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HOST RESPONSE AND INFLAMMATION

# Toll-Like Receptor 2-Tpl2-Dependent ERK Signaling Drives Inverse Interleukin 12 Regulation in Dendritic Cells and Macrophages

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**ABSTRACT** This study investigated responses to Toll-like receptor 2 (TLR2)-driven extracellular signal-related kinase (ERK) signaling in dendritic cells (DCs) versus macrophages. TLR2 signaling was induced with $Pam_3Cys$-Ser-$Lys_4$, and the role of ERK signaling was interrogated pharmacologically with MEK1/2 inhibitor U0126 or genetically with bone marrow-derived macrophages or DCs from *Tpl2*$^{-/-}$ mice. We assessed cytokine production via enzyme-linked immunosorbent assay (ELISA) or V-Plex, and mRNA levels were assessed via reverse transcriptase quantitative PCR (qRT-PCR). In macrophages, blockade of ERK signaling by pharmacologic or genetic approaches inhibited interleukin 10 (IL-10) expression and increased expression of the p40 subunit shared by IL-12 and IL-23 (IL-12/23p40). In DCs, blockade of ERK signaling similarly inhibited IL-10 expression but decreased IL-12/23p40 expression, which is opposite to the effect of ERK signaling blockade on IL-12/23p40 in macrophages. This difference in IL-12/23p40 regulation correlated with the differential expression of transcription factors cFos and IRF1, which are known to regulate IL-12 family members, including IL-12 and IL-23. Thus, the impact of ERK signaling in response to TLR2 stimulation differs between macrophages and DCs, potentially regulating their distinctive functions in the immune system. ERK-mediated suppression of IL-12/23p40 in macrophages may prevent excessive inflammation and associated tissue damage following TLR2-stimulation, while ERK-mediated induction of IL-12/23p40 in DCs may promote priming of T helper 1 (Th1) responses. A greater understanding of the role that ERK signaling plays in different immune cell types may inform the development of host-directed therapy and optimal adjuvanticity for a number of infectious pathogens.

**KEYWORDS** IL-10, IL-27p28, IP-10, IRF1, cFos, nitric oxide synthase

Toll-like receptors (TLRs) on macrophages and dendritic cells (DCs) play important roles in innate and adaptive immune responses by inducing effector molecule production and DC maturation in response to a wide range of microbial molecules (1–4). Depending on the specific TLR and cell type, TLR signaling may induce molecules, such as type I interferons (IFNs), interleukin 6 (IL-6), IL-8, IL-12, and tumor necrosis factor $\alpha$ (TNF-$\alpha$), which are important for protection following infection, and IL-10, IL-4, and IL-13, which prevent excessive inflammation in response to infection. For example, TLR2 can form heterodimers with TLR1 or TLR6 to recognize tri- or di-acylated microbial lipoproteins, respectively. TLR2 recognizes other ligands, such as alarmins, viral particles, and certain fungal infections (1), and this recognition can be enhanced by coreceptors, including cluster of differentiation 14 (CD14) and scavenger receptors. TLR2 signals through myeloid differentiation primary response 88 (MyD88) and Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) (3), and TLR2 signal-

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ing induces most of the cytokines mentioned above (type I IFNs are not typically induced by TLR2 signaling). Our previous studies have indicated an important role for TLR2-specific extracellular signal-related kinase (ERK) pathway signaling in macrophage responses to *Mycobacterium tuberculosis*, including a role in driving macrophage IL-10 expression, inhibiting IL-12 expression, and decreasing T helper 1 (Th1) responses to *M. tuberculosis*-infected macrophages (5). The role of TLR2-tumor progression locus 2 (Tpl2)-ERK signaling in DCs is less clear and requires additional experimentation.

TLR signaling involves the activation of multiple signaling components, including mitogen-activated protein kinases (MAPKs) and the IκB kinase (IKK) complex, which drives nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling (1, 3). The MAPKs include ERK1 and ERK2, Jun N-terminal protein kinases (JNKs), and p38. In macrophages, microtubule-associated protein kinase kinase kinase (MAP3K) Tpl2 mediates TLR activation of ERK1/2 through phosphorylation of mitogen-activated ERK kinase (MEK1/2) but not JNK or p38 (5). The role of Tpl2 in DC MAPK regulation is less clear. ERK1/2 activation is central to the regulation of IL-10 and IL-12 production (5–8) through phosphorylation of transcription factors (9) such as cFos and interferon regulatory factor 1 (IRF1). cFos, a subunit of activator protein 1 (AP-1), promotes NF-κB binding to IL-12 promoters and decreases NF-κB binding to IL-10 promoters. IRF1 associates with MyD88, and the complex translocates into the nucleus (a process termed IRF licensing), resulting in the induction of a subset of TLR-induced genes, including nitric oxide synthase 2 (*Nos2*) and *Il12p40* (*Il12a*) (7). ERK activation has been correlated with IL-10 production, resulting in dampened IL-12 production (5, 8).

While many other cell types express TLRs, macrophages and myeloid DCs have particularly important roles in TLR-mediated regulation of immune responses to pathogens. Macrophages are essential in innate immune responses. Their functions include phagocytosis, secretion of cytokines, and antigen presentation to effector T cells at sites of infection, resulting in the regulation of effector T cell responses. Upon induction of DC maturation, DCs migrate from peripheral tissues to reside in lymph nodes, where they present antigen to naive T cells, inducing primary T cell responses. DCs also secrete cytokines to regulate immune responses. Macrophages and DCs are regulated by TLR signaling, which drives DC maturation, induces macrophage and DC cytokine expression, and influences the spectrum of cytokines expressed, regulating other functions (e.g., the differentiation into functionally distinct macrophage phenotypes with different inflammatory properties and cytokine profiles) (4, 10, 11).

In this study, we investigated the role of TLR2-induced Tpl2-ERK signaling in the regulation of pro- and anti-inflammatory markers (particularly IL-12/23p40 and IL-10) in macrophages and DCs. TLR2-mediated ERK activation was dependent on Tpl2 in both macrophages and DCs. In both cell types, TLR2 induction of IL-10 and Arginase 1 (*Arg1*) was decreased in cells lacking functional ERK signaling. In contrast, expression levels for IL-12/23p40 and *Nos2* mRNA were increased in macrophages by pharmacologic or genetic blockade of ERK signaling (use of U0126 to inhibit ERK activation or use of *Tpl2*$^{-/-}$ macrophages), consistent with our prior observations in an *M. tuberculosis* infection model (5). Surprisingly, DCs exhibited an inverse regulatory pattern, as IL-12/23p40 and *Nos2* levels were decreased by blockade of ERK signaling. Thus, ERK signaling promotes the expression of IL-12/23p40 and *Nos2* mRNA by DCs but inhibits IL-expression of 12/23p40 and *Nos2* mRNA by macrophages. This differing, cell type-specific role of ERK signaling correlated with differences in the regulation of cFos and IRF1, which are known to regulate IL-12 expression.

