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Glucocorticoids Inhibit IRF3 Phosphorylation in Response to Toll-like Receptor-3 and -4 by Targeting TBK1 Activation*

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Phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3) is essential for the induction of promoters which contain the interferon-stimulated response element (ISRE). IRF3 can be activated by Toll-like receptor 3 (TLR3) in response to the double-stranded RNA mimic poly(I-C) and by TLR4 in response to lipopolysaccharide (LPS). Here we have analyzed the effect of the glucocorticoid dexamethasone on this response. Dexamethasone inhibited the induction of the ISRE-dependent gene RANTES (regulated on activation normal T cell expressed and secreted) in both U373-CD14 cells and human peripheral blood mononuclear cells and also an ISRE luciferase construct, activated by either TLR3 or TLR4. It also inhibited increased phosphorylation of IRF3 in its N terminus in response to LPS and in its C terminus on Ser-396 in response to either poly(I-C) or LPS. Several dexamethasone-induced phosphatases were tested for possible involvement in these effects; MKP1 did not appear to be involved, although MKP2 and MKP5 both partially inhibited induction of the ISRE, pointing to their possible involvement in the effect of dexamethasone. Importantly, we found that dexamethasone could inhibit TBK1 kinase activity and TBK1 phosphorylation on Ser-172, both of which are required for IRF3 phosphorylation downstream of TLR3 and TLR4 stimulation. Our study, therefore, demonstrates that TBK1 is a target for dexamethasone, common to both TLR3 and TLR4 signaling.

Interferon-regulatory factor 3 (IRF3)[2] is a transcription factor that is activated through recognition of viral double-stranded RNA by receptors such as Toll-like receptor 3 (TLR3) or by intracellular receptors such as retinoic acid-inducible gene I (RIG-I) (1–5). Recognition of bacterial components such as lipopolysaccharide (LPS) by TLR4 also leads to IRF3 activation (6–8). In response to these stimuli, IRF3 becomes readily phosphorylated, resulting in IRF3 dimerization, association with co-factor cAMP-response element-binding protein (CREB)-binding protein, and subsequent translocation to the nucleus (1, 9). Entry to the nucleus allows IRF3 to bind to consensus DNA sequences known as the interferon (IFN)-stimulated response element (ISRE) found in the promoter regions of genes such as those encoding IFN-β, IFN-α1, CXC-chemokine ligand 10 (CXCL10), and RANTES (10–12). These IRF3-dependent genes play an important role in both the anti-viral and anti-bacterial innate immune response (13).

Multiple phosphorylation sites have been identified on IRF3. Phosphorylation of Ser-396, which lies in a cluster of 5 serine/threonine residues (Ser-396, Ser-398, Ser-402, Thr-404, Ser-405) located in the C terminus is particularly important for IRF3 activation as mutation of this site alone to a phosphomimetic aspartic acid generates a constitutively active form of IRF3 which can strongly induce the ISRE promoter element of genes encoding IFN-β, IFN-α1, and RANTES (9). Phosphorylation of this site is also critical for IRF3 dimerization and association with cAMP-response element-binding protein (CREB)-binding protein as well as nuclear translocation (1, 9, 14). Ser-386, which lies proximal to the C-terminal cluster, also appears to be important as mutation of this residue to an alanine abolishes the ability of IRF3 to dimerize, a function that is critical for the translocation of IRF3 to the nucleus (15). The role for this multitude of IRF3 phosphorylation sites has been clarified in a more recent study detailing that activation of IRF3 appears to be a sequential process of phosphorylation, where phosphorylation of Ser-396 occurs first followed by phosphorylation of Ser-404 and Ser-405, thereby priming IRF3 for phosphorylation on Ser-386, required for dimerization (14). Each of these sites plays a slightly different but equally important role in overall IRF3 activation and transcriptional activation of IRF3-dependent genes. The same study also identified Ser-339 as another important residue that appears to share a redundant role with that of Ser-396 (14). IRF3 can also be phosphorylated within its N terminus by stress inducers such as anisomycin, sorbitol, and DNA-damaging agents such as doxorubicin (4). LPS (but not double-stranded RNA) has also been shown to induce N-terminal phosphorylation (8).

TBK1 and IKKϵ are two serine/threonine kinases that have been shown to lie upstream of IRF3 and are required for phosphorylation of the C-terminal cluster, nuclear translocation and, activation of IRF3-dependent ISRE reporters (16–18). TBK1$^{-/-}$ mouse embryonic fibroblasts were shown to be defective in IRF3 nuclear translocation and IFN-α1, IFN-β, and RANTES gene expression in response to viral infection (both Sendai and Newcastle disease virus), poly(I-C) (a double-stranded RNA mimic), and LPS stimulation (18–20). Solis *et al.* (8) more recently showed that LPS could activate both TBK1

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[2] The abbreviations used are: IRF3, interferon-regulatory factor 3; TLR, Toll-like receptor; ISRE, interferon-stimulated response element; MKP1, MAP kinase phosphatase; RIG-I, retinoic acid-inducible gene I; IFN, interferon; LPS, lipopolysaccharide; RANTES, regulated on activation normal T cell expressed and secreted; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; PBMC, peripheral blood mononuclear cells; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

and IKKε in macrophages but with different kinetics, the effect on TBK1 being much more rapid and pre-dominant. Knockdown of both, however, with small interfering RNA inhibited IFN-β expression in macrophages stimulated with LPS (8). Phosphorylation of IRF3 by TBK1, therefore, appears to be a common component of signaling pathways activated by RIG-I, TLR3, and TLR4. However, evidence has emerged to show that specificity can arise within these pathways. One important observation showed that the nuclear factor κB component p65 can form a complex with IRF3 downstream of TLR4 activation but not TLR3 (21). This observation was supported in an elegant study by Ogawa *et al.* (22), which showed that a subset of ISRE-dependent genes activated by LPS were inhibited by the glucocorticoid dexamethasone through the disruption of this p65/IRF3 complex. The same set of genes when induced by poly(I-C) were, however, insensitive to dexamethasone since p65 is not in the IRF3 complex when activated by this stimulus. In a more recent study, however, Reily *et al.* (23) showed that dexamethasone could in fact inhibit the expression of certain IRF3-dependent genes such as RANTES, IFN-β, IP10, ISG15, and ISG56 downstream of TLR3 stimulation by poly(I-C). They showed that dexamethasone could disrupt the ability of a co-activator protein called glucocorticoid receptor-interacting protein to interact with IRF3, thereby suppressing IRF3-dependent genes (23). The basis for this discrepancy with the study performed by Ogawa *et al.* (22) is unclear.

