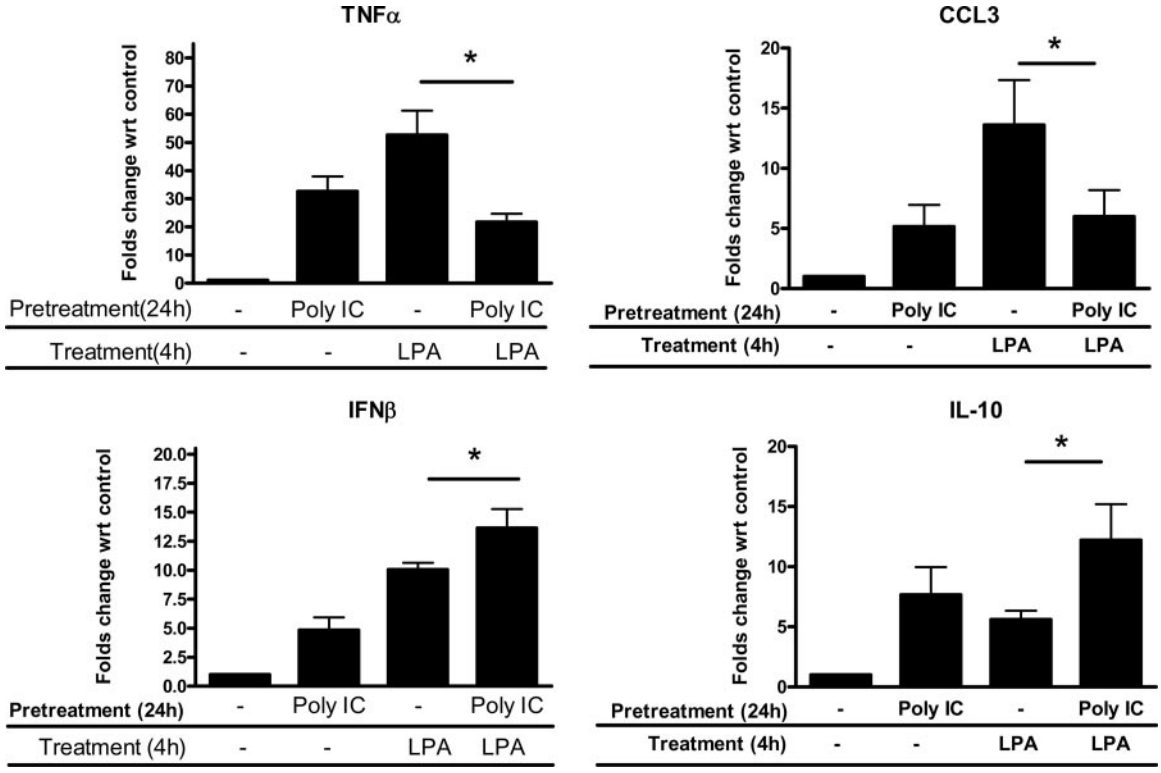

FIGURE 4. Poly(I:C)-induced TRIF pathway activation can induce endotoxin tolerance. Cells were left untreated or pretreated with 50 μg/ml poly(I:C) for 24 h. Thereafter, they were washed and treated with 100 ng/ml LPA for an additional 4 h. RNA was extracted and real-time-PCR was conducted for the expression of the indicated genes. All data are normalized to β-actin gene expression and represented as fold change with respect to the control. Untreated cells incubated in medium alone were taken as the control. Analyzed genes are indicated in bold on the top of each graph. Results are presented as mean ± SD and are representative of three independent experiments. *, $p < 0.05$ LPA-treated vs poly(I:C) plus LPA-treated cells.

from WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice upon 4-h LPA restimulation. We observed a significantly lower tolerance-induced suppression of TNF-α gene expression in tolerized TRIF$^{-/-}$ MEF (4-fold suppression) as compared with their WT counterparts (14-fold suppression) (Fig. 3*A*). This was also true for the CCL3 gene expression, which showed 2-fold suppression in the tolerized TRIF$^{-/-}$ MEF as compared with 7-fold tolerance-induced suppression in WT MEF (Fig. 3*A*). In the MyD88$^{-/-}$ MEF however, LPA-induced expression of both of these genes was very low and, therefore, their further modulation following tolerance could not be studied (Fig. 3*A, inset*). Given that these targets are reported to be primarily induced by the MyD88-dependent pathways (15), the extremely low levels of expression of these genes seen in our experiments are thus not surprising. Similarly, LPA-induced expression of TRIF-dependent IFN-β gene and the anti-inflammatory IL-10 gene was drastically down-regulated in the nontolerized TRIF$^{-/-}$ cells (Fig. 3*A,inset*) and, therefore, their expression during tolerance could not be studied in these cells. Since the effect of MyD88 deficiency on the tolerance-induced up-regulation of both IFN-β and IL-10 gene expression did not show any significant modulation as compared with the tolerized WT cells, no further studies were conducted on these lines.

To characterize the molecular mechanism of tolerance downstream of TRIF, we analyzed the expression of TNF-α and CCL3 genes in IRF3$^{-/-}$ cells. Since IRF3 is a downstream target in the TRIF signaling pathway (16); IRF3$^{-/-}$ MEF serve as important tools in this analysis. Similar to TRIF$^{-/-}$ cells, IRF3 deficiency significantly reduces tolerance-induced suppression of TNF-α to 2-fold as compared with a 22-fold suppression in tolerized WT cells (Fig. 3*B*). A similar trend was observed for the CCL3 gene (Fig. 3*B*). From these results, it is apparent that the deficiency of TRIF or its downstream target IRF3 similarly and significantly reduces tolerance-induced suppression of proinflammatory genes *TNFA* and *CCl3*, providing a strong genetic evidence for the role of this pathway in endotoxin tolerance.

Poly(I:C), a TRIF pathway-specific ligand, induces endotoxin tolerance to LPA

To further validate the involvement of the TRIF pathway in tolerance, we investigated whether pre-exposure to a TRIF-restricted signaling ligand, poly(I:C), for 24 h could tolerize cells to a subsequent LPA stimulation. Real-time PCR results in Fig. 4 show that (50 ng/ml) poly(I:C) pretreatment induced suppression of LPA-induced *TNF-α* and *CCL3* expression, but in contrast led to up-regulation of IFN-β and IL-10 expression. These results are strikingly similar to the cytokine/chemokine profile observed in Fig. 1*C*. Taken together, our results strongly implicate that activation of the TRIF pathway (downstream of TLR3 or TLR4) can be a determinant in inducing endotoxin tolerance in response to LPA.

Involvement of IFN-β in inducing LPA/TLR4- mediated endotoxin homotolerance

To further characterize the tolerance-inducing molecular determinant(s) downstream of TRIF, we focused on the role of IFN-β, since it is up-regulated during tolerance (Fig. 1, *C* and *D*) and since there are efficient ways of testing its loss of function. We designed two sets of experiments. First, the direct effect of neutralizing IFN-β on the induction of tolerance was tested. Second, the effect of rIFN-β pretreatment on the subsequent LPA response in the cells was analyzed. In the first experiment, the cells were pretreated for 24 h with LPA (10 ng/ml) in the presence or absence of