## RESULTS

**TLR2-ERK signaling is dependent on *Tpl2* in macrophages and DCs.** To interrogate the role of ERK signaling in the regulation of macrophages and DC responses to the stimulation of TLR2, we used both pharmacologic and genetic means to block the ERK signaling pathway. Genetic deletion of *Tpl2* was one approach. In some cells, TLR-dependent activation of ERK signaling occurs by Tpl2 acting as a MAP3K (5). The role of Tpl2 in this signaling cascade has not been analyzed in DCs, and some receptors

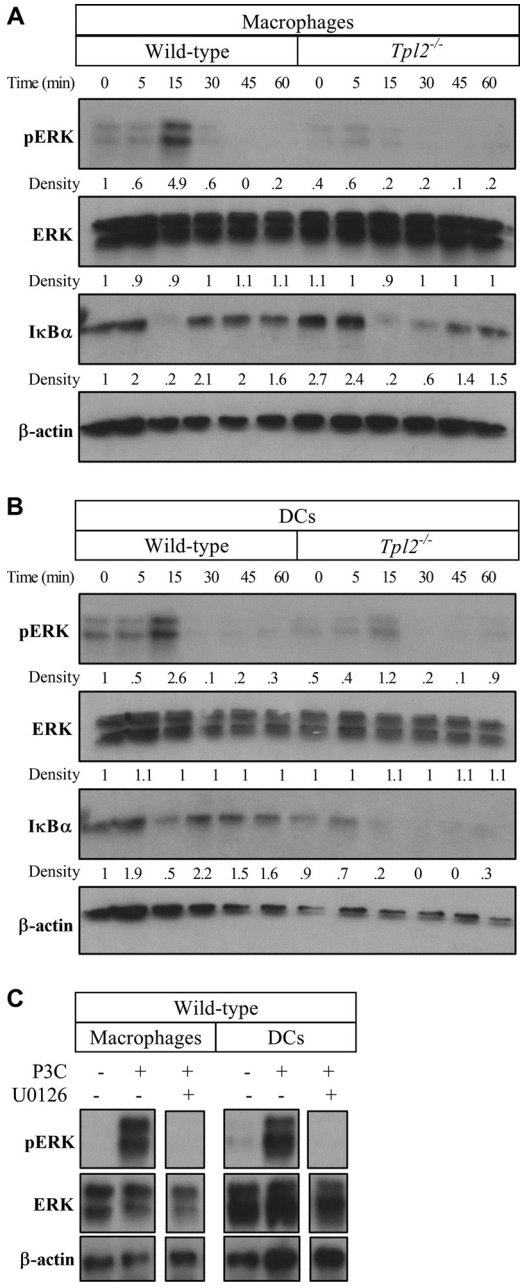


**FIG 1** ERK phosphorylation is dependent on *Tpl2* and MEK1/2. Wild-type and *Tpl2*$^{-/-}$ macrophages (A) and DCs (B) were stimulated with P3C for 0, 5, 15, 30, 45, and 60 min and assessed by Western blotting. The density of bands was quantified by densitometry with results indicated below each panel. For ERK and pERK, both ERK1 and ERK2 were included in the quantification. (C) Wild-type macrophages and DCs were incubated for 30 min with or without P3C in the presence or absence of U0126. Results are representative of three independent experiments.

(e.g., growth factor receptors) can activate MEK/ERK signaling independently of Tpl2 (12–15). To confirm that TLR2-dependent ERK activation is deficient in both macrophages and DCs from *Tpl2*$^{-/-}$ mice, cells were stimulated with 10 ng/ml Pam3Cys-Ser-Lys4 (P3C) for 0, 5, 15, 30, 45, and 60 min, and phosphorylated ERK1/2 was examined by Western blot analysis of cell lysates. In wild-type macrophages and DCs, P3C induced phosphorylation of ERK1 and ERK2 within 15 min of stimulation (Fig. 1A and B, respectively). NF-κB activation (represented by nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha [IκBα] degradation) was also observed in

wild-type macrophages and DCs, peaking at 15 min (Fig. 1A and B). In contrast, TLR2-dependent ERK1/2 phosphorylation was absent or minimal in *Tpl2*$^{-/-}$ macrophages and DCs (Fig. 1A and B). Both wild-type and *Tpl2*$^{-/-}$ DCs showed basal levels of ERK1/2 phosphorylation, as seen previously (16). *Tpl2*$^{-/-}$ macrophages and DCs both showed less recovery of I$\kappa$B$\alpha$ expression relative to wild-type cells through the end of the time course measured (Fig. 1A and B, respectively), but the significance of this observation is unclear. To validate a pharmacologic approach for inhibition of ERK signaling, wild-type macrophages and DCs were treated with MEK1/2 inhibitor U0126 to block ERK activation, which resulted in blockade of TLR2-induced ERK1/2 phosphorylation in both cell types (Fig. 1C). These data establish that TLR2-induced ERK activation is dependent on both *Tpl2* and MEK1/2 in macrophages and DCs, and these findings validate genetic and pharmacologic means to ablate this pathway.

**TLR2-Tpl2-ERK signaling enhances IL-10 expression in both macrophages and DCs but drives inverse regulation of IL-12/23p40 in the two cell types.** To identify the role of ERK signaling in TLR2-mediated cytokine regulation, macrophages and DCs were stimulated with P3C with or without genetic or pharmacologic inhibition of the ERK pathway. Cytokine protein concentrations were measured by ELISA, and mRNA expression levels were evaluated by qRT-PCR to assess the expression of IL-10 and IL-12/23p40 (encoding the p40 subunit shared by IL-12 and IL-23), representing anti-inflammatory and proinflammatory cytokines, respectively.

Following P3C stimulation, macrophages and DCs expressed high levels of *Il10* mRNA, with a peak at 2 h of stimulation and sustained expression through 6 h (Fig. 2A and B). Treatment of macrophages and DCs with U0126 reduced *Il10* transcript levels at all time points examined (Fig. 2A to C). U0126 also reduced IL-10 protein expression in both cell types (Fig. 2D). Thus, IL-10 expression is driven by ERK signaling in both macrophages and DCs. *Il12p40* mRNA was induced with a slower time course, peaking at approximately 6 h in macrophages or 4 h in DCs (Fig. 3A and B, respectively). In DCs, U0126 inhibited P3C induction of *Il12p40* mRNA at all examined time points (Fig. 3B and C) and similarly inhibited protein expression for the p40 subunit that is shared between IL-12 and IL-23 (IL-12/23p40) (Fig. 3D). In contrast, treatment of macrophages with U0126 increased *Il12p40* mRNA (Fig. 3A and C) and IL-12/23p40 protein expression (Fig. 3D). Thus, ERK signaling drives IL-12/23p40 expression in DCs but inhibits IL-12/23p40 expression in macrophages.