The fact that IRF3 is a common component to both the TLR4 and TLR3 pathways led us to examine the effect of glucocorticoids on IRF3 phosphorylation. In this study we have found that dexamethasone can inhibit the induction of an ISRE-dependent reporter gene and the IRF3-dependent gene encoding RANTES by both LPS and poly(I-C). Importantly, we suggest that this is due to a decrease of IRF3 phosphorylation due to the inhibition of TBK1 kinase activity. This demonstrates for the first time that TBK1 is a target for glucocorticoids downstream of TLR4 and TLR3 activation.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The ISRE luciferase plasmid was purchased from Clontech. pcDNA3.1 empty vector was from Invitrogen. pCMV-MKP1 wild-type and in-active MKP1^{C258S} were kind gifts from Andrew Clark (Imperial College London). pEFr-FLAG PAC-1 expression plasmid was obtained from Steve Gerondakis (WEHI, Melbourne). pSG5-myc-tagged DUSP4–7, DUSP8, and DUSP10 plasmids were generous gifts from Stephen Keyse (Ninewells hospital, Dundee, Scotland). Constitutively active Calcineurin pEGFP CN-CaMIA was obtained from Young Jun Kang and Masato Kubo (RIKEN Yokohama Institute) (24). Wild-type GST-IRF3 (380–427), GST-IRF3 (5A), and GST-IRF3 (7A) were kind gifts from Katherine Fitzgerald (University of Massachusetts Medical School). pcDNA3-FLAG kinase dead TBK1 plasmid was obtained from Makoto Nakanishi (Nagoya City University). The following antibodies were used: total IRF3 (Santa Cruz Biotechnology, Inc.), phospho-Ser-396 IRF3, and phospho-p38 MAP kinase (Cell Signaling Technology Inc.), anti-TBK1 (Imgenex), and anti-β-actin (Sigma). Anti-NAK (TBK1) and anti-TBK1 (Ser-172) used for immunoprecipitation assays were from Abcam (Cambridge, UK) and BD Pharmingen, respectively. Non-phosphorylated TBK1 peptide was obtained from Sir Philip Cohen (University of Dundee). LPS from *Escherichia coli*, serotype EH100, was from Alexis (San Diego, CA), poly(I-C) was from Amersham Biosciences, and dexamethasone (D4902) was purchased from Sigma. RANTES enzyme-linked immunosorbent assay kit was from R&D Systems (Abingdon, UK).

Cell Culture and Transient Transfection—Human peripheral blood mononuclear cells (PBMC) were isolated from human blood and maintained in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin solution (v/v). U373 astrocytoma cells stably transfected with CD14 (U373-CD14) were a kind gift from Katherine Fitzgerald (University of Massachusetts Medical School) and were grown in Dulbecco's modified Eagle's medium supplemented as above with the addition of 250 μg/ml neomycin analog G418 to maintain CD14 expression. For transfections, U373-CD14 cells were seeded in 24-well plates at 3×10^4 cells per well (for ISRE luciferase assays) or in 6-well plates at 1.2×10^5 per well (for Western blot analysis), incubated overnight, and transfected using GeneJuice transfection reagent (Novagen, Madison, WI) according to the manufacturer's instructions. For ISRE luciferase assays, 75 ng of ISRE luciferase plasmid, 30 ng of *Renilla* luciferase, and empty pcDNA3.1 vector made up to a total of 220 ng of DNA were transfected into each well of a 24-well plate. For Western blot analysis varying amounts of pCMV-MKP1 plasmid (100 ng, 1 μg, or 2 μg) and empty pcDNA3.1 vector made up to a total of 2 μg of DNA was transfected into each well of a 6-well plate. In both cases cells were transfected for 24 h before treatment with dexamethasone and stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) as indicated in the figure legends.

ISRE Luciferase Assays—Cells were lysed in 100 μl of passive lysis buffer (Promega, Southampton, UK) for 15 min. Firefly luciferase activity was assayed by the addition of 40 μl of luciferase assay mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 $MgSO_4$, 0.1 M EDTA, 33.3 mM dithiothreitol, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to 20 μl of the lysed sample. *Renilla* luciferase was read by the addition of 40 μl of a 1:1000 dilution of Coelentrazine (Argus Fine Chemicals) in phosphate-buffered saline. Luminescence was read using the Reporter microplate luminometer (Turner Designs). The *Renilla* luciferase plasmid was used to normalize for transfection efficiency in all experiments.

Western Blot Analysis—Human PBMC and U373-CD14 cells were seeded in 6-well plates at 3×10^6 and 1.2×10^5 per well, respectively. Cells were treated with dexamethasone and stimulated with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) as indicated in the figure legends. Cells were washed in ice-cold phosphate-buffered saline before being lysed on ice in 100 μl of low stringency lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40 (v/v), 1 mM EDTA containing 1 mM dithiothreitol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cell lysates were centrifuged at 13,000 rpm for 15 min after which the supernatants were removed and deter-

mined for protein concentration using Coomassie Bradford reagent according to manufacturer's instructions (Pierce). Samples containing equal protein concentrations were generated using 5× SDS sample loading buffer (125 mM Tris-HCl, pH 6.8, 15% glycerol (v/v), 2% SDS (v/v), 10 mg bromphenol blue) containing 50 mM dithiothreitol. SDS protein samples (25 μg) were resolved on 8 or 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) dried milk in TBS-T (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.1% (v/v) Tween 20), and incubation of primary antibody was carried out in the same buffer with the exception of phospho-Ser-396 IRF3 and phospho-p38 MAP kinase, which were incubated in 2.5% bovine serum albumin (w/v)/TBS-T. Blots were then incubated with the appropriate secondary antibody in 5% (w/v) dried milk/TBS-T before being developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Cell Signaling Technology, Inc.). Densitometric analysis of band intensities was determined using Multi Gauge Version 2.2 software.

Native PAGE for Analysis of IRF3 Dimerization—Preparation of cell lysates and native gel analysis was performed as previously described (5).

Immunoprecipitation Assays—U373-CD14 cells were seeded at 8×10^5 per 10-cm plate. Once confluent, cells were treated as indicated in the figure legends, washed in ice-cold phosphate-buffered saline, lysed in 300 μl of low stringency lysis buffer, and determined for protein concentration as described above. For immunoprecipitation kinase assays, 500–800 μg of total protein was incubated with 2 μg of anti-NAK (TBK1) overnight at 4 °C. Additional assay controls such as FLAG-tagged kinase dead TBK1 (KD), which had been transfected into U373-CD14 cells 24 h before stimulation, was immunoprecipitated with 2 μg of anti-FLAG (Sigma). 2 μg of anti-rabbit IgG was used as an internal immunoprecipitation control. In each case, 10 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) were added for 2 h at 4 °C. Beads were washed twice in low stringency lysis buffer followed by one wash in kinase buffer (20 mM Hepes, pH7, 10 mM $MgCl_2$, 50 mM NaCl containing 1 mM dithiothreitol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 0.1 mM phenylmethylsulfonyl fluoride). The beads were then incubated for 25 min at 30 °C in a kinase reaction with 1 μg of recombinant substrate GST-IRF3 (380–427), GST-IRF3 (5A) or GST-IRF3 (7A), 1 mM ATP and 5 μCi [γ-^{32}P]ATP (Amersham Biosciences) made up to a total volume of 20 μl with kinase buffer. Samples were then resolved by SDS-PAGE. The upper half of the gel was transferred onto polyvinylidene difluoride and blotted for total TBK1 (Imgenex), whereas the bottom half was stained with Coomassie to detect substrate GST-IRF3 and subsequently exposed to an (X-ray film) overnight at −80 °C. For Ser(P)-172-TBK1 assays, 500–800 μg of total protein was incubated with 2 μg of anti-TBK1 (Ser-172) and 2 μg of non-phosphorylated TBK1 peptide overnight at 4 °C after which 10 μl of protein A/G-agarose beads were added for 2 h at 4 °C. The beads were washed 3 times in low stringency lysis buffer and resuspended in 20 μl of 2× SDS sample loading buffer and analyzed for total TBK1 by Western blot analysis.

