TRIF Signaling Stimulates Translation of TNF-α mRNA via Prolonged Activation of MK2

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TRIF Signaling Stimulates Translation of TNF-α mRNA via Prolonged Activation of MK2

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The adapter protein TRIF mediates signal transduction through TLR3 and TLR4, inducing production of type I IFNs and inflammatory cytokines. The present study investigates the mechanisms by which TRIF signaling controls TNF-α biosynthesis. We provide evidence that, in LPS-stimulated murine dendritic cells, TRIF stimulates TNF-α biosynthesis selectively at the post-transcriptional level by promoting mRNA translation. In the absence of functional TRIF, the production of TNF-α protein was severely impaired, whereas TNF-α mRNA levels and stability, as well as transcriptional activity of the Tnfa gene, were not affected. Similarly, TRIF was required for production of LPS-induced TNF-α protein, but not of mRNA, in bone marrow-derived macrophages. In peritoneal macrophages, however, TRIF was also required for normal induction of TNF-α mRNA, suggesting cell type-related functions of TRIF. The influence of TRIF on dendritic cell TNF-α production was independent of type I IFNs. TRIF was required for prolonged activation of MAPKs in LPS-stimulated dendritic cells but was dispensable for the activation of NF-κB. Inhibition of late p38 activity attenuated LPS-stimulated elevation of TNF-α protein but not mRNA levels. The p38 effector kinase MK2 was directly activated through the TRIF pathway of TLR4. Importantly, stimulation of Mκ2–/– cells through TLR3 or TLR4 severely impaired TNF-α protein production but did not affect TNF-α mRNA induction. Together, these results indicate that the TRIF signaling pathway promotes TNF-α mRNA translation through activation of the protein kinase MK2. The Journal of Immunology, 2010, 184: 000–000.
kinase, MK2. In MK2−/− cells, ligation of TLR4 or TLR3 induced normal levels of TNF-α mRNA, whereas production of TNF-α protein was substantially reduced. Together, these results indicate a critical role for MK2 in the regulation of TNF-α mRNA translation through the TRIF signaling pathway.

Materials and Methods

Mouse strains

Trif−/− homozygous mutant mice were kindly provided by Dr. B. Beutler (9) and Myd88−/− mice by Dr. S. Akira (10). MK2−/− mice were described previously (11). Irf3−/− and control 129 Sv/Ev mice were obtained from B&K Universal Group Limited (North Humberside, U.K.). Control C57BL/6 mice were purchased from Harlan Winkelmann (Borchem, Germany).

Cell preparation and stimulation

Bone marrow cells were cultured in medium supplemented with 20 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) to generate BMDC. Cultures received fresh medium containing GM-CSF every 3 d, and cells were used for experiments at day 10. BMM were generated by differentiation of bone marrow cells in 15% L cell-conditioned medium as a source of M-CSF for 5–7 d. For ex vivo isolation of peritoneal macrophages, mice were injected i.p. with 800 μl 4% sterile thioglycollate, and 4 d later cells were harvested by peritoneal lavage. Cells were stimulated with 100 ng/ml ultrapure LPS from Salmonella minnesota R595 (List Biological Laboratories, Campbell, CA) or 100 μg/ml polynosinic-polycytidylic acid [poly(I:C)] (Invivogen, San Diego, CA) in RPMI 1640 medium containing 10% FCS for the indicated time periods.

Analysis of cytokine mRNA and protein production

Protein concentrations of TNF-α and CCL2 in culture supernatants or cellular lysates were determined by ELISA (R&D Systems, Minneapolis, MN). To measure cell-bound TNF-α protein, cells were lysed in a buffer containing 50 mM Tris, pH 8.0; 1% NP-40; 150 mM NaCl; 1 mM EDTA; and protease inhibitors.

RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg total RNA, using a mixture of oligo(dT)12–18 and random hexamer primers and SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany).