It was important to determine the role of Tpl2 in both macrophages and DCs since it may vary depending on the specific surface receptors, scaffolding proteins, and signaling molecules present in different cell types (12–15). In macrophages and DCs, P3C induction of *Il10* mRNA (Fig. 4A) and IL-10 protein (Fig. 4B) was significantly reduced in *Tpl2*$^{-/-}$ cells. In contrast, *Tpl2* deletion had opposite effects on TLR2-induced IL-12/23p40 expression in macrophages versus DCs, paralleling results with pharmacologic inhibition of MEK1/2 (Fig. 2 and 3). P3C induction of *Il12p40* mRNA (Fig. 4C) and IL-12/23p40 protein (Fig. 4D) was decreased in *Tpl2*$^{-/-}$ DCs but increased in *Tpl2*$^{-/-}$ macrophages. In conclusion, TLR2-Tpl2-ERK signaling drives IL-12/23p40 expression in DCs but inhibits IL-12/23p40 expression in macrophages.

**Different roles for ERK signaling in regulation of *Arg1* and *Nos2* in macrophages versus DCs.** To test whether ERK differentially regulates other proteins in macrophages and DCs, we examined the expression of *Nos2* and *Arg1* following pharmacological inhibition of the ERK pathway. Arg1, like IL-10, is associated with anti-inflammatory responses, whereas NOS2, like IL-12, is associated with proinflammatory responses (IL-12 is also involved in NOS2 regulation). Similar to the pattern seen for *Il10*, U0126 treatment inhibited P3C-induced *Arg1* mRNA in both macrophages and DCs (Fig. 5A). In contrast, U0126 had different effects on *Nos2* mRNA in DCs versus macrophages. Treatment with U0126 decreased *Nos2* mRNA expression in DCs but increased it in macrophages (Fig. 5B), mimicking the pattern seen with IL-12/23p40. These data demonstrate that, like IL-10, *Arg1* expression is driven by ERK signaling in macrophages and DCs. On the other hand, ERK signaling drives *Nos2* expression in DCs

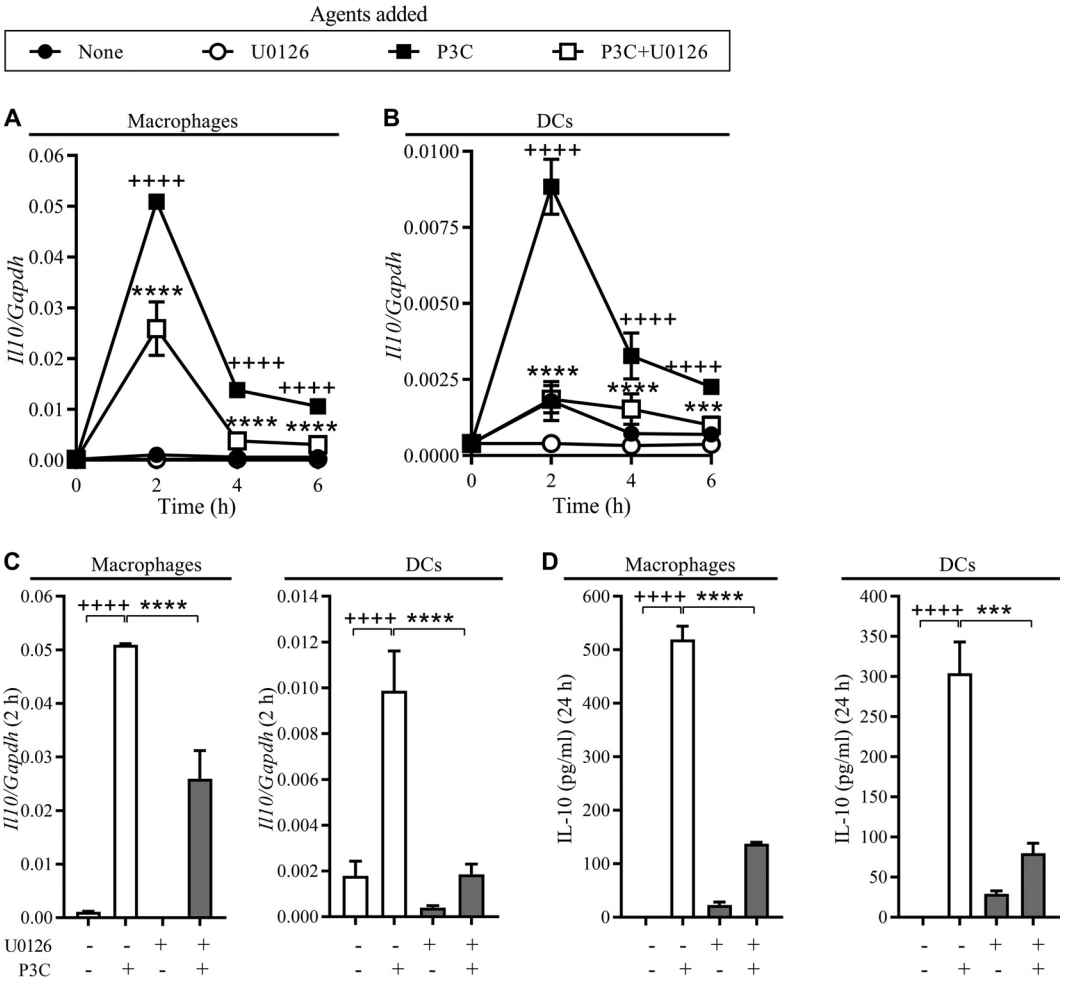


**FIG 2** ERK inhibition by U0126 decreases P3C-induced IL-10 expression in macrophages and DCs. Wild-type macrophages and DCs were incubated with P3C for the indicated time points in the presence or absence of U0126. Macrophage (A) and DC (B) *Il10* mRNA expression were assessed by qRT-PCR after 2, 4, or 6 h of stimulation with P3C. (C) *Il10* mRNA expression after 2 h of stimulation with P3C was assessed by qRT-PCR. (D) Supernatant IL-10 protein after 24 h of stimulation with P3C was assessed by ELISA. Data points indicate mean ± SD for triplicate samples in a single experiment (representative of two independent experiments for A and B and six independent experiments for C and D). Statistical significance is indicated for comparison of treatment with P3C versus control with no P3C (+ symbols) or treatment with P3C plus U0126 versus treatment with P3C without U0126 (* symbols) using 4 symbols for a *P* value of <0.0001 and 3 symbols for a *P* value of <0.001.

but inhibits it in macrophages. The parallel regulation of *Nos2* and IL-12/23p40 in both cell types likely results from the ability of IL-12 to induce *Nos2* expression.