RESULTS

Dexamethasone Inhibits the Induction of RANTES and an ISRE Reporter Gene by TLR4 and TLR3—We first investigated if dexamethasone could affect the functional outcomes of endogenous IRF3 activity in response to both TLR4 and TLR3 stimulation. We, therefore, analyzed the effects of dexamethasone on RANTES expression in response to LPS and poly(I-C) since phosphorylation of IRF3 is essential for RANTES induction (18). As shown in Fig. 1*A*, stimulation of U373-CD14 cells with LPS (*left-hand panel*) or poly(I-C) (*right-hand panel*) induced a 7–8-fold increase in RANTES expression from a basal 100 pg/ml to 700–800 pg/ml. Dexamethasone dose-dependently inhibited this response, with 10 μM decreasing RANTES induction by 90%. We also investigated whether dexamethasone had the same effect on RANTES expression in primary cells. Human PBMC produced a 7-fold increase in RANTES expression in response to LPS (Fig. 1*B*, *left-hand panel*) and a 2–3-fold increase in response to poly(I-C) (*right-hand panel*). In both cases dexamethasone markedly inhibited this response. We next analyzed the effect of dexamethasone on an ISRE luciferase reporter plasmid, which contains five repeats of the ISRE sequence. Stimulation of cells with LPS and poly(I-C) resulted in a 6- and 4-fold increase of ISRE luciferase activity, respectively (Fig. 1*C*). Dexamethasone dose-dependently inhibited both stimuli with an optimal effect evident at 10 μM. These results indicate that the effect of dexamethasone on IRF3-dependent genes is unlikely to be specific to LPS.

Dexamethasone Inhibits IRF3 Phosphorylation in Response to LPS and Poly(I-C)—We next examined whether dexamethasone had any effect on the phosphorylation status of IRF3. Phosphorylated IRF3 exists in multiple forms that have been previously characterized (4). In a resting cell IRF3 can be visualized as a doublet on SDS-PAGE (Fig. 2*A*, *upper panels*) where the lower and upper band have been named form I and form II, respectively. Form I represents non-phosphorylated IRF3, whereas form II represents a basally phosphorylated IRF3 (Fig. 2*A*, *left side*, *upper panel*, *lane 1*). Here we show that treatment of U373-CD14 cells with LPS over time induces a band shift to form II, which is maximal at 60 min (Fig. 2*A*, *left side*, *upper panel*, *lane 7*). This band shift to form II is indicative of IRF3 N-terminal phosphorylation (4, 18). Pretreating the cells with dexamethasone inhibited this response (compare *lane 6* to *lane 5* for 30 min LPS and *lane 8* to *lane 7* for 60 min of LPS). To establish if LPS could cause phosphorylation of any of the C-terminal residues, the same lysates were immunoblotted for Ser-396, a critical residue for IRF3 activation (9, 14). Phosphorylation of IRF3 on Ser-396 occurred from 30 min after LPS stimulation and was maximal at 60 min, which correlated with the band shift observed with the total IRF3 antibody (Fig. 2*A*, *left side*, *second panel*, *lanes 5* and *7*). Pretreating the cells with 1 μM dexamethasone again inhibited this response (compare *lane 6* to *lane 5* for 30 min of LPS and *lane 8* to *lane 7* for 60 min of LPS).

We next examined the effect of dexamethasone on poly(I-C)-induced IRF3 phosphorylation. Despite repeated attempts we were unable to detect a band shift in IRF3 on SDS-PAGE analysis (Fig. 2*A*, *right side*, *upper panel*), which may be explained by

