TPL-2 negatively regulates interferon-β production in macrophages and myeloid dendritic cells

Frank Kaiser,[1] Dorthe Cook,[1,2] Stamatia Papoutsopoulou,[2] Ricardo Rajsbaum,[1] Xuemei Wu,[1] Huei-Ting Yang,[2] Susan Grant,[2] Paola Ricciardi-Castagnoli,[3] Philip N. Tsichlis,[4] Steven C. Ley,[2] and Anne O'Garra[1]

[1]Division of Immunoregulation and [2]Division of Immune Cell Biology, Medical Research Council National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
[3]Singapore Immunology Network, Biopolis, SINGAPORE 138648
[4]Molecular Oncology Research Institute, Tufts-New England Medical Center, Boston, MA 02111

Stimulation of Toll-like receptors (TLRs) on macrophages and dendritic cells (DCs) by pathogen-derived products induces the production of cytokines, which play an important role in immune responses. Here, we investigated the role of the TPL-2 signaling pathway in TLR induction of interferon-β (IFN-β) and interleukin-10 (IL-10) in these cell types. It has previously been suggested that IFN-β and IL-10 are coordinately regulated after TLR stimulation. However, in the absence of TPL-2 signaling, lipopolysaccharide (TLR4) and CpG (TLR9) stimulation resulted in increased production of IFN-β while decreasing IL-10 production by both macrophages and myeloid DCs. In contrast, CpG induction of both IFN-α and IFN-β by plasmacytoid DCs was decreased in the absence of TPL-2, although extracellular signal-regulated kinase (ERK) activation was blocked. Extracellular signal-related kinase–dependent negative regulation of IFN-β in macrophages was IL-10–independent, required protein synthesis, and was recapitulated in TPL-2–deficient myeloid DCs by retroviral transduction of the ERK-dependent transcription factor *c-fos.*

CORRESPONDENCE
Anne O'Garra:
aogarra@nimr.mrc.ac.uk
OR
Steven C. Ley:
sley@nimr.mrc.ac.uk

Abbreviations used: BMDC, BM-derived DC; BMDM, BM-derived macrophage; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IRF, IFN regulatory factor; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKKK, MAP-3 kinase; pDC, plasmacytoid DC; TIR, Toll/IL-1R; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain–containing adaptor inducing IFN-β.

Toll-like receptors (TLRs) on macrophages and DCs respond to pathogen-derived products to induce the production of effector molecules that regulate innate and adaptive immune responses (Medzhitov, 2001; Akira and Takeda, 2004; Beutler et al., 2004). These include the type I IFNs, IFN-α and IFN-β, and the proinflammatory cytokine IL-12, which are important for protective responses to infection (O'Garra and Robinson, 2004; Stetson and Medzhitov, 2006), and IL-10, which suppresses cytokine production by macrophages and DCs, thereby limiting the immune response to infectious agents (Moore et al., 2001).

The cytoplasmic regions of TLRs share the Toll/IL-1R (TIR) domain, leading to associations between TLRs and the TIR-containing adaptors, MyD88, TIRAP (MAL), TIR domain–containing adaptor inducing IFN-β (TRIF; TICAM-1), and TRIF-related adaptor molecule (Akira and Takeda, 2004), upon ligand binding. TLR4, the receptor for LPS, utilizes all four adaptors, whereas TLR9, the receptor for CpG-DNA motifs, uses only MyD88. Differential responses mediated by distinct TLR ligands may be explained in part by selective usage of TIR adaptor molecules (Boonstra et al., 2006; Stetson and Medzhitov, 2006; Colonna, 2007; Schmitz et al., 2007; Rajsbaum et al., 2008).

TLR stimulation induces activation of the major mitogen-activated protein kinase (MAPK) subtypes (extracellular signal-related kinases [ERK]-1/2, Jun N-terminal kinases [JNK], and p38) and the IκB kinase (IKK) complex for activation of NF-κB transcription factors (Akira and Takeda, 2004). TNF receptor-associated factor (TRAF) 6 is required for MyD88-dependent

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activation of MAPKs and NF-κB (Gohda et al., 2004). TRIF-dependent signaling utilizes TRAF3 (Häcker et al., 2006), which couples TRIF and MyD88, to activate TBK1 (Häcker et al., 2006; Oganesyan et al., 2006), a kinase that regulates the activation of IFN regulatory factor (IRF) 3 and IRF7 (Honda et al., 2006). It has been proposed that the TRAF6-dependent pathway, via its activation of MAPKs and IKK, participates in the induction of proinflammatory cytokines, whereas the TRAF3-dependent pathway coordinately regulates the production of type I IFNs and IL-10 (Häcker et al., 2006). Thus, the ratio of TRAF6 versus TRAF3 recruitment to TLRs may dictate the relative production of proinflammatory cytokines, such as IL-12, versus type I IFNs and IL-10. However, the observation that LPS induction of IFN-β in macrophages and DCs is dependent on TRIF, but not MyD88 (Akira and Takeda, 2004; Boonstra et al., 2006), whereas induction of IL-10 requires both adaptors (Boonstra et al., 2006), indicates that IFN-β and IL-10 may not always be coordinately regulated. Furthermore, the signals regulating the induction of type I IFN differ according to the activating TLR and the cell type being stimulated (Colonna, 2007; Schmitz et al., 2007). Thus, TLR4-mediated induction of IFN-β in macrophages and myeloid DCs involves activation of IRF3 (for review see Akira and Takeda, 2004; Stetson and Medzhitov, 2006; Colonna, 2007), whereas TLR9 stimulation of IFN-β in these cells is mediated via IRF1 (Schmitz et al., 2007). However, TLR9 induction of type I IFN in plasmacytoid DCs (pDCs) involves activation of IRF7 (for review see Akira and Takeda, 2004; Stetson and Medzhitov, 2006; Colonna, 2007).