**Inverse roles of ERK signaling in macrophages versus DCs for regulation of IL-12/23p40 parallel differential regulation of cFos and IRF1.** We considered the hypothesis that the inverse role of ERK in regulating IL-12/23p40 in macrophages versus DCs may be related to the differential regulation of transcription factors that control IL-12 and IL-23 expression (7). To assess this hypothesis, macrophages and DCs were stimulated with P3C in the presence or absence of U0126, followed by qRT-PCR to assess the expression of mRNA for transcription factors known to regulate IL-12 expression. Expression of mRNA for CCAAT enhancer binding protein $\beta$ (*c/EBP$\beta$*), spi-1/SFFV provirus integration site 1 (*Pu.1*), signal transducer and activator of transcription (*Stat1*), and *Stat3* was unaffected by U0126 treatment in macrophages and DCs (see Fig. S2 in the supplemental material). Expression of mRNA for *cJun* was enhanced by U0126 treatment, and expression of mRNA for *cRel* and *Irf4* was inhibited by U0126 in both cell types (Fig. S2). None of these transcription factors showed inverse regulation in macrophages versus DCs in response to inhibition of the ERK pathway. On the other hand, U0126 significantly inhibited P3C induction of *Irf1* and *cFos* mRNA

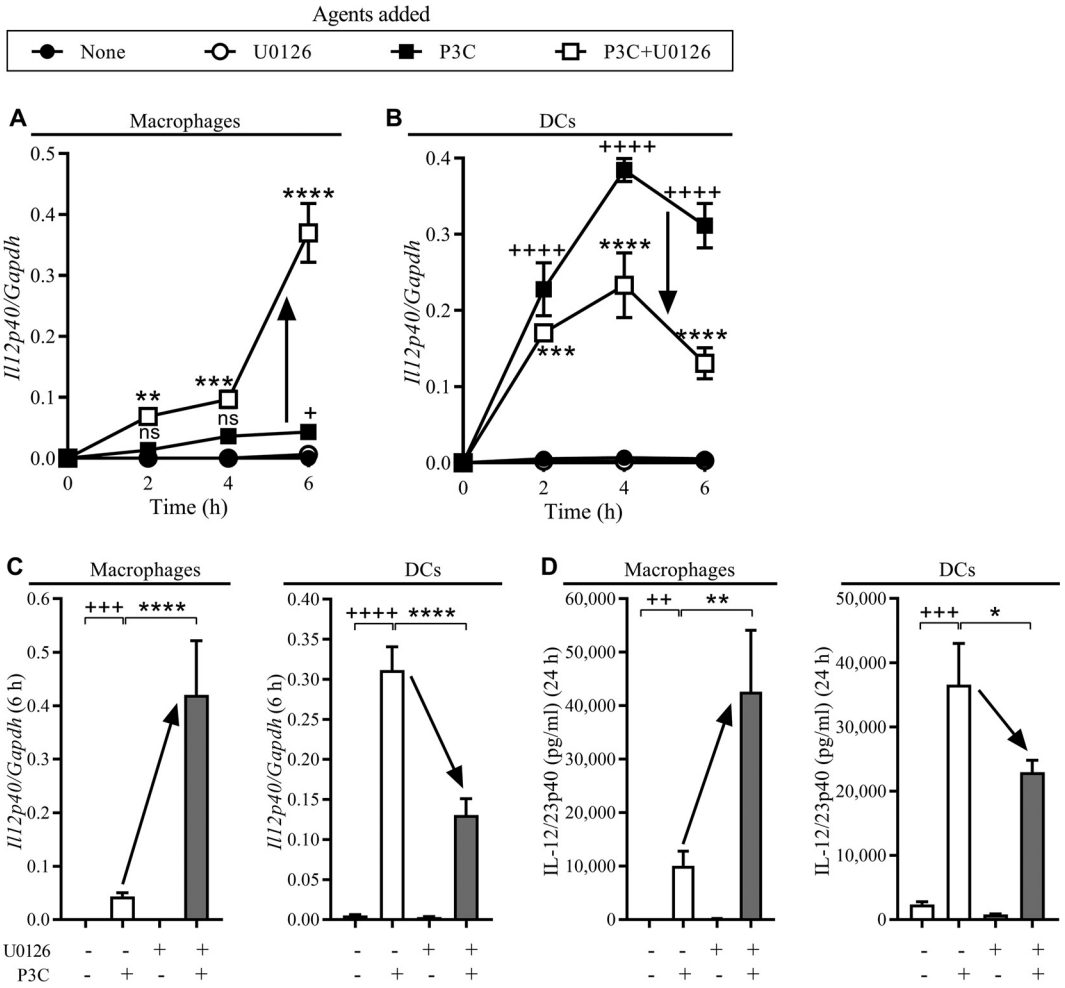


**FIG 3** ERK inhibition by U0126 enhances IL-12/23p40 levels in macrophages and decreases IL-12/23p40 levels in DCs. Wild-type macrophages and DCs were stimulated with P3C in the presence or absence of U0126 for the indicated times. Macrophage (A) and DC (B) *Il12p40* mRNA expression were assessed by qRT-PCR after 2, 4, or 6 h of stimulation with P3C. (C) *Il12p40* mRNA expression after 6 h of stimulation with P3C was assessed by qRT-PCR. (D) Supernatant IL-12/23p40 protein expression after 24 h of stimulation with P3C was assessed by ELISA. Data points indicate mean ± SD for triplicate samples in a single experiment (representative of two independent experiments for A and B and six independent experiments for C and D). Statistical significance is indicated for comparison of treatment with P3C versus control with no P3C (+ symbols) or treatment with P3C plus U0126 versus treatment with P3C without U0126 (* symbols) using 4 symbols for a *P* value of <0.0001, 3 symbols for a *P* value of <0.001, 2 symbols for a *P* value of <0.01, or 1 symbol for a *P* value of <0.05. Arrows call attention to the inverse regulatory role of U0126 in P3C-stimulated macrophages versus DCs for *Il12p40* mRNA or the IL-12/23 p40 protein that it encodes.

expression in DCs but increased P3C induction of *Irf1* and *cFos* mRNA expression in macrophages (Fig. 6A and B), a pattern similar to that seen for IL-12/23p40. This inverse regulatory pattern was also seen at the level of IRF1 and cFos protein expression. U0126 enhanced P3C-induced cFos and IRF1 expression in macrophages but inhibited cFos and IRF1 expression in DCs (Fig. 6C). The U0126-induced decrease in IRF1 expression in DCs is slight but is clearly not an increase as that seen with macrophages. Thus, ERK signaling drives IRF1 and cFos expression in DCs but inhibits IRF1 and cFos expression in macrophages. IRF1 and cFos are known to enhance IL-12 expression (7, 17, 18), and this aspect is incorporated in our model. We confirmed the importance of *Irf1* in this system, as *Irf1*$^{-/-}$ macrophages showed reduced *Il12p40* and *Il27p28* mRNA expression (see Fig. S3 in the supplemental material). Our data suggest a role for IRF1 and cFos in determining the inverse effects of ERK signaling on IL-12/23p40 expression in DCs versus macrophages and suggest the need for further research in this area.