For quantitative RT-PCR analysis of TNF-α mRNA levels, Mastermix Plus for SYBR Green (Eurogentec, Seraing, Belgium) was used. RNA expression levels of TNF-α were normalized to those of β-actin and were displayed as fold-change relative to samples of unstimulated cells used as calibrator. The primers were as follows: actb sense, 5'-ACC CAC ACT GTG CCC TTC A TC-3'; actb antisense, 5'-AGC CAA GTG CAG ACC CAG G-3'; Tnfα sense, 5'-AAA ATG CGA ATC AGC TAG CAG C-3'; and Tnfα antisense, 5'-AGC CAA GTG CAG ACC CAG G-3'. Accumulation of PCR products was quantified on an ABI 7300 cycler (Applied Biosystems, Foster City, CA).

Primary transcript RT-PCR

The rate of transcription of the Tnfa gene was determined by real-time quantitative primary transcript RT-PCR. The principle of the assay is to use primers that are placed in adjacent exons and introns of a given gene, thereby generating amplicons from the unspliced populations of RNAs. Isolation of total cellular RNA was performed as described above. For cDNA synthesis, the QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany) was used. DNA contamination of RNA preparations was monitored by omitting reverse transcriptase from control reactions and was not detectable in any of the experiments reported. Expression levels of primary Tnfa transcripts were normalized to those of Gapdh and were displayed as fold-change relative to samples of unstimulated wild-type cells used as the calibrator. The primers were as follows: Tnfa sense, 5'-CCG GGA CCT CAT AGC CA-3'; Tnfa antisense, 5'-GCA AAT CCT CTC ACG GTG TG-3'; Gapdh sense, 5'-TCC AGT ATG ACT CCA CTT TCC-3'; and Gapdh antisense, 5'-ATC CCT CGT GGT TCA CAC-3'. Accumulation of PCR products was quantified on an ABI 7300 cycler (Applied Biosystems, Foster City, CA).

Western blot analysis of TLR signaling

Cells were stimulated as indicated and lysed in cell extraction buffer containing 50 mM Tris, pH 8.0; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 10 mM NaF; 2 mM Na3VO4, and protease inhibitors. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with Abs specific for phospho-p38 MAPK, phospho-SAPK/JNK, phospho-Erk-1/2, phospho-MK2, phospho-Mnk1, phospho-eIF-4E, IκBα, and phospho-IκBα (all from Cell Signaling Technology, Beverly, MA) and α-tubulin (Calbiochem, Darmstadt, Germany).

The p38 kinase activity was determined using the nonradioactive p38 MAPK assay kit (Cell Signaling Technology). Briefly, an immobilized Ab directed against phosphorylated p38 was used for immunoprecipitation, and the in vitro kinase assay was performed using recombinant ATF-2 as a substrate. ATF-2 phosphorylation was detected by Western blotting.

Ab binding to nitrocellulose membranes was visualized with the ECL Western blotting Detection System (Thermo Fisher Scientific, Rockford, IL). Chemiluminescence signals were recorded using the Kodak Image Station 2000R (Kodak, New Haven, CT).

Statistical analysis

Statistical analysis of the data was performed using the two-tailed Student t test. All data are presented as mean values ± SEM, with the number of independent experiments indicated in the figure legends. Differences between experimental groups were considered significant for p < 0.05.

Results

The TRIF pathway of TLR4 controls dendritic cell TNF-α production at the posttranscriptional level

The present study examines the mechanisms by which the TRIF-dependent signaling pathway of TLR regulates the production of TNF-α in murine BMDC and macrophages. BMDC derived from wild-type or Trif−/− mice were stimulated with LPS for various time periods, and the amounts of TNF-α protein secreted in the supernatants were measured. The results in Fig. 1A show that, throughout the observation period of 20 h, the release of TNF-α by Trif−/− BMDC was markedly reduced, compared with wild-type cells. To further delineate the mechanisms by which TRIF may promote TNF-α production, mRNA levels were quantified by real-time RT-PCR at various time points after LPS stimulation. In marked contrast to protein levels, the amounts of TNF-α transcripts in BMDC were not significantly altered by the absence of functional TRIF (Fig. 1B). Moreover, we found that the stability of TNF-α mRNA did not differ between LPS-stimulated Trif−/− and wild-type BMDC (Fig. 1C). To directly examine the transcriptional activity of the Tnfa gene, quantitative primary transcript RT-PCR analyses were performed. Consistent with the results for total TNF-α mRNA levels, we found that LPS-stimulated Trif−/− and wild-type BMDC generated comparable amounts of primary TNF-α transcripts (Fig. 1D).