The signaling pathways coupling TLRs to the activation of MAPKs diverge downstream of TRAF6 and involve distinct MAPKKKs (MAP 3-kinases; Symons et al., 2006). In macrophages, LPS activation of ERK, but not JNK or p38 MAPK, is mediated by the MAP 3-kinase TPL-2 (Dumitru et al., 2000), which phosphorylates and activates the ERK-specific MAPKKs MEK-1/2 (Salmeron et al., 1996). A role for ERK activation in the induction of IL-10 and down-regulation of IL-12 production has previously been reported (Yi et al., 2002; Agrawal et al., 2003; Dillon et al., 2004; Banerjee et al., 2006). However, it is still unclear whether the inhibition of IL-12 is a direct effect of TLR ligand-induced ERK activation or results as a consequence of IL-10 induction by this signaling pathway (Yi et al., 2002; Agrawal et al., 2003; Dillon et al., 2004).

In this study, we investigated the role of the TPL-2 signaling pathway in the induction of IFN-β and IL-10 in macrophages, myeloid DCs, and pDCs. We demonstrate that TLR4 and TLR9 activation of ERK was dependent on TPL-2 in each of these cell types. Furthermore, we show that TLR induction of IL-10 was decreased in *Tpl-2*$^{-/-}$ macrophages and myeloid DCs compared with WT control cells; pDCs do not produce this cytokine upon TLR stimulation. Levels of IFN-β and IL-12 were substantially increased in *Tpl-2*$^{-/-}$ macrophages and myeloid DCs stimulated with LPS or CpG, as compared with controls. In contrast, production of IFN-α and IFN-β was significantly reduced in *Tpl-2*$^{-/-}$ pDCs stimulated with CpG, as compared with controls, demonstrating that TPL-2 signaling has different consequences depending on the cell type activated. Negative regulation of IFN-β in macrophages and myeloid DCs required protein synthesis and could be restored in *Tpl-2*$^{-/-}$ or *IL-10*$^{-/-}$ myeloid DCs by retroviral expression of the ERK-dependent transcription factor *c-fos*.

RESULTS AND DISCUSSION

IFN-β is up-regulated and IL-10 is down-regulated in *Tpl-2*$^{-/-}$ macrophages and myeloid DCs stimulated with LPS

To determine the role of the TPL-2 signaling pathway in TLR regulation of IL-10 and IFN-β production, BM-derived macrophages (BMDMs) and BM-derived DCs (BMDCs/myeloid DCs) were generated from *Tpl-2*$^{-/-}$ or control WT mice. Induction of *IFN-β* mRNA and IFN-β protein in response to LPS stimulation were significantly increased in *Tpl-2*$^{-/-}$ BMDMs compared with control cells (Fig. 1 a). In contrast, *IL-10* mRNA and IL-10 protein were significantly decreased in the absence of TPL-2. Increased production of *IL-12p40* and *p35* mRNA, and in IL-12p70 and p40 protein, was also observed in *Tpl-2*$^{-/-}$ BMDMs (Fig. 1 a).

Similarly, BMDCs from *Tpl-2*$^{-/-}$ mice produced elevated levels (mRNA and protein) of IFN-β, but reduced amounts of IL-10 (Fig. 1 b). However, the increase in IFN-β production in *Tpl-2*$^{-/-}$ relative to WT cells was less in BMDCs than in BMDMs. The elevated amounts of IFN-β produced by *Tpl-2*$^{-/-}$ BMDMs and BMDCs may result in part from decreased production of IL-10 because IL-10 inhibits IFN-β production (Moore et al., 2001). This may explain why IFN-β production is less affected by TPL-2 deficiency in BMDCs compared with BMDMs because the BMDCs produce lower concentrations of IL-10. Consistent with the results using *Tpl-2*$^{-/-}$ BMDMs, *Tpl-2*$^{-/-}$ BMDCs produced elevated levels of IL-12 p40 and p70 protein and *IL-12 p35* and *p40* mRNA (Fig. 1 b) compared with control BMDCs.

LPS induction of ERK phosphorylation in macrophages and myeloid DCs is TPL-2 dependent

To determine whether impaired LPS-induced ERK activation accounted for the altered cytokine production by *Tpl-2*$^{-/-}$ BMDMs, activation of ERK was monitored by specific immunoblotting. In WT cells, LPS-stimulated rapid ERK phosphorylation, which peaked at 15 min (Fig. 1 a). In contrast, LPS-induced ERK phosphorylation was not detected in *Tpl-2*$^{-/-}$ BMDMs (Fig. 1 a), whereas JNK and p38 phosphorylation were unaffected (Fig. 1 a), as reported previously (Dumitru et al., 2000). To confirm that the effects of TPL-2 deficiency on TLR-induced cytokine production resulted from abrogation of ERK activation, WT cells were treated with the MEK inhibitor U0126 to block ERK activation while leaving p38 or JNK activation unaffected (Favata et al., 1998; Davies et al., 2000). U0126 increased IFN-β and IL-12 production and reduced production of IL-10 by LPS-stimulated BMDMs (Fig. S1), similar to the effects of TPL-2 deficiency in this cell type (Fig. 1 a). Comparable results were obtained with a structurally distinct inhibitor of MEK-1/2, PD184352