**Inverse regulation of TLR2-Tpl2-ERK signaling response in macrophages versus DCs is restricted to IL-12 and a small subset of molecules.** To investigate whether

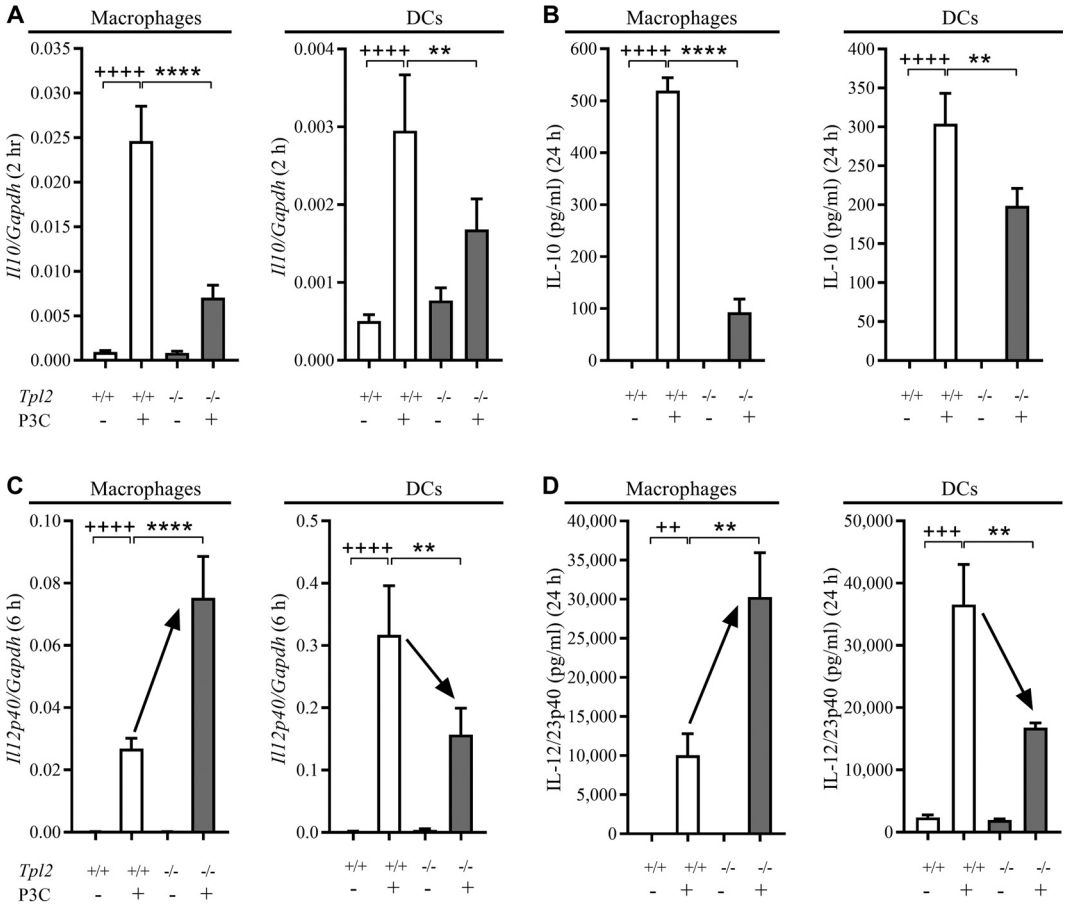


**FIG 4** Inhibition of TLR2-ERK signaling by genetic deletion of *Tpl2* enhances IL-12/23p40 levels in macrophages and decreases IL-12/23p40 levels in DCs. Wild-type and *Tpl2*$^{-/-}$ macrophages and DCs were stimulated with P3C for the indicated times. (A) *Il10* mRNA expression after 2 h of stimulation with P3C was assessed by qRT-PCR. (B) Supernatant IL-10 protein after 24 h of stimulation with P3C was assessed by ELISA. (C) *Il12p40* mRNA expression after 6 h of stimulation with P3C was assessed by qRT-PCR. (D) Supernatant IL-12/23p40 protein after 24 h of stimulation with P3C was assessed by ELISA. Data points indicate mean ± SD for triplicate samples in a single experiment (representative of six independent experiments). Statistical significance is indicated for the comparison of treatment with P3C versus control with no P3C (+ symbols) or *Tpl2*$^{-/-}$ cells versus wild-type cells (* symbols) using 4 symbols for a *P* value of $<0.0001$, 3 symbols for a *P* value of $<0.001$, and 2 symbols for a *P* value of $<0.01$. Arrows call attention to the inverse regulatory role of Tpl2 in P3C-stimulated macrophages versus DCs for *Il12p40* mRNA or the IL-12/23p40 protein that it encodes.

TLR2-Tpl2-ERK signaling results in the differential expression of additional proteins in DCs versus macrophages, we used V-Plex technology to study the expression of additional immune regulatory molecules by wild-type versus *Tpl2*$^{-/-}$ cells. Three MSD V-Plex plates (proinflammatory panel 1, cytokine panel 1, and Th17 panel 1) were run with supernatants from cells cultured with P3C for 24 h. The IL-10 protein production was reduced in *Tpl2*$^{-/-}$ macrophages and DCs compared with that of wild-type cells, confirming the role for ERK in regulating IL-10 in both cell types (see Fig. S1A in the supplemental material, consistent with results in Fig. 4B). As predicted by our earlier results, a deficiency of *Tpl2* decreased the expression of IL-12p70 (the heterodimer containing IL-12/23p40 and IL-12p30) in DCs but increased it in macrophages (Fig. S1B), confirming the trend seen with IL-12/23p40 (Fig. 4D). The majority of other proteins (15 of 20 examined) studied showed a regulatory pattern similar to that of IL-10, with production decreased by *Tpl2* deficiency in both macrophages and DCs, delineating a role for TLR2-Tpl2-ERK signaling in the induction of multiple cytokines and chemokines, including IL-6 and TNF-$\alpha$ (Fig. 7A and B, respectively), as well as IL-1$\beta$, monocyte chemoattractant protein-1 (MCP-1), IL-15, and keratinocyte-derived cytokine (KC/GRO) (Fig. S1C to F).