These results suggested that the TRIF signaling pathway of TLR4 does not affect Tnfa transcription but instead may influence either translation of the TNF-α mRNA or release of TNF-α protein from BMDC or both. To address the question of whether Trif−/− BMDC may exhibit normal TNF-α protein synthesis, but may be impaired in their ability to secrete cell-bound protein, TNF-α levels were determined in cellular lysates of LPS-stimulated BMDC. The results in Fig. 1E show that the amounts of cell-bound TNF-α were also markedly lower in Trif−/− than in wild-type BMDC, indicating that de novo synthesis rather than secretion of TNF-α protein may be impaired in the absence of functional TRIF.

To further analyze the role of TRIF for TNF-α production, BMDC were stimulated with the TLR3 agonist poly(I:C). TLR3 signaling is mediated by TRIF and does not involve MyD88 (2, 4, 5). However, consistent with a previous report (12), BMDC did not release significant amounts of TNF-α in response to TLR3 ligation, thereby precluding further analysis of TLR3-induced TNF-α production by BMDC (data not shown).
BMDC by both IFN-β found that TRIF may mediate TLR4-induced gene expression in expression of IFN-regulated genes (9, 13). In a previous study, we production would be indirect and related to reduced IFN-α production. To address this possibility, we compared the production of IFN-β from wild-type or Trif<sup>lps2/lps2</sup> mice were stimulated with LPS for the indicated time periods, and the release of TNF-α protein in the supernatants (A) and the induction of TNF-α mRNA (B) were measured (n = 5–6). C. To determine TNF-α mRNA stability, BMDC were stimulated with LPS for 1 h and subsequently treated with 10 μg/ml actinomycin D. TNF-α mRNA levels were measured at various time points thereafter (n = 3). D. Transcriptional activity of the Trif gene was determined by quantitative primary transcript RT-PCR (n = 5). E. BMDC were stimulated with LPS for the indicated time periods, and cell-bound TNF-α protein levels were measured in cellular lysates (n = 5–6). *p < 0.05; **p < 0.01.

Cell type-related functions of TRIF for LPS-stimulated TNF-α production

Previous studies have shown that TRIF is required for the production of normal amounts of TNF-α protein by peritoneal macrophages (9, 13). However, specifically which molecular processes during TNF-α biosynthesis may be influenced by TRIF has not been investigated. To address this question, thioglycollate-elicited peritoneal macrophages were stimulated with LPS for various time periods and protein as well as mRNA levels of TNF-α were measured. As expected, the absence of functional TRIF resulted in a marked reduction of TNF-α protein release (Fig. 2A). In contrast to the results obtained with BMDC, however, Trif<sup>lps2/lps2</sup> peritoneal macrophages also exhibited significantly reduced levels of TNF-α mRNA when compared with wild-type cells (Fig. 2B).

Macrophages are well known to represent a heterogeneous lineage of hematopoietic cells (14). We therefore generated BMM to study the role of TRIF in TLR4-induced TNF-α production in different macrophage populations. The results depicted in Fig. 2C show that the release of TNF-α protein by Trif<sup>lps2/lps2</sup> BMM was substantially reduced, compared with wild-type BMM. In contrast, LPS stimulation induced comparable levels of TNF-α mRNA in both Trif<sup>lps2/lps2</sup> and wild-type BMM (Fig. 2D). In additional experiments, TNF-α production was analyzed in poly(I:C)-stimulated BMM. Consistent with previous findings showing that signaling of TLR3 is entirely dependent on TRIF (2, 4, 5), we found that poly(I:C)-induced production of both TNF-α protein and mRNA was abrogated by the lack of functional TRIF (Fig. 2E, 2F). These results reveal that, depending on the cell type, TRIF may control TLR-stimulated TNF-α production at distinct steps of biosynthesis.