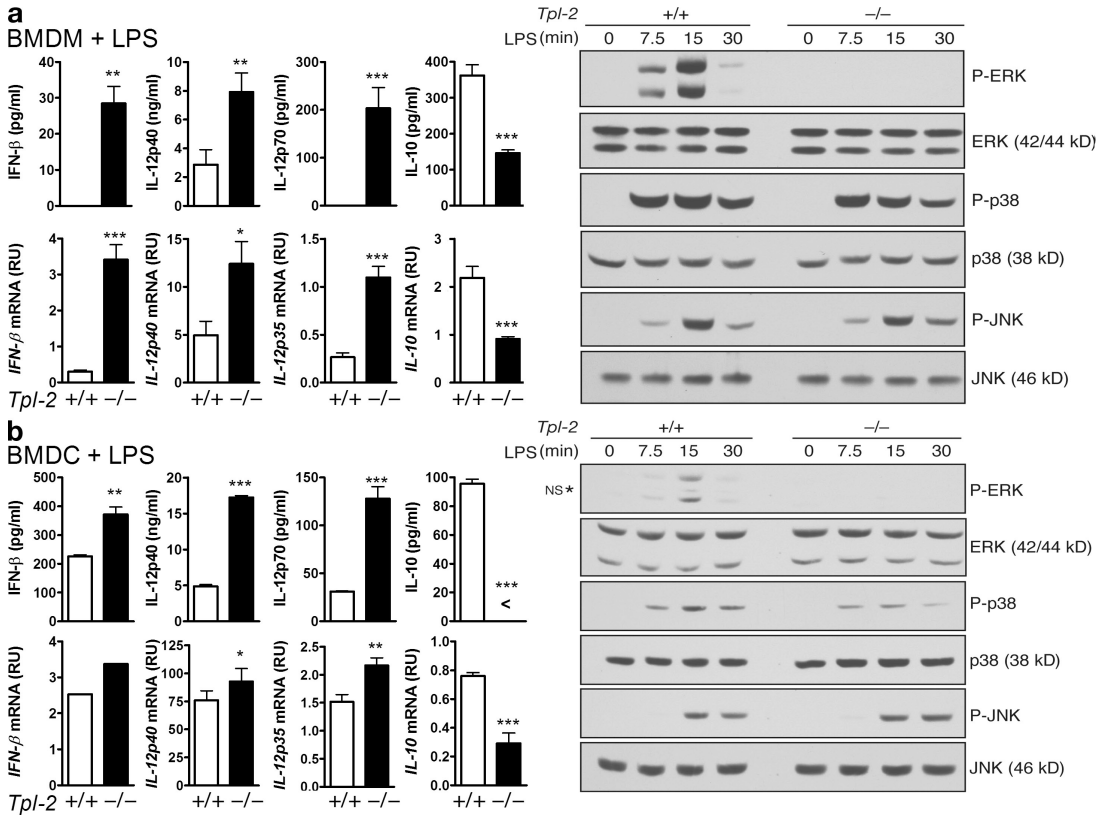

Figure 1. TPL-2 activation in BMDMs and BMDCs after LPS stimulation negatively regulates IFN-β and IL-12, but is required for optimal IL-10 production. BMDMs (a) and BMDCs (b) generated from wild-type (+/+) and *Tpl-2*$^{-/-}$ (−/−) mice were stimulated with 100 ng/ml LPS. Cytokine levels in culture supernatants were determined by ELISA after 24 h stimulation. Total RNA was extracted from cells stimulated for 3 h, and cytokine mRNA expression was measured by quantitative PCR (normalized to ubiquitin mRNA). Graphs show mean of individual cell cultures ± SD (protein, $n = 4$; mRNA, $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test. Data are representative of at least three independent experiments with similar results. Whole-cell extracts of BMDMs (a, right) and BMDCs (b, right) were generated after indicated stimulation times and analyzed by immunoblotting with the antibodies shown. Results are representative of at least four independent experiments.

(a gift from P. Cohen, University of Dundee, Scotland, UK; unpublished data). These data support the hypothesis that TPL-2 promotes IL-10 production while decreasing IL-12 and IFN-β production via its activation of the ERK MAPK pathway.

We show that activation of ERK by TLR4 in macrophages required TPL-2, in agreement with previous findings (Dumitru et al., 2000; Banerjee et al., 2006; Papoutsopoulou et al., 2006). Our data also suggest that activation of the TPL-2 signaling pathway was required for optimal IL-10 production, but negatively regulated IL-12 production. These findings are in accordance with published data using pharmacological inhibitors to block ERK activation in macrophages (Feng et al., 1999; Häcker et al., 1999; Yi et al., 2002; Banerjee et al., 2006). Moreover, our data suggest that TLR4 activation of the TPL-2 pathway negatively regulates the production of IFN-β, in contrast to its positive effects on IL-10 production.

Although the consequences of TPL-2 deficiency on cytokine production after TLR stimulation were very similar in BMDMs and BMDCs (Fig. 1), it has been suggested previously that LPS does not stimulate ERK activation in BMDCs (Häcker et al., 1999). Hence, we reinvestigated whether TLR4 was actually able to induce ERK phosphorylation in BMDCs. In our hands, LPS clearly stimulated ERK phosphorylation in WT BMDCs (Fig. 1 b), although at a reduced intensity compared with WT BMDMs (Fig.1 a). This may explain the previous failure to detect ERK phosphorylation in BMDCs after LPS stimulation (Häcker et al., 1999). We show that LPS-induction of ERK phosphorylation was blocked in BMDCs from *Tpl-2*$^{-/-}$ mice (Fig. 1 b), similarly to BMDMs. p38 MAPK phosphorylation was marginally reduced in *Tpl-2*$^{-/-}$ BMDCs compared with WT cells after LPS stimulation, but JNK phosphorylation was unaffected. Thus, altered cytokine production in *Tpl-2*$^{-/-}$ BMDCs after TLR stimulation was associated predominantly with a block in ERK activation.

Our data that TPL-2 negatively regulates IL-12 in BMDCs are consistent with reports from the Pulendran group that pharmacological inhibition of ERK activation increases IL-12 production by human and mouse DCs (Agrawal et al., 2003; Dillon et al., 2004). The negative regulatory effect of TPL-2 on IFN-β production that we observe in BMDCs stimulated with LPS contrasts to the requirement of TPL-2 for optimal IL-10 induction (Fig. 1 b). Thus, TLR-induction of IL-10 and IFN-β is oppositely regulated by TPL-2 in both macrophages and myeloid DCs.

CpG stimulation results in increased IFN-β production in *Tpl-2*$^{-/-}$ macrophages and myeloid DCs, but decreased IFN-α and IFN-β in *Tpl-2*$^{-/-}$ pDCs

Because pDCs are a major source of type I IFNs during immune responses (Stetson and Medzhitov, 2006), we determined the role of the TPL-2 signaling pathway in TLR-induction of IFN-α and IFN-β in this cell type. As pDCs express TLR9, but not TLR4 (Boonstra et al., 2003), we stimulated pDCs with CpG rather than LPS in comparison to macrophages and myeloid DCs. BMDMs and BMDCs obtained from *Tpl-2*$^{-/-}$ mice produced elevated levels of IFN-β protein, IL-12p40 and IL-12p70 upon CpG stimulation, as compared with the same cell types from WT mice (Fig. 2, a and b). Ex vivo *Tpl-2*$^{-/-}$ splenic CD11b^{+} macrophages purified directly from *Tpl-2*$^{-/-}$ mice also showed highly elevated levels of IFN-β and IL-12p40 and p70 as compared with equivalent cells from control mice after CpG stimulation (Fig. S2). Furthermore, CpG-induced IL-10 production was significantly reduced in *Tpl-2*$^{-/-}$ BMDMs (Fig. 2 a) and BMDCs (Fig. 2 b), similar to results observed after LPS stimulation. IL-10 production by purified splenic macrophages was also reduced compared with control WT cells after CpG stimulation (Fig. S2).