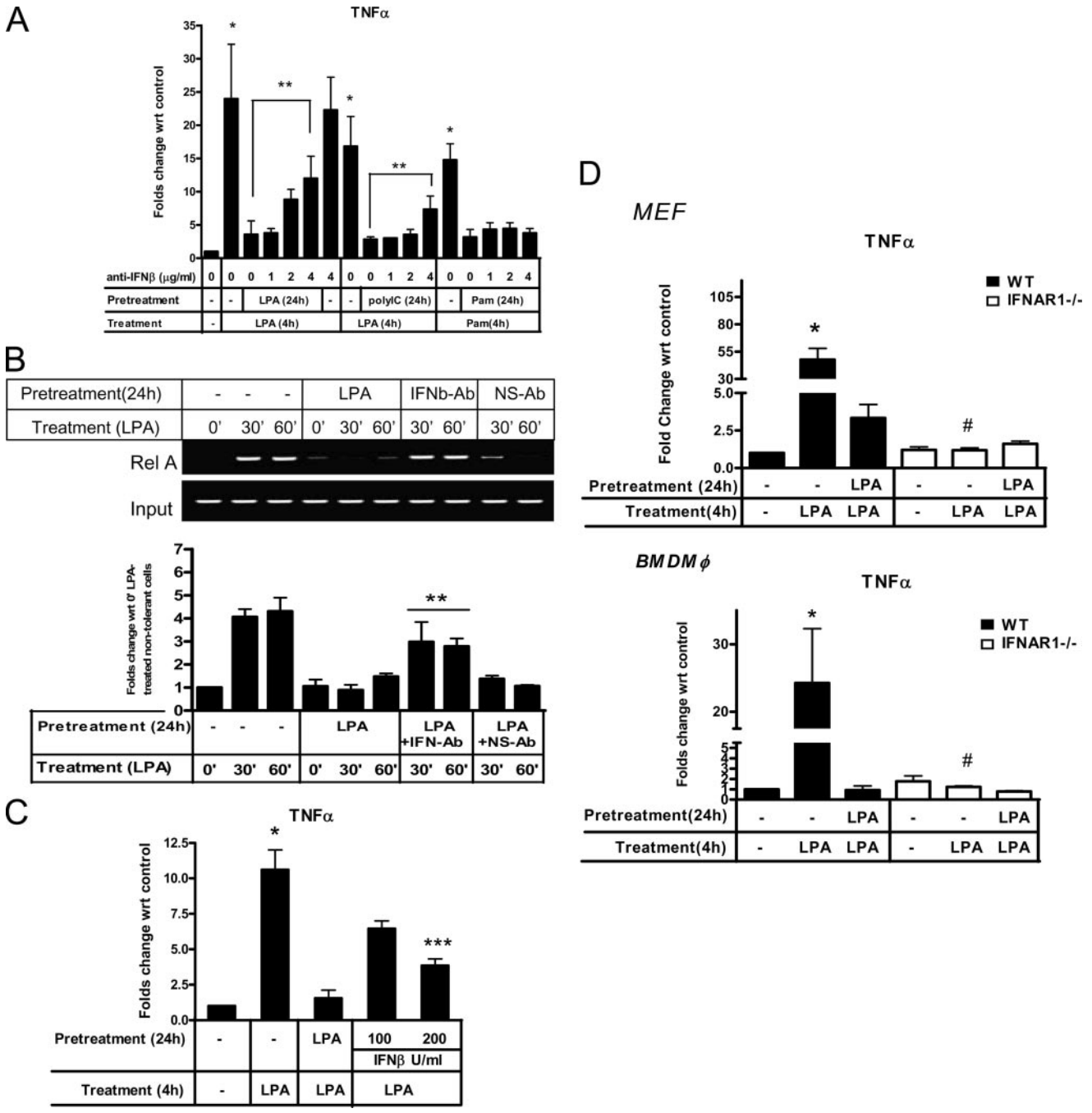

FIGURE 5. Involvement of IFN-β in inducing endotoxin tolerance. *A,* Cells pretreated with 10 ng/ml LPA, 50 μg/ml poly(I:C), or 10 ng/ml Pam_3Cys_4 (Pam) were incubated for 24 h in the presence or absence of varying doses of IFN-β-neutralizing Ab (IFN-Ab). The cells were washed and restimulated with 100 ng/ml LPA or 100 ng/ml Pam_3Cys_4 for 4 h. Real-time-PCR for TNF-α gene expression for these samples are indicated as fold change with respect to the control. Cells that did not receive any pretreatments and were incubated in medium alone were taken as control. All gene expression values were normalized to β-actin gene expression and represented as mean $\pm$ SD. *, $p < 0.05$ LPA (4 h)-treated cells vs LPA (24 h) + LPA (4 h)-treated cells; **, $p < 0.05$ LPA (24 h) + LPS (4 h)-treated cells vs anti-IFN-β + LPA (24 h) + LPA (4 h)-treated cells (*B*). ChIP data on RelA binding to TNF-α gene promoter in LPA-treated nontolerant and tolerant cells. Cells were tolerized by pretreatment with 10 ng/ml LPA for 24 h either in the absence or presence of 4 μg/ml neutralizing IFN-β Ab or nonspecific isotype-matched Abs (NS-Ab). Thereafter, the cells were washed and again treated with 100 ng/ml LPA for the indicated time periods and processed for ChIP using RelA Ab. PCR (*upper panel*) and real-time PCR (*lower panel*) were performed using TNF-α promoter-specific primers. Input controls for genomic DNA are shown for equal loading in the PCR experiments. Real-time PCR data are indicated as fold change with respect to 0′ LPA-treated nontolerant cells. Results are presented as mean $\pm$ SD and are representative of three independent experiments. *C,* Cells were pretreated with medium alone or IFN-β (100–200U/ml) for 24 h. Cells were then washed and treated again with 100 ng/ml LPA for 4 h. Cells that did not receive any pretreatments and were incubated in medium alone were taken as control. Real-time PCR for TNF-α gene expression is represented as fold change with respect to control. Values are normalized to β-actin gene expression and shown as mean $\pm$ SD. *, $p < 0.03$ LPA (4 h)-treated cells vs LPA (24 h) + LPA (4 h)-treated cells; **, $p < 0.03$ LPA (4 h)-treated cells vs IFN-β (24 h) + LPA (4 h)-treated cells. *D,* WT or IFNAR1$^{-/-}$ MEF and BMDM were left unstimulated or tolerized by pretreatment with 100 ng/ml LPA for 24 h. Thereafter, these