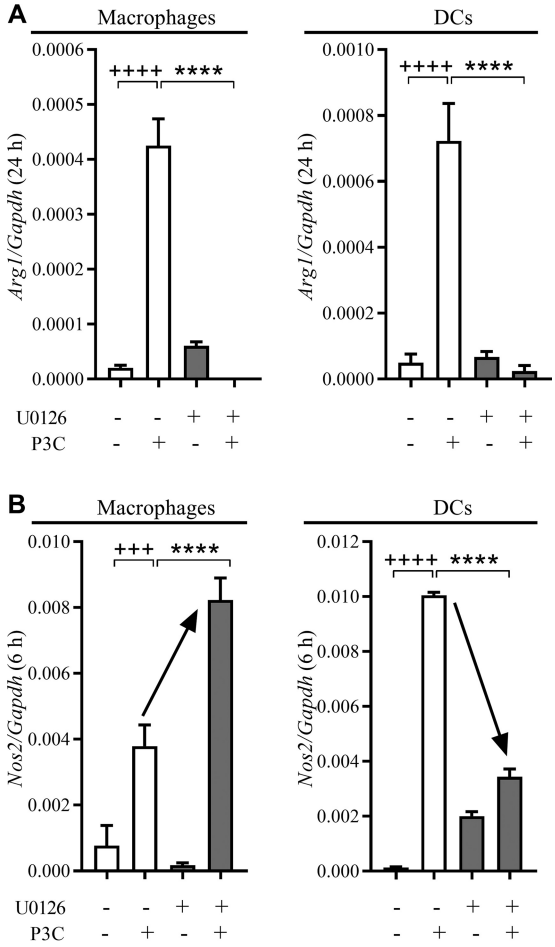


**FIG 5** Inhibition of TLR2-ERK signaling by U0126 enhances *Nos2* levels in macrophages and decreases *Nos2* levels in DCs. Wild-type macrophages and DCs were incubated with P3C in the presence or absence of U0126 for the indicated times. qRT-PCR was used to assess mRNA expression of *Arg1* after 24 h of stimulation with P3C (A) and *Nos2* after 6 h of stimulation with P3C (B) in macrophages and DCs. Data points indicate mean ± SD for triplicate samples in a single experiment (representative of two independent experiments). Statistical significance is indicated for the comparison of treatment with P3C versus control with no P3C (+ symbols) or treatment with P3C plus U0126 versus treatment with P3C without U0126 (* symbols) using 4 symbols for a *P* value of $<0.0001$ and 3 symbols for a *P* value of $<0.001$. Arrows call attention to the inverse regulatory role of U0126 in P3C-stimulated macrophages versus DCs for *Nos2*, similar to the pattern seen in other figures for *Il12p40*.

A limited number of proteins showed the differential pattern seen with IL-12/23p40, with *Tpl2* deficiency causing decreased expression in DCs but increased expression in macrophages. These proteins included IL-27p28 (Fig. 7C), a member of the IL-12 cytokine family that upregulates natural killer (NK) cell activation (19) and acts as an antagonist of IL-27 and IL-6 signaling in murine cells (20). Interferon gamma-induced protein 10 (IP-10), a chemokine produced by macrophages after challenge (21), which may have a role in DC migration to lymph nodes (22), was also differentially regulated (Fig. 7E). Protein expression levels for these molecules were significantly lower in DCs than in macrophages, which was recapitulated at the mRNA level (Fig. 7D and F). While P3C-stimulated *IL27p28* and *Ip10* mRNA were significantly higher in *Tpl2*$^{-/-}$ macrophages than those in wild-type cells, the change in *Il27p28* and *Ip10* expression in DCs was not significant (Fig. 7D and F, respectively). Since macrophages generate considerably larger quantities of these molecules, ERK signaling in macrophages may have a larger impact on their secretion. Thus, for these genes, the IL-12/23p40 regulation model may be slightly modified with ERK inhibiting expression in macrophages (as for IL-12/23p40) but having little effect in DCs (as opposed to driving expression in DCs for IL-12/23p40). Nonetheless, IL-27p28 and IP-10 share much