Influence of TRIF on TNF-α production by BMDC is independent of type I IFN

Following engagement of TLR4, the TRIF signaling pathway stimulates the production of IFN-β, which, in turn, leads to the expression of IFN-regulated genes (9, 13). In a previous study, we found that TRIF may mediate TLR4-induced gene expression in BMDC by both IFN-β-dependent and –independent mechanisms (15). It was therefore conceivable that the effect of TRIF on TNF-α production would be indirect and related to reduced IFN-β induction. To address this possibility, we compared the production of TNF-α by LPS-stimulated BMDC derived from wild-type or Ifnar<sup>−/−</sup> mice. The results demonstrate that TNF-α production by BMDC was not affected by the lack of IFNAR1 (Fig. 3A), whereas the release of CCL2 was strongly impaired (Fig. 3B). These findings suggest that TRIF does not regulate TNF-α production through an indirect pathway mediated by IFN-β but rather through direct interference with TLR4 signaling.

FIGURE 2. Cell type-related functions of TRIF for regulating TNF-α production in macrophages. Thioglycollate-elicited peritoneal macrophages (A, B) and BMM (C–F) were derived from wild-type or Trif<sup>lps2/lps2</sup> mice. Cells were stimulated with LPS (A–D) or poly(I:C) (E, F). Peritoneal macrophages were stimulated for the indicated time periods. BMM were incubated with LPS or poly(I:C) for 6 h (TNF-α protein production) or 1 h (TNF-α mRNA induction). The release of TNF-α protein in the supernatants (A, C, E) or the induction of TNF-α mRNA (B, D, F) was measured (n = 3–4). *p < 0.05; **p < 0.01.
MAPK. In this case, inhibition of late p38 activity and the absence was almost completely abolished in wild-type BMDC. In contrast, the kinase activity of p38 was observed in lps2/lps2 BMDC at a late, but not for NF-κB or MAPKs. Cells were stimulated with LPS, and the release of TNF-α or CCL2 protein was measured in the supernatants (n = 6).

**p38 MAPK is activated by TRIF and controls translation of Tnfa mRNA**

To elucidate the role of TRIF for TLR4 signaling in dendritic cells, we examined the LPS-induced activation of NF-κB and MAPKs. Consistent with our data indicating that TRIF does not influence Tnfa transcription, we found that IkBα was phosphorylated and degraded to a similar extent and with comparable kinetics in wild-type and Trif−/− BMDC (Fig. 4A). In contrast, we consistently observed a reduced phosphorylation of the MAPKs p38, JNK, and Erk1/2 at late time points after LPS stimulation (Fig. 4B). At early time points after LPS stimulation, however, the phosphorylation levels of p38, JNK, and Erk1/2 did not differ between Trif−/− and wild-type BMDC (Fig. 4B), suggesting that the TRIF signaling pathway of TLR4 is important for prolonged activation of MAPKs in dendritic cells.

The p38 MAPK pathway has been implicated in the post-transcriptional regulation of numerous inflammatory genes (16). Therefore, the role of TRIF for the activation of p38 in BMDC following engagement of TLR4 was examined in more detail. The experiments depicted in Fig. 4C revealed that stimulation with LPS caused a strong and prolonged elevation of p38 kinase activity in wild-type BMDC. In contrast, the kinase activity of p38 was almost completely abolished in Trif−/− BMDC at a late, but not early, time point after LPS stimulation (Fig. 4C).

These results suggested that impaired p38 MAPK activation in Trif−/− BMDC may be related to the reduced activation of p38 MAPK. In this case, inhibition of late p38 activity and the absence of functional TRIF would be expected to cause similar alterations of TNF-α biosynthesis. To achieve inhibition of late p38 activity, the p38 inhibitor SB203580 was added to BMDC 5 min after initiation of LPS treatment. Control BMDC cultures received the SB203580 inhibitor 30 min prior to stimulation with LPS. As depicted in Fig. 5A, pharmacological blockade of early as well as late induction of p38 MAPK activity strongly impaired TNF-α protein production by BMDC. However, consistent with a role of the p38 pathway for the translational control of TNF-α production, neither treatment caused any significant reduction of LPS-stimulated TNF-α mRNA levels (Fig. 5B). Thus, delayed inhibition of p38 MAPK is sufficient to block TNF-α protein production without influencing mRNA induction.