cells were washed and treated with 100 ng/ml LPA for 4 h. Gene expression for TNF-α was accessed using real-time PCR. Results are presented as fold change with respect to the control. Untreated WT cells incubated in medium alone were taken as control. All data are normalized to β-actin gene expression and values are represented as mean $\pm$ SD. *, $p < 0.05$ LPA (4 h)-treated cells vs LPA (24 h) + LPA(4 h)-treated cells; #, $p < 0.02$ LPA (4 h)–treated WT cells vs LPA (4 h)-treated IFNAR1$^{-/-}$ cells. All data in *A–D* are representative of three independent experiments.

various concentrations of IFN-β-neutralizing Ab (and isotype-matched control). Thereafter, the cells were washed, resuspended in fresh medium, and treated with LPA (100 ng/ml) for an additional 4 h. Tolerance was studied by assessing the expression of the TNF-α gene using real-time PCR. As compared with tolerized cells that did not receive the anti-IFN-β Ab, cells treated with anti-IFN-β Ab during tolerization showed a dose-dependent augmentation in TNF-α gene expression (Fig. 5*A*). This implies that the addition of IFN-β-neutralizing Ab abrogated the induction of LPA homotolerance in these cells (Fig. 5*A*). Incubation with isotype-matched Abs did not relieve the tolerance-mediated suppression of the TNF-α gene, demonstrating the specificity of blocking IFN-β (data not shown). However, when the IFN-β neutralization experiment was conducted for cells undergoing a homotolerance induced by the TLR2 agonist Pam_3Cys_4, no effect on the tolerance-induced TNF-α gene expression was observed (Fig. 5*A*). In contrast, addition of IFN-β-neutralizing Abs during heterotolerance treatment, following pre-exposure to poly(I:C) followed by LPA, partially augmented the TNF-α gene expression (Fig. 5*A*). These observations clearly indicate a role for IFN-β in TLR4- or TLR3-induced tolerance but not in TLR2-induced tolerance. Reversal of TLR4 homotolerance by treatment with neutralizing IFN-β Ab was also reflected in the ChIP assay which demonstrated recovery of RelA binding to the TNF-α gene promoter in the LPA-treated tolerant cells (Fig. 5*B*). The tolerized samples that were treated with nonspecific Abs (as control) did not show significant RelA binding to the TNF-α gene promoter. A quantitative estimate of RelA binding to the TNF-α promoter was derived by real-time PCR using the ChIP DNA (Fig. 5*B, lower panel*).