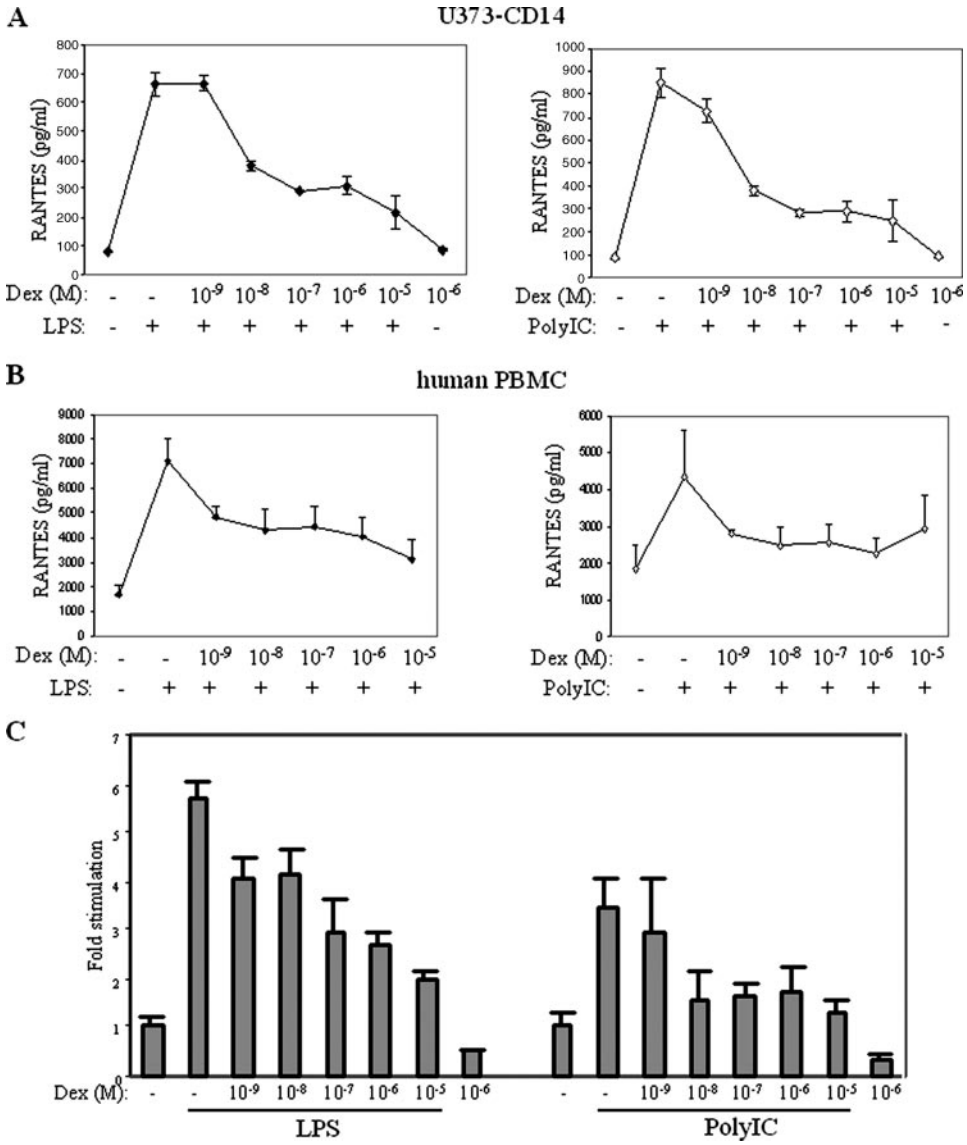

FIGURE 1. **Dexamethasone inhibits the induction of RANTES and an ISRE reporter gene by TLR4 and TLR3.** U373-CD14 cells (*A*) and human PBMC (*B*) were treated with 10-fold increasing doses of dexamethasone (*Dex*) from 1 nM to 10 μM as indicated for 2 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for a further 24 h. Cell supernatants were removed and assayed for RANTES expression by enzyme-linked immunosorbent assay. RANTES concentrations were calculated by comparison to a RANTES standard curve and measured as pg/ml. Results are expressed as the mean ± S.D. for triplicate determinations and are representative of two separate experiments. *C*, U373-CD14 cells were transiently transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng). 24 h after transfection cells were treated with 10-fold increasing doses of dexamethasone from 1 nM to 10 μM for 2 h as indicated before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for 6 h. The cells were lysed, and luciferase activity was subsequently measured. Results were normalized for *Renilla* luciferase activity and represented as -fold stimulation over the unstimulated control. Results are expressed as the mean ± S.D. for triplicate determinations and are representative of three separate experiments.

the fact that poly(I-C) has never been found to induce N-terminal phosphorylation of IRF3. However, we were able to detect increased phosphorylation of Ser-396 after a treatment time of 90 min (Fig. 2*A*, *right side*, *second panel*, *lane 9*). Importantly this was inhibited with dexamethasone pretreatment (*lane 10*). As a positive control for dexamethasone, lysates were also immunoblotted for phospho-p38, which has previously shown to be inhibited by dexamethasone (25). Both LPS and poly(I-C) strongly induced p38 phosphorylation where the LPS effect was evident at 15 min (Fig. 2*A*, *left side*, *third panel*, *lane 3*), and poly(I-C) caused an effect from 60 min (Fig. 2*A*, *right side*, *third panel*, *lane 7*). As expected, dexamethasone could inhibit p38 phosphorylation in response to both stimuli at all time points (*e.g.* compare *lane 4* to *lane 3* for 15 min LPS, and compare *lane 8* to *lane 7* for 60 min poly(I-C)).

The effects of dexamethasone were further analyzed by performing both a time course and a dose response (Fig. 2, *B* and *C*). In the time course, cells were incubated with dexamethasone for either 2, 6, or 24 h before being stimulated with LPS for 60 min (Fig. 2*B*, *left side*) or poly(I-C) for 90 min (Fig. 2*B*, *right side*). At all incubation time points, dexamethasone could reduce the ability of LPS to cause a band shift in IRF3 to form II (Fig. 2*B*, *left side*, *upper panel*), and this appeared to be maximal when the cells were pretreated with dexamethasone for 24 h (*lane 8*). Optimal inhibition of LPS-induced phosphorylation of Ser-396 was also maximal at a pretreatment time of 24 h (Fig. 2*B*, *left side*, *second panel*, *lane 8*). As mentioned previously, poly(I-C) failed to induce an IRF3 band shift; however, activation could be monitored by immunoblotting for Ser-396. Dexamethasone dramatically reduced Ser-396 phosphorylation at each time point, with pretreatment for 24 h causing the greatest effect (Fig. 2*B*, *right side*, *second panel*, *lane 8*). For the dose response, cells were treated with increasing doses of dexamethasone (1 nM to 10 μM) for 24 h before stimulation with LPS for 60 min or poly(I-C) for 90 min (Fig. 2*C*). This resulted in a dose-dependent reduction of an LPS-induced band shift (Fig. 2*C*, *left side*, *upper panel*). Ser-396 phosphorylation was also dose-dependently reduced in response to poly(I-C) (Fig. 2*C*, *right side*, *second panel*). These results, therefore, suggested that dexamethasone inhibits IRF3 phosphorylation in response to both LPS and poly(I-C).

To corroborate that the effects of dexamethasone on IRF3 phosphorylation were not restricted to one cell type, similar experiments were performed in primary human PBMC (Fig. 3). Dexamethasone could reduce Ser-396 phosphorylation at 30 and 60 min LPS stimulation (Fig. 3*A*, *left side*, *second panel*, compare *lane 4* to *lane 3* and *lane 6* to *lane 5*) and could reduce Ser-396 phosphorylation at 90 and 120 min of poly(I-C) stimulation (Fig. 3*A*, *right side*, *second panel*, compare *lane 8* to *lane*