Opposite effects of TPL-2 deficiency were observed after stimulation of pDCs with CpG. Production of both IFN-α and IFN-β in *Tpl-2*$^{-/-}$ BM-pDCs were significantly reduced, as compared with pDCs from WT mice (Fig. 2 c). Furthermore, again in contrast to *Tpl-2*$^{-/-}$ BMDMs and BMDCs, a small but significant decrease in IL-12p40 and IL-12p70 was observed in *Tpl-2*$^{-/-}$ pDCs stimulated with CpG, as compared with controls (Fig. 2 c). Similar effects were obtained with directly purified ex vivo splenic pDCs (unpublished data). Consistent with the hypothesis that pDCs are wired differently to macrophages and myeloid DCs, pDCs did not produce IL-10 in response to CpG stimulation (unpublished data). Conversely, whereas pDCs produced high levels of IFN-α after stimulation with CpG, IFN-α was not detectable in supernatants of BMDMs and BMDCs stimulated similarly, even in the absence of TPL-2 (unpublished data).

Our findings reveal cell-intrinsic differences in the requirements for TPL-2 in TLR-induced signaling in pDCs versus macrophages and myeloid DCs. Whereas TPL-2 negatively regulated the induction of optimal IFN-β and IL-12 in macrophages and myeloid DCs after TLR-9 stimulation, TPL-2 was required for TLR9 induction of both IFN-α and IFN-β by pDCs. Furthermore, these data extend previous studies showing that the signaling requirements for induction of type I IFNs in pDCs are distinct to macrophages and myeloid DCs (Colonna, 2007; Schmitz et al., 2007).

TPL-2 is required for CpG-induced ERK phosphorylation in macrophages, myeloid DCs, and pDCs

Because CpG stimulation of *Tpl-2*$^{-/-}$ pDCs resulted in an opposite effect on type I IFN induction than that observed in macrophages and myeloid DCs, it was important to determine whether ERK was phosphorylated in pDCs in response to CpG in a TPL-2–dependent fashion. It was also important to determine whether TLR9 induction of ERK phosphorylation in BMDMs and BMDCs required TPL-2, as it has been recently proposed that TLR9 stimulation does not induce ERK phosphorylation in this cell type (Häcker et al., 1999) and that CpG activation of ERK is not affected by TPL-2 deficiency in macrophages (Sugimoto et al., 2004).

We show here that CpG induced a more gradual increase in ERK phosphorylation in WT BMDMs (Fig. 3 a) than LPS did (Fig. 1 a), and that this was abrogated in BMDMs from *Tpl-2*$^{-/-}$ mice (Fig. 3 a). CpG clearly stimulated ERK phosphorylation in WT BMDCs (Fig. 3 b), although at a reduced intensity compared with WT BMDMs, possibly explaining data from Sugimoto et al. (2004), who failed to detect any reduction in ERK phosphorylation after CpG stimulation of *Tpl-2*$^{-/-}$ macrophages. The

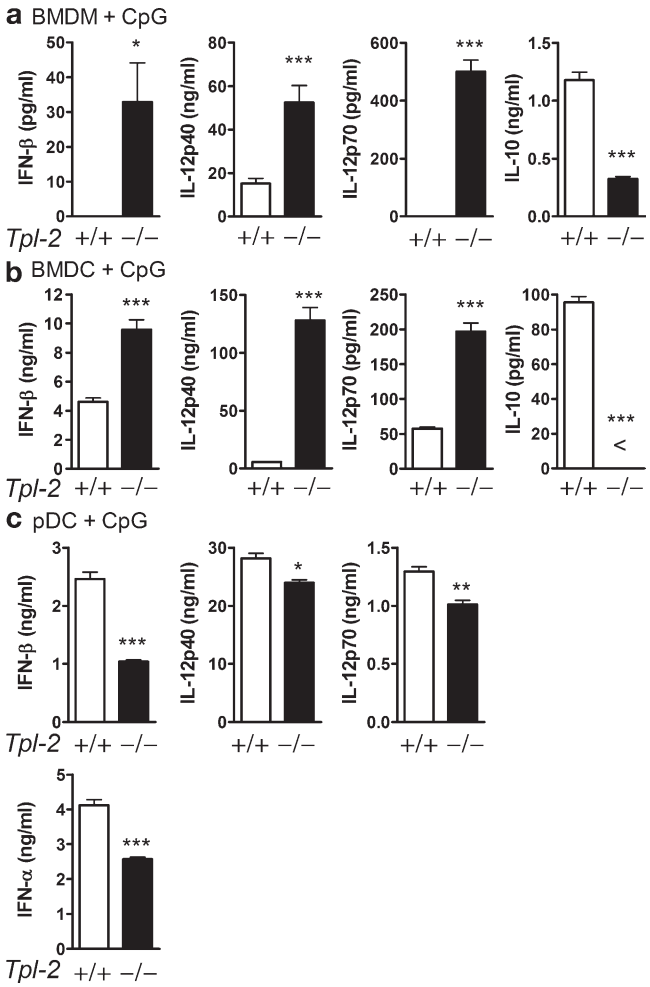

Figure 2. Upon stimulation with CpG, TPL-2 negatively regulates IFN-β and IL-12 in macrophages and conventional DCs, but not in pDCs. BMDMs, BMDCs, and pDCs generated from WT and *Tpl-2*$^{-/-}$ mice were stimulated with CpG (500 nM) for 24 h. Cytokine production in culture supernatants was determined by ELISA (mean ± SD; $n = 3$).
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test. Results are representative of at least three independent experiments.

reduced signal may also explain the previous failure to detect CpG stimulation of ERK phosphorylation in BMDCs (Häcker et al., 1999). CpG-induced ERK phosphorylation in BMDCs was blocked in the absence of TPL-2 (Fig. 3 b). Using a high sensitivity substrate for chemiluminescent detection of phospho-ERK antibody, CpG was also found to induce phosphorylation of ERK in pDCs, which was dependent on TPL-2. CpG-induced p38 and JNK phosphorylation in pDCs were minimally affected by the absence of TPL-2 (Fig. 3 c).