Further support for the role of IFN-β in mediating tolerance was obtained by pretreatment of cells with recombinant murine IFN-β for 24 h, followed by washing and retreatment with LPA (100 ng/ml) for 4 h (Fig. 5*C*). Dose-dependent down-regulation of TNF-α gene expression in response to IFN-β pretreatment provided direct evidence for its role in inducing endotoxin tolerance.

Since IFN-β mediates the induction of several TRIF-specific genes like *CXCL10* and *CCl5* through an autocrine cycle involving its interaction with the IFNAR1 (48), we also studied tolerance in $IFNAR1^{-/-}$ murine bone-marrow derived macrophages (BMDM) and MEF (Fig. 5*D*). Compared with the WT counterparts, IFNAR1 deficiency in the MEF as well as BMDM caused a complete abrogation of LPA-induced *TNF-α* expression in tolerized and nontolerized cells (Fig. 5*D*). These experiments demonstrate the crucial requirement for IFN-β-IFNAR1 interaction in the expression of inflammatory cytokines like TNF-α and, therefore, in the induction of tolerance. Taken together, our experiments clearly suggest a crucial role for the TRIF-pathway-mediated IFN-β production in the induction of tolerance.

Discussion

Dissecting the TLR4 signaling pathway using genetic and biochemical approaches in WT-, MyD88-, or TRIF-deficient cells, this study provides direct evidence on the role of the TRIF-IRF3 pathway and its downstream cytokine, IFN-β, in mediating LPA-induced endotoxin homotolerance. Studying the expression of well-known cytokine/chemokine target genes downstream of MyD88 (e.g., *TNF-α, CCL3*) and TRIF (e.g., *IFN-β*) (10, 15, 16), we demonstrate their differential regulation in LPA-homotolerized cells. Tolerized cells showed drastic down-regulation of MyD88-dependent cytokines TNF-α/CCL3, upon stimulation with LPA, as compared with their normal counterparts (Fig. 1, *C* and *D*). In contrast, the anti-inflammatory cytokine IL-10 and the MyD88-independent, TRIF-induced cytokine IFN-β (16) were found to be significantly up-regulated at message and protein levels upon LPA restimulation of the tolerized cells (Fig. 1, *C* and *D*). Although the down-regulation of proinflammatory cytokines like TNF-α, IL-1, and IL-6 and the up-regulation of IL-10 has been reported by several earlier studies on tolerized human monocytes and murine macrophages (3, 4, 22, 24, 49), the up-regulation of the TRIF-specific gene IFN-β in endotoxin-tolerized cells was unexpected.

Like TLR4, TLR2 is also an important detector of Gram-negative endotoxins and is reported to induce tolerance (2). Therefore, we studied whether the cytokine/chemokine profile observed for LPA-induced tolerance was also evident in TLR2 homo- and heterotolerized cells. However, up-regulated IFN-β expression was only specific to the LPA/TLR4-induced homotolerance and TLR3/TLR4 heterotolerance but not TLR2 homotolerance (Fig. 1*D*), indicating the involvement of the TRIF pathway (triggered by TLR3 or TLR4). Indeed, signaling studies in the LPA-homotolerant cells demonstrated a preferential utilization of the TRIF-dependent STAT1 pathway, as opposed to a defective NF-κB activation (Fig. 2). Further study on the role of the TRIF pathway in LPA homotolerance using $MyD88^{-/-}$, $TRIF^{-/-}$, and $IRF3^{-/-}$ MEF was instrumental in demonstrating that TRIF and IRF3 deficiency causes a drastic decrease in the degree of tolerance-induced suppression of proinflammatory genes *TNFA* and *CCl3* (Fig. 3). Conversely, prolonged activation of TRIF by 24-h poly(I:C) pretreatment was demonstrated to tolerize cells to LPA with a similar phenotype as the LPA-homotolerant cells (Fig. 4). Supporting this data, it has been reported recently that Kupffer cells pre-exposed to poly(I:C) also fail to respond to LPS (39). These observations together constituted the first evidence for the involvement of the MyD88-independent/TRIF pathway in LPA-induced endotoxin tolerance.