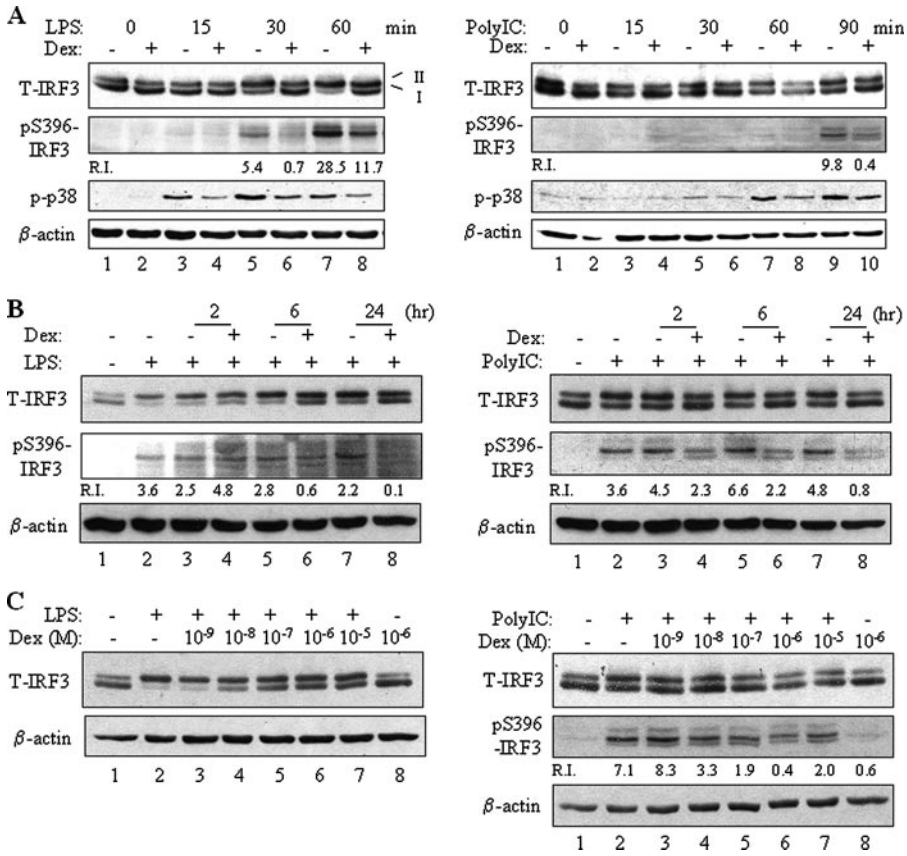

FIGURE 2. **Dexamethasone inhibits IRF3 phosphorylation in U373-CD14 cells in response to LPS and poly(I-C).** *A*, U373-CD14 cells were pretreated with dexamethasone (*Dex*) (1 μM) for 24 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for the times indicated. *B*, U373-CD14 cells were pretreated with dexamethasone (1 μM) for various times as indicated before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. *C*, U373-CD14 cells were pretreated for 24 h with 10-fold increasing doses of dexamethasone from 1 nM to 10 μM as indicated before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. In all cases, cells were lysed and immunoblotted for total IRF3 (*T-IRF3*), phospho-Ser-396 IRF3 (*pS396-IRF3*), and β-actin as a loading control. Densitometric analysis of band intensities was determined for pS396-IRF3 blots, where relative intensity (*R.I.*) values were calculated by normalizing each band to its β-actin control and are represented as -fold change over unstimulated control. Lysates were also immunoblotted for phospho-p38 (*p-p38*) in *A*. Results are representative of two to three separate experiments.

7 and *lane 10* to *lane 9*). PBMC were also pretreated with dexamethasone for 2, 6, and 24 h before stimulation with LPS and poly(I-C). Consistent with the U373-CD14 data, pretreatment with dexamethasone for 24 h had the greatest effect, and for simplicity this time point alone is illustrated in Fig. 3*B* (compare *lane 3* to *lane 2* and *lane 6* to *lane 5*). In addition, PBMC were sensitive to a dexamethasone dose response with inhibition evident from 10 nM as shown for poly(I-C) stimulation in Fig. 3*C*.

Dexamethasone Inhibits IRF3 Dimerization in Response to LPS and Poly(I-C)—The phosphorylation of IRF3 at its C terminus, particularly on Ser-386 and Ser-396, is required for its dimerization and subsequent translocation to the nucleus (9, 14, 15). The fact that dexamethasone can reduce IRF3 phosphorylation on Ser-396 led us to examine whether dexamethasone could affect IRF3 dimerization. As shown in Fig. 3*D*, pretreatment of both U373-CD14 cells and human PBMC with dexamethasone could abolish the induction of IRF3 dimerization in response to LPS (compare *lane 4* to *lane 3* for U373-CD14 and *lane 3* to *lane 2* for PBMC) and poly(I-C) (compare *lane 8* to *lane 7* for U373-CD14 and *lane 5* to *lane 4* for PBMC).

MKP1 Overexpression Does Not Affect ISRE Luciferase Activity or IRF3 Phosphorylation—To understand how dexamethasone affects IRF3 phosphorylation, we turned our attention *DUSP1*, a gene that encodes the dual-specificity phosphatase MAP kinase phosphatase 1 (MKP1). MKP1 has the ability to dephosphorylate serine/threonine and tyrosine residues and is the phosphatase responsible for dephosphorylating both the threonine and tyrosine residues within the activation loop of p38 MAPK (26). Interestingly, dexamethasone has been shown to strongly induce MKP1 expression, which subsequently resulted in p38 dephosphorylation (25). Therefore, we tested whether MKP1 might be responsible for dephosphorylating IRF3 by first determining the effect of MKP1 on the ISRE luciferase system. Transfection of cells with increasing amounts of plasmid encoding MKP1 had no effect on ISRE luciferase activity in U373-CD14 cells stimulated with either LPS or poly(I-C), whereas dexamethasone alone could dramatically reduce luciferase activity in response to both stimuli (Fig. 4*A*). To analyze the effect of MPK1 overexpression in more detail, IRF3 phosphorylation was examined (Fig. 4*B*). MKP1 expression had no effect on the LPS-induced band shift (Fig. 4*B*, *upper panel*) or Ser-396 phosphorylation (*second panel*). In contrast, increasing amounts of MKP1 expression could reduce LPS-induced phosphorylation of p38 (Fig. 4*B*, *third panel*, compare *lanes 4* and *5* to *lane 2*), whereas an inactive mutant of MKP1 had no effect (*lane 7* and *lane 8*). This implies that MKP1 is unlikely to be targeting IRF3.

To investigate the potential roles of other candidate phosphatases, we turned our attention to other dual-specificity phosphatases encoded by DUSP genes, PAC-1 (DUSP2), MKP2 (DUSP4), VH3 (DUSP5), MKP3 (DUSP6), PYST2 (DUSP7), VH5 (DUSP8), and MKP5 (DUSP10). Of particular interest were the phosphatases PAC-1, MKP2, and MKP5, whose expression has also been shown to increase upon dexamethasone treatment (25). We also focused on calcineurin, a serine/threonine phosphatase that has recently been shown to negatively regulate TLR pathways by inhibiting the adaptor proteins MyD88 and TRIF (24). To determine whether any of these phosphatases could play a negative role in the IRF3 pathway, increasing concentrations (1, 10, and 100 ng) of plasmids encoding these phosphatases were tested in the ISRE luciferase