Our data show that ERK activation after TLR9 ligation required TPL-2 in macrophages, myeloid DCs, and pDCs, although the effects of TPL-2 deficiency on cytokine induction were distinct in pDCs compared with the other two cell types. The very low levels of ERK phosphorylation induced by CpG stimulation of pDCs compared with BMDMs and BMDCs may explain this differential requirement for TPL-2 in cytokine induction in these different cell types; it has been established in PC12 cells and fibroblasts that the amplitude and duration of ERK signaling can dictate the biological effect of this signaling pathway (Marshall, 1995; Murphy et al., 2002). Alternatively, it is possible that ERK signaling is interpreted differently in pDCs compared with macrophages and myeloid DCs as a consequence of integration of ERK activation with other signaling pathways triggered by TLR9. In accordance with the latter possibility, pDCs have been shown to use unique signaling pathways for the induction of type I IFNs (Honda et al., 2006; Stetson and Medzhitov, 2006; Colonna, 2007; Schmitz et al., 2007; Cao et al., 2008; Guiducci et al., 2008). Consistent with cell type–specific differences for ERK in regulating IFN-β production, a previous study has shown that ERK positively regulates IFN-β production by mouse embryonic fibroblasts in response to myxoma virus infection (Wang et al., 2004), similar to CpG induction of IFN-α and IFN-β in pDCs, but opposite to its negative effects on IFN-β production in macrophages and myeloid DCs (Fig. 2).

Negative regulation of IFN-β by TPL-2 in macrophages requires protein synthesis

To determine the requirement for protein synthesis in the negative regulation of *IFN-β* mRNA induction by TPL-2, *Tpl-2*$^{-/-}$ and WT control BMDMs were stimulated with CpG in the presence or absence of cycloheximide (CHX). Induction of *IFN-β* mRNA was increased when WT BMDMs were stimulated in the presence of CHX compared with untreated control, indicating that *IFN-β* expression was negatively regulated by a protein synthesis–dependent mechanism (Fig. 4 a). The elevated expression of *IFN-β* mRNA observed in the *Tpl-2*$^{-/-}$ BMDMs, however, was not affected by CHX, demonstrating that the negative regulation of IFN-β production by protein synthesis is TPL-2 dependent. Similar results were obtained for IL-12p40 expression (Fig. 4 a); however, CHX increased IL-12p40 mRNA not only in WT BMDMs but also, to a lesser extent, in *Tpl-2*$^{-/-}$ BMDMs, suggesting TPL-2–dependent and –independent mechanisms for IL-12p40 mRNA regulation. Similar data were obtained with BMDMs stimulated with LPS (unpublished data). This protein synthesis requirement for negative regulation of IFN-β mRNA expression could indicate the action of a TPL-2–dependent cytokine or transcription factor that negatively regulates IFN-β transcription.

ERK signaling negatively regulates IFN-β and IL-12 production independent of IL-10

Because IL-10 has profound inhibitory effects on cytokine production by TLR-stimulated macrophages (Moore et al., 2001), it was possible that the increased levels of IFN-β and IL-12 in *Tpl-2*$^{-/-}$ BMDMs resulted from diminished IL-10 production. However, we show that stimulation of *IL-10*$^{-/-}$ BMDMs with LPS (not depicted) or CpG (Fig. 4 b) in the presence of U0126 inhibitor resulted in elevated levels of IFN-β and IL-12, as compared with controls. In addition, in the absence of U0126, the production of IFN-β and IL-12 was increased in *IL-10*$^{-/-}$ BMDMs compared with WT cells.

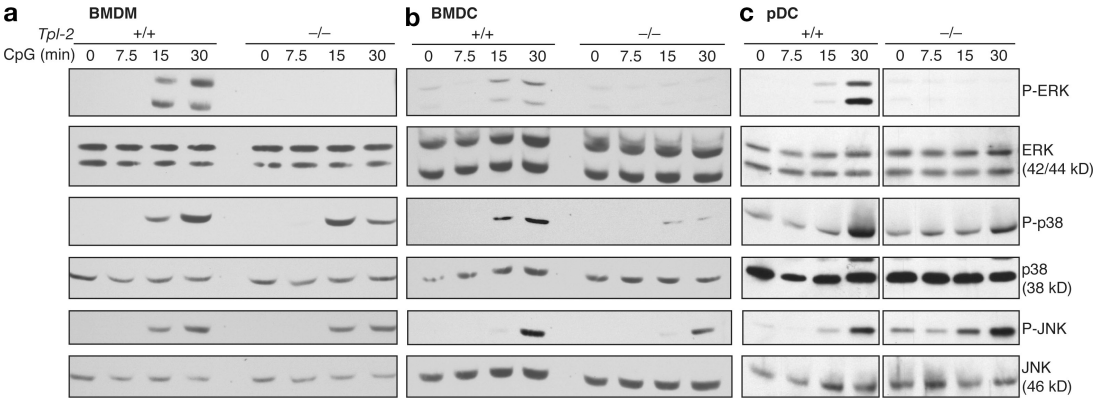

Figure 3. TPL-2 is essential for TLR-induced ERK signaling in pDCs. Macrophages (a), myeloid DCs (b), and pDCs (c) were generated from *WT* and *Tpl-2*$^{-/-}$ mice and stimulated with 500 nM CpG for times indicated. Whole-cell extracts were analyzed by immunoblotting with the indicated antibodies. Luminescence signals in pDCs blots were visualized using a high sensitivity substrate. Results are representative of three independent experiments with similar results.