**Translational control of TNF-α production by TRIF signaling is dependent on MK2**

MK2 is a serine/threonine kinase activated through direct phosphorylation by p38 MAPK and regulates TNF-α biosynthesis mainly at the level of mRNA translation (11, 17, 18). Because Trif−/− and MK2−/− cells exhibit similar defects in LPS-induced TNF-α translation, we next sought to examine whether TRIF may be required for the activation of MK2 in response to TLR4 engagement. To this end, Western blot analyses were performed with Abs against the Thr222 and Thr334 phosphorylation sites of MK2, both of which were found to be important for activation of the MK2 kinase activity (19). The results in Fig. 6A demonstrate that MK2 was readily phosphorylated on Thr222 and Thr334 after stimulation of wild-type BMDC with LPS. In marked contrast, phosphorylation of MK2 on both sites was almost completely abolished in Trif−/− BMDC at late and, to a lesser extent, at early time points after LPS stimulation (Fig. 6A). To directly demonstrate activation of MK2 through the TRIF pathway of TLR4, BMDC and BMM were generated from Myd88−/− mice and stimulated with LPS. In these cells, TLR4 signals are transduced exclusively through the adapter protein TRIF. We found that MK2 was readily phosphorylated both in Myd88−/− BMDC (Fig. 6B) and BMM (Fig. 6C) and that the extent of MK2 phosphorylation was similar in Myd88−/− and wild-type cells. Together, these data indicate that the TRIF signaling pathway of TLR4 mediates prolonged activation of MK2.

To directly address the role of MK2 in the translational control of TNF-α production through TRIF signaling, BMM derived from Mk2−/− mice were generated. We found that, in LPS-stimulated BMM, TNF-α protein, but not mRNA production, was markedly impaired by MK2 deficiency (Fig. 7A). Importantly, poly(I:C)-stimulated BMM from Mk2−/− mice also showed a substantial reduction of TNF-α protein release, whereas induction of TNF-α mRNA was not altered (Fig. 7B). Thus, these results...
demonstrate that MK2 is required for the normal production of TNF-α protein in response to TRIF signaling but is dispensable for TNF-α mRNA induction.

In addition to MK2, Mnk1 may be involved in the post-transcriptional regulation of TNF-α by inducing the phosphorylation of target proteins, such as eIF-4E and hnRNP A1 (20–24).

The results in Fig. 8A demonstrate that BMDC exhibited high constitutive levels of Mnk1 phosphorylation, which were elevated upon LPS stimulation. In addition, phosphorylation of eIF-4E was found to be induced by LPS treatment of BMDC (Fig. 8A). However, LPS increased the phosphorylation of both Mnk1 and eIF4E in Trif<sup>−/−</sup> mice and wild-type BMDC to a similar extent (Fig. 8A). To further investigate the role of Mnk proteins for TNF-α production in BMDC, the influence of the Mnk-specific inhibitor CGP57380 (24) on TNF-α biosynthesis in LPS-stimulated BMDC was examined. We found that CGP57380 dose dependently reduced both protein and mRNA levels of TNF-α to a similar extent (Fig. 8B). Thus, these findings suggest that the lack of functional

FIGURE 5. Inhibition of late p38 MAPK activity in LPS-stimulated BMDC impairs production of TNF-α protein, but not of mRNA. BMDC were generated from wild-type mice and stimulated with LPS for 16 h. Activation of p38 MAPK was blocked by incubation of cells with 20 μM SB203580. SB203580 was added to the cells either 30 min before or 5 min after administration of LPS, to achieve inhibition of p38 kinase activity at the onset or during the late phase of LPS stimulation, respectively. The release of TNF-α protein in the supernatants (A) and the induction of TNF-α mRNA (B) were measured (n = 3). *p < 0.01.

FIGURE 6. TRIF mediates activation of MK2 in LPS-stimulated BMDC and BMM. BMDC (A, B) or BMM (C) were generated from Trif<sup>−/−</sup> mice and stimulated with LPS for the indicated time periods. Phosphorylation of MK2 at position Thr222 or Thr334 was determined. α-Tubulin was used as protein loading control. The blots depicted are representative of at least three independent experiments yielding comparable results.