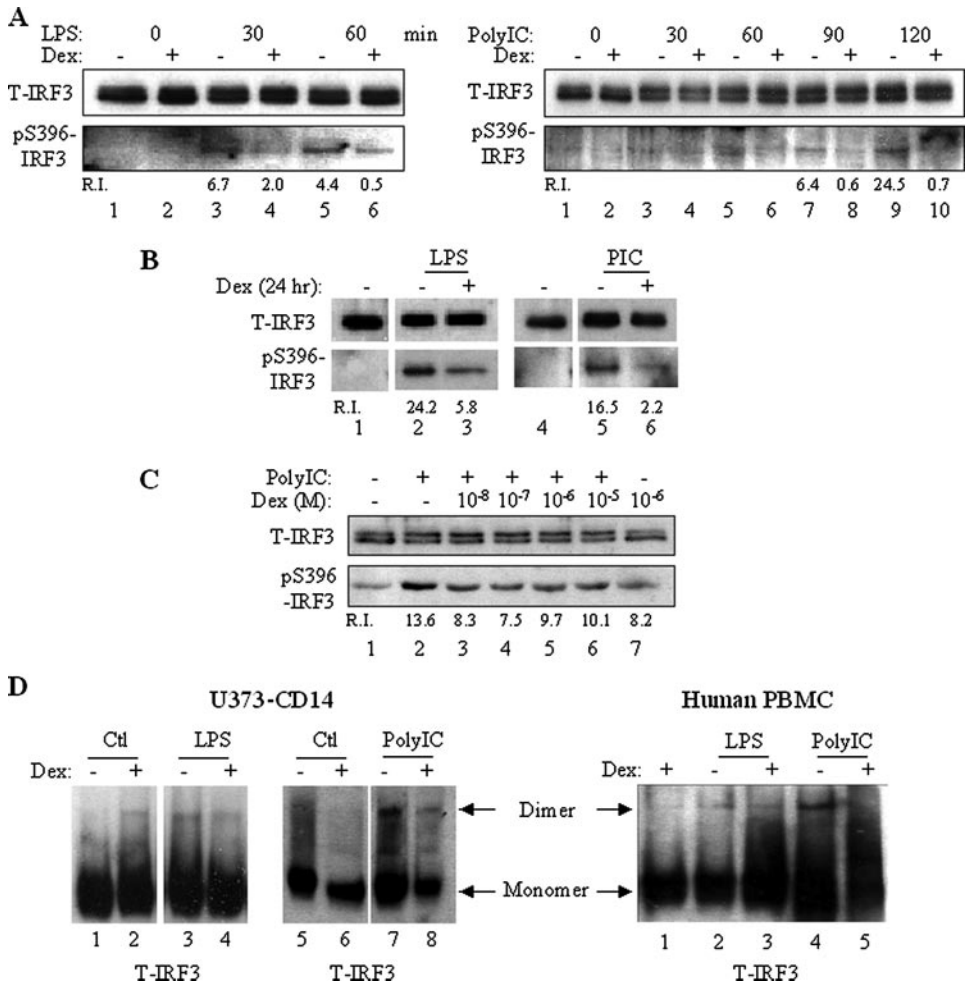

FIGURE 3. **Dexamethasone inhibits IRF3 phosphorylation in human PBMC and IRF3 dimerization in response to LPS and poly(I-C).** *A*, human PBMC were pretreated with dexamethasone (*Dex*, 500 nM) for 24 h before stimulation with LPS (100 ng/ml) or poly(I-C) (50 μg/ml) for the times indicated. *T-IRF3*, total IRF3; *pS396*, phospho-Ser-396. *B*, human PBMC were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. *PIC*, poly(I-C). *C*, human PBMC were pretreated for 24 h with 10-fold increasing doses of dexamethasone from 10 nM to 10 μM as indicated before stimulation with poly(I-C) (50 μg/ml) for 90 min. In all cases cells were lysed and immunoblotted for T-IRF3 and pSer-396-IRF3. Densitometric analysis of band intensities was determined for pS396-IRF3 blots, where relative intensity (*R.I.*) values were calculated by normalizing each band to T-IRF3 and are represented as -fold change over unstimulated control. *D*, U373-CD14 cells and human PBMC were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min. Lysates were run on native gels and immunoblotted with T-IRF3 to visualize IRF3 monomers and dimers. Results are representative of two to three separate experiments. *Ctl*, control.

system upon LPS and poly(I-C) stimulation (Fig. 4*C*). Both LPS and poly(I-C) alone could increase ISRE luciferase activity 8- and 6-fold, respectively, and this -fold change did not dramatically alter when increasing concentrations of any of the phosphatase plasmids were transfected into this system. However, of potential interest were the phosphatases MKP2 and MKP5, which could reduce ISRE activity by half when transfected at the highest plasmid concentration (Fig. 4*C*, *upper* and *lower graph*). These may, therefore, play a role in the effects of dexamethasone.

Dexamethasone Can Inhibit TBK1 Kinase Activity—Finally we examined the effect of dexamethasone on TBK1 activation. To test this we immunoprecipitated TBK1 from LPS- or poly(I-C)-stimulated U373-CD14 cells and examined its kinase activity by observing the amount of ^{32}P-labeled ATP incorporated into the wild-type recombinant GST-IRF3 (380–427) substrate in the absence or presence of dexamethasone. We first performed a time course of activation, demonstrating that a treatment time of 60 min for LPS resulted in maximal ^{32}P incorporation, whereas poly(I-C) required 90 min of treatment time (Fig. 5*A*). These time points correlated with phosphorylation of endogenous IRF3 (Fig. 2*A*). Dexamethasone dose-dependently inhibited the response to both LPS and poly(I-C), the effect being optimal at 500 nM for LPS (Fig. 5*B*, *upper panel*, *upper blot*, *lane 5*) and 100 nM for poly(I-C) (Fig. 5*B*, *lower panel*, *upper blot*, *lane 5*). To ensure that equal amounts of TBK1 were being immunoprecipitated, the samples were also analyzed for total TBK1 (Fig. 5*B*, *upper panel*, *lower blot* and *lower panel*, *lower blot*). The specificity of TBK1 to phosphorylate IRF3 in this assay was confirmed when it was shown that TBK1 failed to phosphorylate a GST-IRF3 (5A) substrate where the 5 C-terminal residues responsible for IRF3 activation Ser-396, Ser-398, Ser-402, Thr-404, and Ser-405 were mutated to an alanine, and this was further confirmed by a GST-IRF3 (7A) mutant (5A plus S385A/S386A) (Fig. 5*B*, *lane 6* and *7*, *upper panel* and *lane 7* and *8*, *lower panel*). Immunoprecipitation of a FLAG-tagged kinase dead TBK1 (KD) also failed to phosphorylate GST-IRF3 (Fig. 5*B*, *lane 9*, *upper panel* and *lane 10*, *lower panel*).

Phosphorylation of TBK1 on Ser-172 within its activation loop is essential for its kinase activity (27). We, therefore, decided to investigate if dexamethasone had any effect on Ser-172 phosphorylation in response to both LPS and poly(I-C) (Fig. 5*C*). LPS and poly(I-C) both induced phosphorylation of TBK1 on Ser-172, and this phosphorylation was completely abolished in the presence of dexamethasone (Fig. 5*C*, *upper panel*, compare *lane 4* to *lane 3* and *lane 6* to *lane 5*). A fraction of the same lysates was also used to immunoprecipitate total TBK1 to ensure equal TBK1 protein levels (Fig. 5*C*, *second panel*). Samples were also blotted for phospho-p38 to ensure that dexamethasone was active (*third panel*).

Taken together these data, therefore, confirm that TBK1 is the kinase responsible for IRF3 phosphorylation. In addition, it predicts that dexamethasone inhibits the phosphorylation of IRF3 by LPS and poly(I-C) via the inhibition of TBK1 kinase activity.