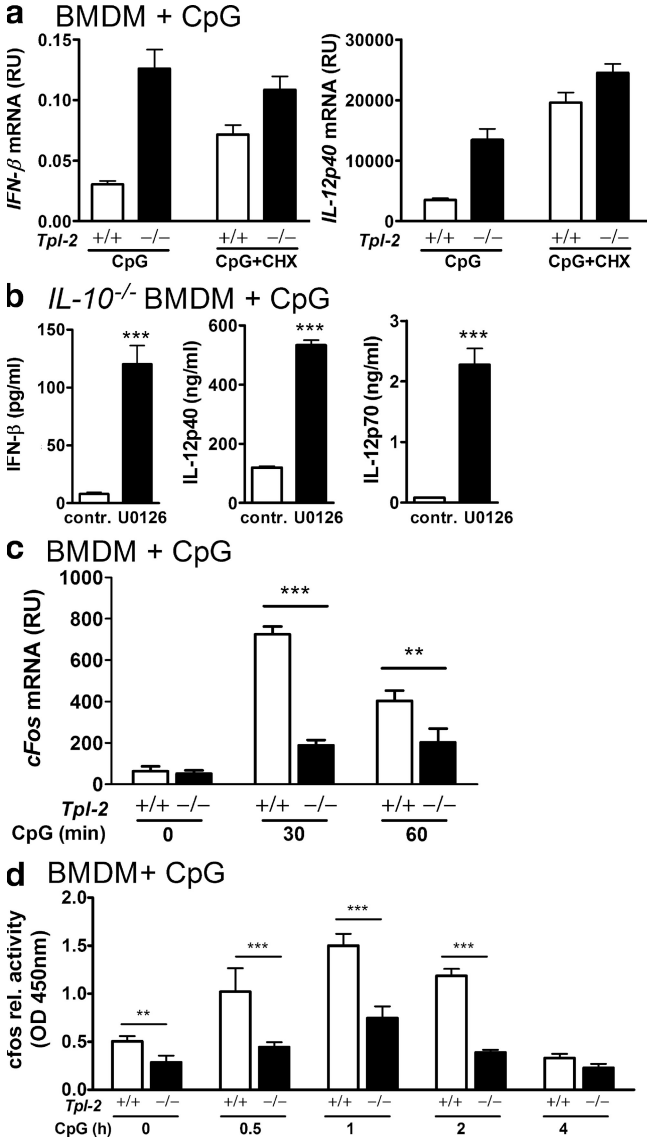

Figure 4. TPL-2 negatively regulates TLR-induced *IFN-β* mRNA expression via a de novo protein synthesis–dependent mechanism and independent of IL-10. (a) Before stimulation with 500 nM CpG for 3 h, BMDMs generated from WT and *Tpl-2*$^{-/-}$ mice were incubated with cycloheximide or left untreated. Total RNA was extracted and the indicated cytokine mRNA expression was measured by quantitative PCR (normalized to *HPRT* mRNA). The graph shows mean ± SD from two individual mice assayed in triplicate cell cultures. Results are representative of two independent experiments. (b) Before stimulation with 500 nM CpG, BMDMs derived from *IL10*$^{-/-}$ mice were incubated for 30 min with 2.5 µM U0126 or vehicle control (DMSO). After 24 h stimulation, cytokine production in culture supernatants was assayed by ELISA (mean ± SD; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test. (c) BMDMs generated from WT and *Tpl-2*$^{-/-}$ mice were stimulated with 500 nM CpG for the times indicated and total RNA was extracted. *c-fos* mRNA expression was measured by quantitative PCR (normalized to *HPRT* mRNA). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; two-way analysis of variance (ANOVA) with Bonferroni correction. (d) BMDMs generated from WT and *Tpl-2*$^{-/-}$ mice were stimulated with 500 nM CpG for the times indicated, and nuclear extracts were prepared. c-fos binding activity was measured by ELISA. Graph shows mean ± SD; $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni correction. Data in b–d are representative of at least three independent experiments.

Thus, ERK negatively regulates the production of IFN-β and IL-12 independent of IL-10, but TLR-induced production of these cytokines is additionally regulated by the autocrine effects of IL-10, which is itself positively regulated by ERK.

It has been suggested that TLR stimulation coordinately induces the TRAF3-dependent production of IFN-β and IL-10 (Häcker et al., 2006). However, we have shown previously that IFN-β and IL-10 are not always co-regulated and that LPS activates signaling pathways that have different effects on the expression of these two cytokine genes (Boonstra et al., 2006). In keeping with this, we show that TPL-2 activation of ERK is required for TLR4 and TLR9 induction of IL-10, but negatively regulates IFN-β production via both IL-10–dependent and –independent mechanisms.

Abrogation of ERK signaling in *Tpl-2*$^{-/-}$ macrophages significantly reduces both *c-fos* expression and c-Fos DNA binding activity

Our results indicate that negative regulation of *IFN-β* expression by TPL-2–mediated activation of ERK was dependent on protein synthesis. To identify which transcription factors might regulate *IFN-β* transcription in an ERK-dependent fashion, LPS and CpG-induced gene expression in BMDMs stimulated in the presence or absence of the MEK inhibitor UO126 was analyzed by Affymetrix gene array. Transcription of the *IFN-β* gene is known to be regulated by NF-κB, AP-1, and IRFs (for review see Colonna, 2007). Analysis of the gene array data revealed that expression of mRNAs encoding NF-κB and IRF family members was not affected by U0126 (unpublished data). However, U0126 substantially reduced LPS and CpG induction of mRNA encoding the AP-1 transcription factor *c-fos* (unpublished data), which was shown to regulate IL-10 and IL-12 expression (Dillon et al., 2004). Previous studies with MEK inhibitors have shown that ERK signaling is required for IL-10 production by myeloid cells triggered via TLR (Yi et al., 2002; Dillon et al., 2004). This was suggested to be mediated by transcriptional induction of IL-10 by c-Fos, whose expression is positively regulated by ERK signaling. It has also been suggested that negative regulation of IL-12p40 by ERK was mediated indirectly as a consequence of reduced IL-10 production (Yi et al., 2002). Although ERK down-regulation of IL-12p40 production has also been proposed to be mediated via c-Fos (Dillon et al., 2004), the regulation of IFN-β production was not addressed. Our data obtained with IL-10$^{-/-}$ macrophages demonstrate that ERK negatively regulates IL-12 and IFN-β production independent of IL-10 (Fig. 4 b).