FIGURE 7. MK2 is required for production of TRIF-mediated TNF-α protein, but not of mRNA. BMM were generated from wild-type or Mk2<sup>−/−</sup> mice and stimulated with LPS (A) or poly(I:C) (B). Production of TNF-α protein (left panels) or mRNA (right panels) was determined (n = 5–6). *p < 0.01; n.s., not significant (p = 0.309).

FIGURE 8. TRIF is dispensable for LPS-induced phosphorylation of Mnk1 and eIF-4E. A. BMDC were generated from wild-type or Trif<sup>−/−</sup> mice and stimulated with LPS for the indicated time periods. Phosphorylation of Mnk1 and eIF-4E was determined. α-Tubulin was used as protein loading control. The blots depicted are representative of at least three independent experiments yielding comparable results. B. Wild-type BMDC were stimulated with LPS for 16 h in the presence of the indicated concentrations of the Mnk inhibitor CGP57380. The release of TNF-α protein in the supernatants and the induction of TNF-α mRNA were measured. Results of protein and mRNA determinations are normalized relative to the respective values of LPS-stimulated BMDC in the absence of inhibitor (n = 5–6). **p < 0.01.
TRIF does not influence the activation of Mnk1 and eIF-4E in LPS-stimulated BMDC.

Discussion

The signaling pathways emanating from TLR4 use the adapter proteins MyD88 and TRIF for regulating gene expression (5, 25). The TRIF pathway was shown to be essential for the phosphorylation of IRF-3, leading to the transcriptional activation of the *Ifnb* gene (9, 13). TRIF-dependent signaling of TLR4 also contributes to the production of TNF-α, but the underlying mechanisms are largely unknown. In this paper, we show that LPS-stimulated production of TNF-α protein required functional TRIF, whereas neither transcriptional activity of the *Tnfa* gene nor induction or stability of TNF-α mRNA was altered in *Trif*-defective cells. In addition, the lack of functional TRIF did not cause retention of TNF-α protein in LPS-exposed cells. The activation of NF-κB, which is crucial for transcriptional activation of the *Tnfa* gene, was also not reduced in *Trif* ^ΔΔ/ΔΔ^ cells. Together, these results indicate that, in dendritic cells, the TRIF-dependent signaling pathway of TLR4 selectively contributes to the post-transcriptional control of TNF-α biosynthesis by promoting mRNA translation. A model for the contribution of the TRIF signaling pathway of TLR4 and TLR3 to TNF-α biosynthesis is shown in Fig. 9.

Posttranscriptional mechanisms are considered important for regulating the expression of inflammation-promoting genes. Whereas posttranscriptional regulation of COX-2 and IL-6 occurs mostly at the level of mRNA stability, translational activation is an important mechanism accounting for the rapid induction of TNF-α protein production after LPS administration (17, 26, 27). AU-rich elements in the 3′-untranslated region of TNF-α transcripts are reported to mediate translational control of TNF-α biosynthesis (17, 28, 29). When administered simultaneously with LPS, IL-10 targets the 3′ AU-rich elements of the TNF-α mRNA to inhibit its translation by a mechanism that involves blockade of the p38 and MK2 protein kinases (18). Moreover, MK2-deficient macrophages show reduced production of TNF-α protein, but not mRNA, following LPS challenge, indicating that the p38/MK2 kinase cascade is essential for activation of TNF-α translation by TRIF (11). In the present report, we show that late activation of p38 and MK2 kinases by LPS was severely reduced in *Trif*-defective dendritic cells. In *Myd88^−/−^* BMDC and BMM, LPS-stimulated MK2 phosphorylation was normal, directly demonstrating activation of MK2 through the TRIF pathway. We also show that pharmacological inhibition of late p38 activity and genetic inactivation of TRIF have similar effects on TNF-α biosynthesis, with production of TNF-α protein, but not mRNA, being substantially reduced. Finally, when MK2-deficient BMM were treated with LPS or poly(I:C), the production of TNF-α protein was severely impaired, whereas induction of TNF-α mRNA was not significantly affected. Considered together, these results indicate that TRIF-dependent signaling promotes TNF-α mRNA translation by mediating activation of the MK2 kinase.