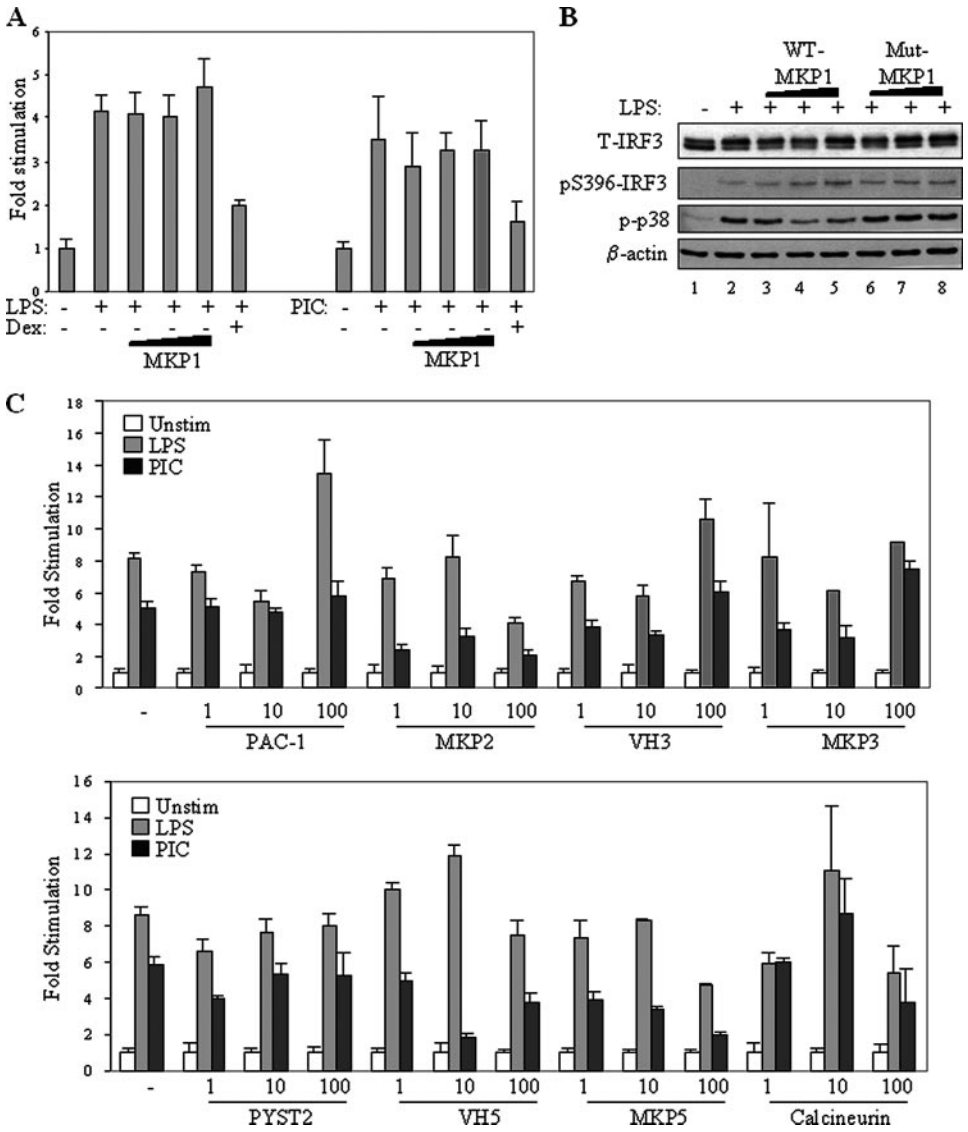

FIGURE 4. **Analysis of various phosphatases on ISRE luciferase activity and IRF3 phosphorylation.** *A*, U373-CD14 cells were transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng) along with increasing amounts of plasmid expressing wild-type (*WT*) MKP1 (25, 100, or 200 ng). 24 h after transfection cells were stimulated with LPS (100 ng/ml) or poly(I-C) (*PIC*, 50 μg/ml) for 6 h. Pretreatment of cells with dexamethasone (*Dex*, 1 μM) for 2 h was used as a control. ISRE luciferase activity was subsequently measured. Results were normalized for *Renilla* luciferase activity and are represented as -fold stimulation over the unstimulated control. *B*, U373-CD14 cells were transfected with increasing amounts (100 ng or 1 or 2 μg) encoding wild-type MKP1 or inactive (*Mut*)-MKP1 before stimulation with LPS (100 ng/ml) for 60 min. Cells were lysed and immunoblotted for total IRF3 (*T-IRF3*), phospho-Ser-396 (pS396)-IRF3, phospho-p38 (*p-p38*), and β-actin as a loading control. *C*, as in *A*, except that increasing amounts of phosphatase plasmids expressing PAC-1, MKP2, VH3, MKP3, PYST2, VH5, MKP5, and constitutively active calcineurin (1, 10, and 100 ng) were co-transfected with ISRE luciferase (75 ng) and *Renilla* luciferase (30 ng).

DISCUSSION

In this study we demonstrate that dexamethasone targets IRF3 in response to both TLR4 and TLR3 stimulation mediated by LPS and poly(I-C), respectively. We propose that this is due to the ability of dexamethasone to reduce IRF3 phosphorylation via inhibition of TBK1 activation. This in turn inhibits activation of the ISRE and thereby blocks induction of IRF3-dependent gene expression, as exemplified by decreased expression of the chemokine RANTES. The effect on IRF3 phosphorylation was first indicated from the observation that dexamethasone can reduce a band shift in IRF3 from form II to form I, an event that has been characterized as N-terminal phosphorylation in previous studies (4). In addition, dexamethasone can reduce C-terminal phosphorylation as indicated by a reduction of Ser-396 phosphorylation in response to both LPS and poly(I-C) in both U373-CD14 cells and human PBMC. This reduction in phosphorylation could have been due to two mechanisms. Either dexamethasone induces a phosphatase, which could de-phosphorylate IRF3, or it targets an upstream kinase such as TBK1, again possibly via induction of a phosphatase.

To date very few phosphatases have been identified that may target components of the TLR signaling cascades. Instead, polyubiquitination and proteasomal degradation of these components have been studied in far greater detail (28, 29). Indeed, IRF3 itself has been shown to become phosphorylated by an unknown kinase on Ser-339 and Thr-440 in response to poly(I-C), resulting in the recruitment of a peptidyl-prolyl isomerase called Pin1. This association with Pin1 results in polyubiquitination and proteasome-dependent degradation of IRF3, thereby terminating the IRF3 signal (30). The polyubiquitination of IRF3 in response to Sendai virus infection, mediated by Cullin-based ligases, has also been demonstrated where phosphorylation of the C-terminal residues by TBK1 is critical (31). These results highlight that IRF3 is not only positively regulated by phosphorylation but that phosphorylation is also required for negative regulation, a fact that makes IRF3 a likely target for phosphatases. Indeed it has been shown that treatment of cells with a general phosphatase, calf intestinal phosphatase, has the ability to abolish IRF3 phosphorylation on the C-terminal cluster but also on the N terminus in response to various stimuli, illustrating that IRF3 phosphorylation sites are sensitive to phosphatase treatment (1, 4). However, to date no effort has been made to try and identify specific phosphatases that may be responsible. The fact that dexamethasone can reduce an LPS-induced band shift and can also reduce Ser-396 phosphorylation in response to both LPS and poly(I-C) provides us with a tool to explore and identify a phosphatase which dexamethasone may induce to down-regulate IRF3 phosphorylation.