Molecules responsible for mediating inflammatory responses are also known to suppress it, depending on the timing and context (50). One of the reasons believed to explain the occurrence of endotoxin-tolerant phenotype in septicemia and systemic inflammatory response syndrome patients (1, 5, 6) is the high levels of inflammatory cytokines in the circulating serum which desensitize the blood-borne inflammatory cells. Markedly elevated systemic levels of type I IFNs, especially, macrophage-associated IFN-β, has been noted in cases of septic peritonitis (42). Our observations on up-regulated IFN-β production by LPA-homotolerant cells support this fact (Fig. 1, *B* and *C*). Further evidences for the role of IFN-β, downstream of TRIF, in mediating LPA homotolerance was provided from 1) the induction of LPA tolerance by 24-h pretreatment of cells with rIFN-β (Fig. 5*C*), 2) the ability of IFN-β-neutralizing Abs to relieve LPA tolerance both in terms of cytokine production and NF-κB activation (Fig. 5, *A* and *B*), and 3) severe defect in TNF-α gene expression and complete absence of endotoxin tolerance in IFNAR1-deficient MEF and BMDM (Fig. 5*E*).

The observation that IFN-β-neutralizing Abs could only partial recover the LPA-induced *TNF-α* expression in these tolerized cells supports the existence of other molecular pathways that induce endotoxin tolerance. This is plausible since endotoxin tolerance is a complex phenomenon and several mechanisms have been proposed (7, 17, 18, 51). These involve defective activation of several signaling molecules like MyD88, MAPKs, IRAK4, and RelB (18, 21, 32), disruption of chromatin remodeling (33) and up-regulation of negative regulators of TLR signaling like IRAK-M, Src2-containing inositol phosphatase, and FLN90 (26, 27, 52). Gene knockouts of these negative regulators have enhanced lethality toward LPS shock and therefore are implicated in endotoxin tolerance. Our unpublished observations support the up-regulation of IRAK-M and Src2-containing inositol phosphatase in LPA- homotolerant cells and their regulation through TRIF. Up-regulation of anti-inflammatory cytokines like TGF-β and IL-10 (24, 25)

have been previously reported to be yet another mechanism for down-regulation of proinflammatory cytokines during endotoxin tolerance. Recent evidences show the expression of IL-10 to be regulated through the TRIF pathway via the adaptor TNF receptor-associated factor 3 (53). This might explain the up-regulation of IL-10 along with IFN-β during LPA-induced tolerance, as observed here.

Furthermore, our data on IFNAR1$^{-/-}$ cells (MEF and BMDM) support the importance of a functional IFN-β/IFNAR1 autocrine loop in the induction of inflammatory gene expression and tolerance in response to LPA (Fig. 5*E*). This is in line with recent data from IFN-β knockout mice which show defective inflammatory cytokine production (e.g., TNF, IL-6, and IL-12p40) and resistance to endotoxin shock (41). Another study using IFNAR1 knockout mice in the septic peritonitis model indicated that IFNAR1 deficiency can strongly attenuate late, but not early, hyperinflammation (42). These evidences collectively suggest the importance of the TRIF/IFN-β pathway in mediating the late-phase (sustained) expression of inflammatory cytokines like TNF-α, as suggested recently (37, 54). A natural consequence of sustained TNF-α expression under chronic inflammatory situations would most likely lead to tolerance.

In conclusion, our study presents several lines of evidence that support a role for the TRIF pathway in mediating LPA-induced endotoxin homotolerance. The results point out that the differential modulation of MyD88 and TRIF signaling and their effect on downstream cytokines, such as up-regulation of TRIF-dependent IFN-β, play an important role in mediating tolerance. However, the existence of other TRIF-dependent mechanisms that contribute to tolerance remains to be investigated. Similarly, the notion as to whether the differential modulation of MyD88 and TRIF pathways could be used in developing a more selective therapeutic strategy is an open question. Interestingly, our characterization of the molecular phenotype of LPA-tolerized cells (NF-κB defective but a functional TRIF/STAT1) is strikingly similar to the phenotype of macrophages in other pathologies like tumor-associated macrophages (43), suggesting that this could be a molecular paradigm for "tolerant" macrophages in general. The complex and multilayered nature of tolerance still needs to be investigated thoroughly.

Disclosures

The authors have no financial conflict of interest.

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