Previous studies have shown that dexamethasone impairs induction of JNK activity, but not p38 or Erk1/2 activity, in LPS-stimulated macrophages (30). Dexamethasone also inhibited translation of the TNF-α mRNA, and this effect was reversed by overexpression of JNK. Moreover, a kinase-defective mutant of JNK blocked LPS-induced translation of TNF-α, suggesting that, in addition to p38, JNK may be involved in the induction of TNF-α translation. Subsequent studies using macrophages from genetically modified mice have shown that translational repression by dexamethasone requires the presence of the 3′ AU-rich element in the TNF-α mRNA (29). In the present report, we show that prolonged activation of JNK by LPS is impaired in dendritic cells lacking functional TRIF. It is therefore conceivable that the TRIF signaling pathway of TLR4 may also contribute to the translational induction of TNF-α expression by promoting prolonged activation of JNK.

The protein kinases Mnk1 and Mnk2 are phosphorylated and activated by the p38 and Erk1/2 MAPKs (20, 31). Mnk proteins have been shown to phosphorylate substrate proteins, such as eIF-4E and hnRNP A1, and thereby promote posttranscriptional mechanisms of gene expression (20–22, 24). Notably, pharmacological inhibition of Mnk proteins and knockdown of Mnk1 were reported to impair TNF-α production by Jurkat T lymphoid cells (24). Mnk2 mainly contributes to the basal phosphorylation of eIF-4E, whereas Mnk1 mediates the inducible phosphorylation of eIF-4E in response to various stimuli, including LPS (23). Because late phosphorylation of p38 and Erk1/2 MAPKs was reduced in LPS-stimulated *Trif* ^ΔΔ/ΔΔ^ BMDC, we addressed the question of whether TRIF-dependent signaling may influence TNF-α mRNA translation by activating the Mnk1 and its substrate eIF-4E. However, the LPS-induced phosphorylation of Mnk1 and eIF-4E was not altered by the lack of functional TRIF in BMDC. Furthermore, inhibition of Mnk proteins impaired the induction of TNF-α protein and mRNA to a similar extent. It therefore appears that TRIF-dependent signaling of TLR4 is not required for the activation of Mnk1 and eIF-4E.

TRIF is known to be required for TNF-α protein production in peritoneal macrophages upon stimulation of TLR3 and TLR4 (9, 13). However, the influence of TRIF signaling on TNF-α mRNA induction was not investigated in these studies. In the present report, we show that, in contrast to BMDC and BMM, in peritoneal macrophages, the lack of functional TRIF is associated with impaired induction of both TNF-α protein and mRNA. It therefore appears that TRIF signaling contributes to transcriptional regulation in peritoneal macrophages, but not in BMDC or BMM. Moreover, we found severely reduced activation of p38 kinase activity and impaired phosphorylation of the MAPKs p38, Erk-1/2, and JNK at late time points after LPS stimulation of BMDC. In *Trif*-defective peritoneal macrophages, however, there was an early decline of Erk-1/2 phosphorylation but essentially normal...
activation of p38 and JNK (9). Similarly, LPS-stimulated phos- phorylation of JNK was not altered in Trif-deficient embryonic fibroblasts (13). Of note, previous studies have provided additional evidence that the function of TRIF signaling for regulating gene expression may vary depending on the cell type investigated. For example, macrophage subpopulations that respond to LPS in either a TRIF-dependent or TRIF-independent manner may be distinguished (9). In addition, the adapter protein TRADD was found to be required for TRIF-dependent TLR signaling in embryonic fibroblasts and dendritic cells, but not macrophages (7, 8). Considered together, the results of the present and previous reports therefore strongly suggest that TRIF regulates expression of im- mune response genes in a cell type-dependent manner.

Deregulated production of TNF-α is thought to be a common mechanism involved in the pathogenesis of various inflammatory disorders. In clinical trials, antagonists of TNF-α are being found to be required for TRIF-dependent TLR signaling in embryonic fibroblasts and dendritic cells, but not macrophages (7, 8). Considered together, the results of the present and previous reports therefore strongly suggest that TRIF regulates expression of immune response genes in a cell type-dependent manner.

References