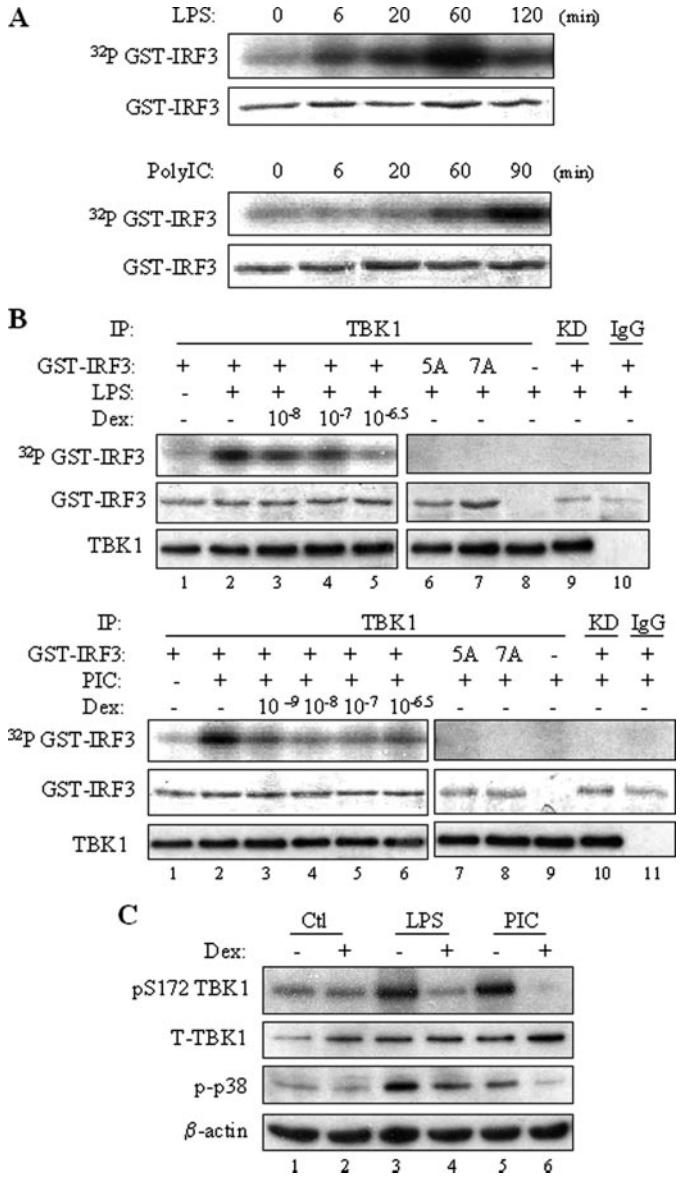

FIGURE 5. **Dexamethasone inhibits TBK1 kinase activity.** *A*, U373-CD14 cells were stimulated for various times as indicated with LPS (100 ng/ml) or poly(I-C) (50 μg/ml). *B*, cells were pretreated with 10-fold increasing doses of dexamethasone (*Dex*) from 1 nM to 10 μM for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (50 μg/ml) for 90 min as indicated. In both cases, TBK1 was immunoprecipitated and assayed for kinase activity against recombinant GST-IRF3 (380–427), GST-IRF3 (5A), or GST-IRF3 (7A) as indicated. Additional assay controls such as FLAG-tagged kinase dead TBK1 (*KD*) and anti-rabbit IgG were assayed for kinase activity against recombinant GST-IRF3 (380–427). The *upper blots* illustrate ^{32}P incorporation into GST-IRF3, *middle blots* were stained with Coomassie Blue to demonstrate equal loading of GST-IRF3 (*A* and *B*) and *lower blots* were analyzed for total TBK1 to ensure equal immunoprecipitation (*IP*, *B*). *C*, U373-CD14 were pretreated with dexamethasone (500 nM) for 24 h before stimulation with LPS (100 ng/ml) for 60 min or poly(I-C) (*PIC*, 50 μg/ml) for 90 min. 55% of the lysate was immunoprecipitated with anti-phospho-Ser-172 (*pS172*) TBK1, and 35% was immunoprecipitated with total-TBK1 (*T-TBK1*). The immunoprecipitation samples were then blotted for total-TBK1. The remaining 10% of the lysate was used to blot for phospho-p38 (*p-p38*) and β-actin. Results are indicative of two to three separate experiments. *Ctl*, control.

We, therefore, first turned our attention to MKP1, a phosphatase expressed by DUSP1, because it has been shown to become induced by dexamethasone (25). However, overexpression of MKP1 had no effect on the ISRE luciferase system or on the phosphorylation status of IRF3, suggesting that IRF3 is not a substrate for this phosphatase. We next turned our attention to other potential phosphatases such as those expressed by other DUSP genes, particularly PAC-1, MKP-2, and MKP-5, which were also shown to become induced upon dexamethasone treatment (25). Calcineurin was also an interesting candidate and has been shown to negatively regulate TLR pathways (24). Overexpression of seven DUSP expressed phosphatases (PAC-1, MKP2, VH3, MKP3, PYST2, VH5, and MKP5) and calcineurin had no dramatic effect on the ISRE luciferase system. However, of potential interest are the phosphatases MKP2 and MKP5, which showed a significant reduction of ISRE luciferase when expressed at the highest concentration. Ongoing studies will continue to examine if these phosphatases may targeting IRF3 for dephosphorylation.

We did, however, find that dexamethasone could block the activation of the upstream kinase, TBK1, as judged by its kinase activity against recombinant GST-IRF3. In addition, dexamethasone abolished the phosphorylation of Ser-172 within the activation loop of TBK1, indicating for the first time that this kinase is a target for dexamethasone. This is an interesting observation as the importance of TBK1 as a central player in innate immune pathways is only recently becoming more apparent. The TBK1-IRF3 axis is not only crucial for TLR3-, TLR4-, and RIG-I-mediated signaling (16, 17, 19, 20) but is also critical for TLR-independent pathways such as those activated by intracellular B-DNA (32, 33). More recently, this dependence for TBK1 downstream of B-DNA signaling was shown to be essential for generating the adjuvant effect of DNA vaccines (33). The knowledge that dexamethasone can abolish TBK1 kinase activity is, therefore, an important observation that could impact on DNA vaccine administration. In addition, this observation may provide a basis for why steroids are contraindicated for certain viral infections which would trigger TBK1 activation as a key process in anti-viral host defense.

The effects of dexamethasone as an anti-inflammatory agent are highly complex, and many molecular targets have been identified to date (34). Ogawa *et al.* (22) analyzed the effects of dexamethasone on a range of 543 and 572 genes that were induced by LPS and poly(I-C), respectively. Interestingly they identified a subset of identical genes (~80) which contained ISRE sites that were sensitive to dexamethasone in response to LPS but were resistant in response to poly(I-C). The fact that p65 exists in a complex with IRF3 downstream of the TLR4 pathway but not the TLR3 pathway led them to examine whether dexamethasone could disrupt an IRF3·p65 complex by sequestering p65 out of the complex, which they demonstrated to be the case (21, 22). Because IRF3 activation downstream of TLR3 is p65-independent, they concluded that the targeting of the IRF3·p65 complex was the basis for the specific effect of dexamethasone. However, in our hands dexamethasone can affect both LPS and poly(I-C) pathways, indicating that TBK1 and IRF3 may be the common target. This was supported by the study where Reily *et al.* (23) could show that certain IRF3-dependent genes are in fact sensitive to dexamethasone in response to poly(I-C). The mechanism involved the ability of dexamethasone to disrupt an IRF3-glucocorticoid receptor-in-

teracting protein complex thought to be required for the expression of these genes (23).

Our study, therefore, indicates that dexamethasone can target both TLR4 and TLR3 signaling to the ISRE rather than being specific for TLR4 alone. We identify the phosphorylation of IRF3 and the kinase activity of TBK1 as important additional targets for dexamethasone in response to activation of both these pathways. TBK1 is, therefore, another important target for dexamethasone, and ongoing studies will aim to elucidate the process determining whereby dexamethasone inhibits TBK1 activation, namely through the identification of a candidate phosphatase that may be targeting the TBK1-IRF3 pathway.

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