We show that *c-fos* mRNA expression in macrophages is positively regulated by TPL-2 because CpG induced reduced levels of *c-fos* mRNA in *Tpl-2*$^{-/-}$ compared with WT control BMDMs (Fig. 4 c). Similar results were obtained with

LPS (unpublished data). At the peak time of *c-fos* mRNA induction, however, no differences in mRNA expression of AP-1 transcription factors, *Jund1* or *ets-1,* were observed between WT and *Tpl-2*$^{-/-}$ BMDMs (Fig. S3). Reduced *c-fos* mRNA in *Tpl-2*$^{-/-}$ BMDMs stimulated with CpG translated into reduced c-Fos binding activity, since c-Fos binding to a specific AP-1 DNA oligonucleotide was found to be significantly reduced in the absence of TPL-2 (Fig. 4 d).

Retroviral transduction of *c-fos* into *Tpl-2*$^{-/-}$ and *IL-10*$^{-/-}$ myeloid DCs reduces CpG-induced IFN-β and IL-12 expression

To investigate the potential role of c-Fos in negative regulation of IFN-β and IL-12, BMDCs from *Tpl-2*$^{-/-}$, *IL-10*$^{-/-}$, and WT mice were transduced with a retroviral construct containing *c-fos*-IRES-*GFP* or Mock-IRES-*GFP* and flow cytometry purified on the basis of GFP expression. Expression of *c-fos* decreased the levels of IFN-β and IL-12 in *Tpl-2*$^{-/-}$, *IL-10*$^{-/-}$, and WT cells (Fig. 5) induced after CpG stimulation. Whereas negative regulation of IL-12 by c-Fos had been proposed previously, it remained unclear whether this was independent of effects on IL-10 expression (Agrawal et al., 2003; Dillon et al., 2004). We show that c-Fos regulates IL-12 production in the presence and absence of IL-10. Furthermore, the negative regulation of IFN-β by c-Fos suggests a potential mechanism by which the TPL-2 signaling pathway negatively regulates IFN-β production.

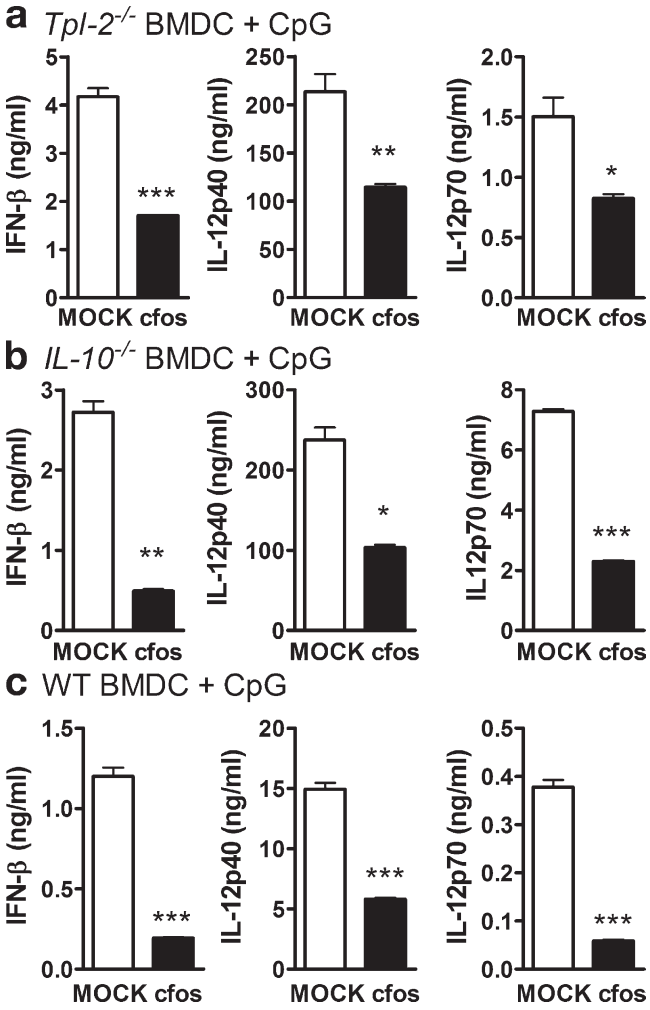

Figure 5. *c-fos* reduces TLR up-regulation of IFN-β and IL-12 in *Tpl-2*$^{-/-}$, *Il-10*$^{-/-}$, and WT BMDCs, independent of IL-10. BMDCs were generated from *Tpl-2*$^{-/-}$ (a), *IL-10*$^{-/-}$ (b), and WT (c) mice and transfected with control GFP or *c-fos*-IRES-*GFP* encoding vectors. GFP^{+} BMDCs were sorted by MoFlo and stimulated with 500 nM CpG for 24 h. Cytokine production in culture supernatants was assayed by ELISA (mean ± SD; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; Student's *t* test. Results are representative of five (a and b) or two (c) independent experiments with similar results.

The TPL-2 MAPK signaling pathway has attracted considerable interest as a possible target for antiinflammatory drugs (Hall et al., 2007). Because of its critical role in the induction of TNF (Dumitru et al., 2000; Rousseau et al., 2008), several large pharmaceutical companies are developing small molecule inhibitors of TPL-2 as potential drugs to treat inflammatory diseases (Hall et al., 2007). However, our work suggests that TPL-2 inhibition may have undesirable proinflammatory effects caused by its complex regulation of cytokine production. Thus, although TPL-2 inhibition might decrease TNF production, our data suggest that the production of the proinflammatory cytokines IL-12 and IFN-β would be increased in macrophages and myeloid DCs, whereas production of the antiinflammatory cytokine IL-10 would be decreased. We also show that TPL-2 regulates cytokine production in a cell-specific fashion, positively regulating TLR-induced IL-12 and IFN-β production in pDCs. Our data, therefore, raise questions about the validity of TPL-2 as an antiinflammatory drug target.