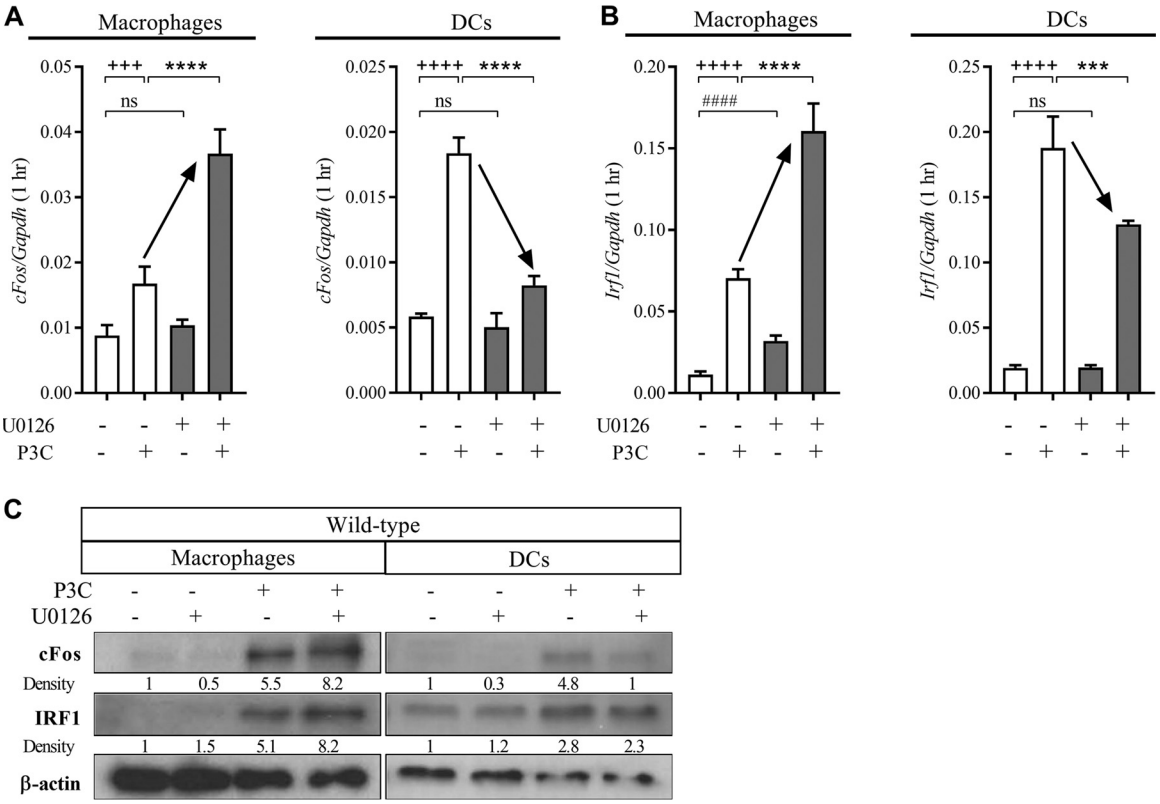


**FIG 6** Inhibition of TLR2-ERK signaling by U0126 enhances *Irf1* and *cFos* expression in macrophages and decreases *Irf1* and *cFos* expression in DCs. Wild-type macrophages and DCs were incubated with P3C in the presence or absence of U0126. Expression of *cFos* (A) and *Irf1* (B) mRNA was assessed by qRT-PCR after stimulation for 1 h with P3C (with or without U0126). (C) cFos and IRF1 protein expression were examined by Western blotting after 2 h of incubation with or without P3C in the presence or absence of U0126. The density of bands was quantified by densitometry with results indicated below each panel. Data points indicate mean ± SD for triplicate samples in a single experiment (representative of three independent experiments). Statistical significance is indicated for the comparison of treatment with P3C versus control with no P3C (+ symbols), treatment with P3C plus U0126 versus treatment with P3C without U0126 (* symbols), or treatment of U0126 versus untreated control (# symbols), using 4 symbols for a *P* value of <0.0001 and 3 symbols for a *P* value of <0.001; ns, not significant. Arrows call attention to the inverse regulatory role of U0126 in P3C-stimulated macrophages versus DCs for *cFos* and *Irf1*, similar to the pattern seen in other figures for *Il12p40*.

of the IL-12/23p40 regulatory pattern. Since IL-27p28 and IP-10, like IL-12, are regulated by IRF1 (17, 18, 23) and cFos (24), these transcription factors may be the drivers behind the differential outcomes of TLR2-Tpl2-ERK signaling seen in macrophages versus DCs.

## DISCUSSION

TLR2 regulates macrophage and DC functions that are important for immune responses to infectious pathogens. How TLR2 may differentially regulate their functions remains largely unexplored. TLR2 dimers respond to bacterial lipoproteins/lipopeptides, alarmins, viral particles, and certain fungal infections (1). Given the broad roles ascribed to TLR2, it is important to understand its downstream signaling and associated outcomes in cell types that are important in immunity to infections. Our previous studies revealed an important role for TLR2-driven ERK pathway signaling in macrophage responses to *M. tuberculosis*, including a role for TLR2-ERK signaling in driving macrophage IL-10 expression, inhibiting macrophage IL-12 expression, and decreasing Th1 responses to *M. tuberculosis*-infected macrophages (5). Other studies have implied an important regulatory relationship between IL-10 and IL-12, where high IL-10 expression can dampen IL-12 expression (25, 26). In this study, we assessed and compared the outcomes of TLR2-ERK signaling in macrophages and DCs. In both cell types, TLR2 contributes to the detection and clearance of pathogens via induction of cytokines and chemokines that recruit and activate other inflammatory cell types (3), as well as by the induction of defense mechanisms intrinsic to macrophages and DCs. We observed both