MATERIALS AND METHODS

Mice and primary cells. C57BL/6, *Tpl-2*$^{-/-}$ (Dumitru et al., 2000), and *IL-10*$^{-/-}$ (Kühn et al., 1993) mice were bred at the National Institute for Medical Research under specific pathogen–free conditions. All protocols for breeding and experiments with animals were performed and approved by the Home Office, UK, Animals (Scientific Procedures) Act 1986 (Project License Number: PPL 80/2236). Macrophages, myeloid DCs, and pDCs were generated from BM as previously described (Boonstra et al., 2006). BMDM (F4/80^{+}) and BMDC (CD11c^{+}) purities were ≥95%, and pDC (CD11b^{-} CD11c^{+} B220^{+} or 120G8^{+}) purities were ≥98% (MoFlo cytometer; Cytomation). Similarly, spleen cell suspensions were depleted of T cells, and DCs and macrophages were purified as CD11b^{+} (≥95%; Fiorentino et al., 1991).

Antibodies, cDNA, plasmids, and reagents. The antibodies used for immunoblotting have been previously described (Papoutsopoulou et al., 2006). mAbs used for cell purification were as follows: anti-F4/80-PE (Invitrogen), anti-CD11c-PE (BD), anti-CD11b-APC (eBioscience), and 120G8-Alexa Fluor 488 (Boonstra et al., 2006). Cells were stimulated with *Salmonella Minnesota* LPS (Enzo Biochem, Inc.) or phosphorothioate CpG DNA (CpG1668: TCCATGACGTTCCTGATGCT; TriLink Biotech). GM-CSF was obtained from Schering-Plough, and Flt3 ligand was purchased from Shanghai Genomics. The MEK-1 inhibitor U0126 (Favata et al., 1998) was obtained from BIOMOL International; DMSO, cycloheximide, and polybrene were purchased from Sigma-Aldrich; Liberase Cl and FuGENE-6 were obtained from Roche. The cDNA encoding *c-fos* was donated by A. Behrens (Cancer Research Institute, London, UK).

In vitro stimulation of macrophages and DCs, and quantitation of cytokine production. Supernatants from BMDMs and BMDCs (2×10^5/200 µl) stimulated with LPS (100 ng/ml) or CpG1668 (500 nM) for 24 h were analyzed by commercial ELISA kits for IFN-β (PBL), IL-12p70, and IL-10 (eBioscience). IL-12p40 was detected as previously described (Boonstra et al., 2006).

Protein analyses. BMDMs and BMDCs were cultured in medium containing 1% FCS for 5 h before stimulation with LPS or CpG and washed in PBS before lysis (1% NP-40, 0.1% SDS, 0.5% deoxycholate acid, 50 mM Tris HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 2 mM sodium-pyrophosphate, 50 mM sodium fluoride, 100 mM vanadate [all from Sigma-Aldrich], and complete EDTA-free protease inhibitor cocktail [Roche]). Immunoblotting of proteins was performed as previously described (Saraiva et al., 2005; Papoutsopoulou et al., 2006) and visualized with ECL (GE Healthcare) or SuperSignal West Femto Substrate (Thermo Fisher Scientific). pDCs showed low background levels of MAPK phosphorylation without resting and were used directly, but stimulated in 1% FCS.

RT-PCR. Total RNA was isolated from BMDMs, BMDCs, and pDCs, using the RNeasy kit (QIAGEN) and reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of *IFN-β*, *IL-12p35*, *IL-12p40*, *IL-10*, *c-fos*, *JunD1*, and *ets-1* was quantified by Real-Time PCR (7900HT; Applied Biosystems) and normalized against ubiquitin or HPRT mRNA levels, as previously described (Rajsbaum et al., 2008). The following primers and probes were obtained from Applied Biosystems: *c-fos* (Mm00487425); *Jund1* (Mm00495088); *ets-1* (Mm00468970).

Measurement of transcription factor DNA-binding activity. Nuclear extracts of BMDMs stimulated with CpG (500 nM) were prepared with the Nuclear Extract kit and assayed with the TransAM ELISA kit (both from Active Motif).

Retroviral infection of DCs. Amphoteric c-fos recombinant retrovirus was produced by transfection of *c-fos* cDNA (subcloned into pMXI-IRES-eGFP vector and sequenced to verify correct insertion; Shoemaker et al., 2006) into the Plat-E packaging cell line (Morita et al., 2000) using FuGene-6 transfection reagent (Roche). Transfected cells were cultured at 37°C for 48 h (DMEM plus 10% FCS and antibiotics). Culture supernatants were centrifuged at 48,000 *g* for 4 h at 4°C, sediments resuspended and filtered (0.4 µm) as concentrated virus. BMDCs from WT, *Tpl-2*$^{-/-}$, or *IL-10*$^{-/-}$ mice were prepared as previously described (Boonstra et al., 2006), with minor modifications. BM cells were cultured in 6-well plates (Corning) in complete RPMI containing GM-CSF as previously described (Boonstra et al., 2006) at 2 ml/well. After 48 h of incubation, 0.5 µg/ml polybrene and 200 µl of concentrated virus was added per well and plates were centrifuged at 2,000 *g* for 1 h at 25°C. On days 3, 4, and 5, each well was further supplemented with 1 ml complete BMDCs medium containing 100 µl of virus. On day 7, nonadherent cells were harvested and sorted for GFP$^+$ (MoFlo; Cytomation) and 96% of cells were CD11c$^+$, and 99% GFP$^+$.

Statistical analyses. Analyses were performed using Prism software (GraphPad).

Online supplemental material. Fig. S1 shows the contribution of ERK activation to LPS-mediated cytokine production. Fig. S2 shows that TPL-2 negatively regulates TLR-induced IFN-β and IL-12 production, but is required for optimal IL-10 production in splenic macrophages stimulated with CpG. Fig. S3 shows that transcription of *Jund1* and *Ets1* is not regulated by TPL-2. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091059/DC1.

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