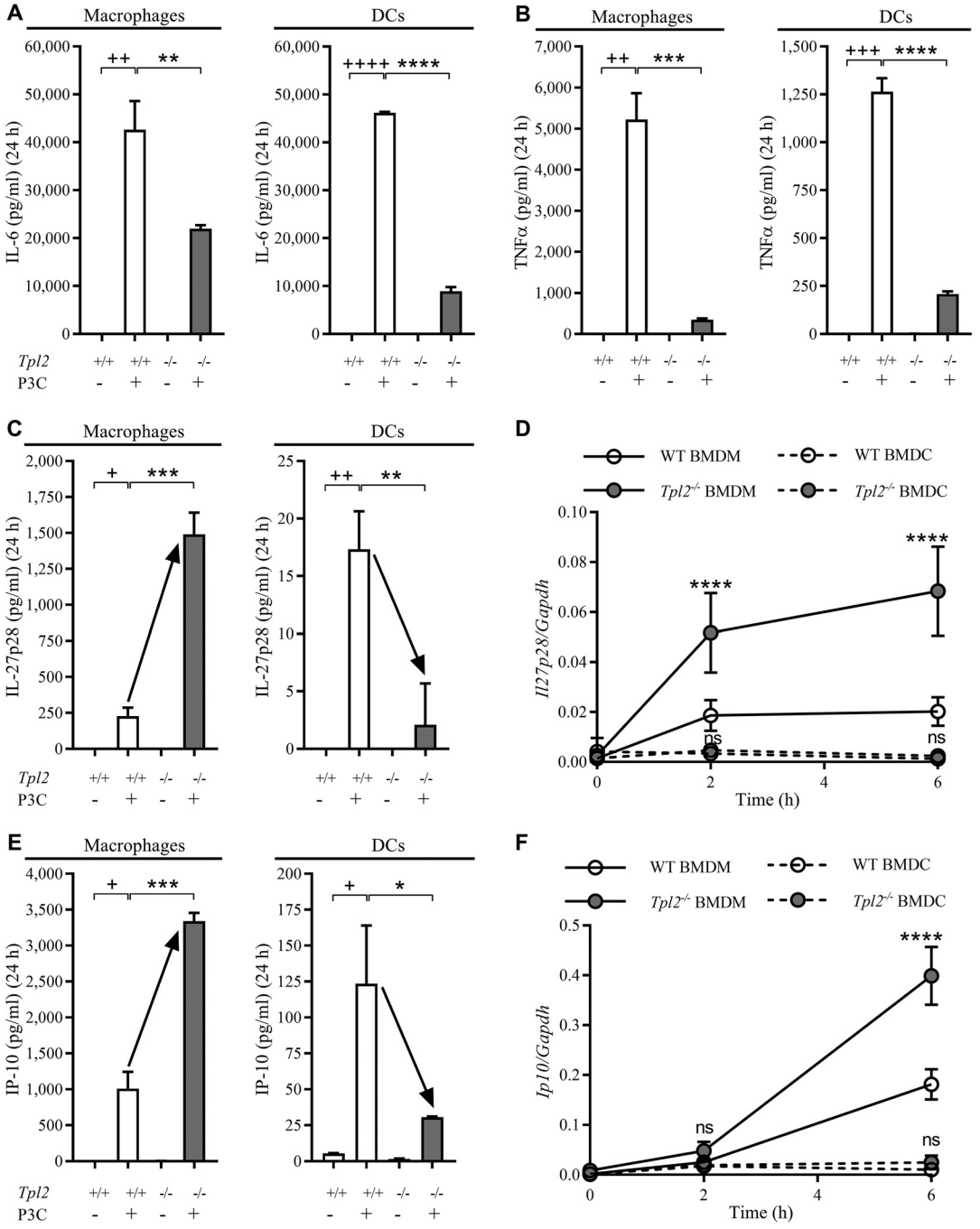


**FIG 7** Inhibition of TLR2-ERK signaling by genetic deletion of *Tpl2* differentially regulates a small number of cytokines in macrophages and DCs. Wild-type and *Tpl2*$^{-/-}$ macrophages and DCs were stimulated with P3C for the indicated times. Supernatant IL-6 (A), TNF-$\alpha$ (B), and IL-27p28 (C) protein after 24 h of stimulation with P3C was assessed via ELISA. (D) *Il27p28* mRNA expression was assessed by qRT-PCR after 2, 4, or 6 h of stimulation with P3C. (E) Supernatant IP-10 protein after 24 h of stimulation with P3C was assessed via ELISA. (F) *Ip10* mRNA was assessed by qRT-PCR after 2, 4, or 6 h of stimulation with P3C. Data points indicate mean ± SD of triplicate samples for a single experiment. Statistical significance is indicated for the comparison of treatment with P3C versus control with no P3C (+ symbols) or *Tpl2*$^{-/-}$ cells versus wild-type cells (* symbols) using 4 symbols for a *P* value of $<0.0001$, 3 symbols for a *P* value of $<0.001$, 2 symbols for a *P* value of $<0.01$, or 1 symbol for a *P* value of $<0.05$. Arrows call attention to the inverse regulatory role of Tpl2 in P3C-stimulated macrophages versus DCs for IL-27p28 and IP-10, similar to the pattern seen in other figures for IL-12/23p40.

similarities and important differences in the TLR2 regulation of gene and protein expression in DCs relative to macrophages.

Our studies revealed inverse roles for TLR2-Tpl2-ERK signaling in the regulation of IL-12 in macrophages and DCs. These roles were dissected using both a genetic

knockout of *Tpl2* and pharmacologic inhibition of MEK1/2 with U0126. In both macrophages and DCs, TLR2-mediated ERK1/2 phosphorylation was dependent on *Tpl2* (Fig. 1A and B) and inhibited by U0126 (Fig. 1C). Other studies have shown that ERK1/2 phosphorylation is also dependent on *Tpl2* downstream of TLR3, TLR4 (14, 27), TLR7 (14, 28), and TLR9 (14, 27, 28) signaling. However, the MAPKs controlled by Tpl2 vary, not just within these TLR pathways, but also by cell type, temporal and spatial regulation, and differences in scaffolding proteins present during signaling (15).

TLR2-Tpl2-ERK signaling enhanced IL-10 expression in both cell types (Fig. 2) but had an inverse role in regulating IL-12/23p40 in DCs versus macrophages. In macrophages, IL-12/23p40 expression was enhanced by blockade of TLR2-induced ERK signaling with U0126 (Fig. 3A, C, and D) or genetic deletion of *Tpl2* (Fig. 4C and D) (all ELISA data for IL-12/23p40 represent the p40 subunit that is shared by IL-12 and IL-23 and is encoded by *Il12p40*). In DCs, IL-12/23p40 expression was decreased by blockade of ERK signaling by U0126 or *Tpl2* genetic deletion. Other data (not shown) demonstrated that *Il23* mRNA expression under these conditions follows an expression pattern similar to that of *Il12p40* for both cell types. These data, in addition to the enhanced IL-12p70 expression seen in *Tpl2*$^{-/-}$ macrophages and decreased IL-12p70 expression seen in *Tpl2*$^{-/-}$ DCs (Fig. S1B), indicate that regulatory differences between macrophages and DCs may broadly affect the IL-12 family. As mentioned previously, throughout all experiments, the effect of U0126 and/or *Tpl2*$^{-/-}$ is of greater magnitude in macrophages versus DCs, potentially indicating a more significant role for ERK signaling in the regulation of macrophage cytokine expression.

Other studies have observed Tpl2-mediated inhibition of IL-12 in macrophages downstream of TLR4 and TLR9 (27); our studies demonstrate that this also occurs downstream of TLR2. Preliminary data from our lab show that *M. tuberculosis* produces similar, albeit more complex, trends regarding cytokine regulation in macrophages and DCs. While TLR2 is a potent regulator of macrophages and DCs, future studies should extend the understanding of the cytokine regulatory mechanisms identified in this study, the roles of implicated transcription factors, and differences between macrophages and DCs to a clinically relevant model, such as *M. tuberculosis* infection.

Studies have shown that induction of IL-12 expression by DCs follows a pattern similar to that of macrophages, with increased induction following ablation of Tpl2-ERK signaling (27–29). We have considered two explanations for why our results differ. First, these studies investigated IL-12 production by DCs downstream of TLR4 (27, 29), TLR9 (27, 29), and TLR11 (28), as opposed to TLR2, suggesting the possibility of a unique role for TLR2-dependent Tpl2-ERK signaling in promoting IL-12 production in DCs. A second hypothesis concerns differences due to distinct DC culture systems. The granulocyte macrophage colony-stimulating factor (GM-CSF)/IL-4 growth system utilized in the other studies results in a mixed population of cells more closely resembling macrophages. GM-CSF-derived DCs are much larger and more granular with lower major histocompatibility complex class II (MHC-II) expression than Flt3-derived DCs (30). When stimulated through a variety of TLRs, GM-CSF-derived DCs produced a proinflammatory panel of cytokines and showed a distinct lack of migration to draining lymph nodes (30). Furthermore, GM-CSF-derived cells include two distinct major populations, namely, an MHC-II-high, F4/80-low DC-like population that makes up 8% to 37% of cultured cells, and an MHC-II-low, F4/80-high macrophage-like population that makes up approximately 34% to 67% (31). In addition, in preliminary flow cytometry studies, we observed that GM-CSF-derived DC size and granularity values were in between the values seen for Flt3-derived DCs and LADMAC-cultured macrophages. Our GM-CSF-derived DC population had very few CD11c$^{+}$ cells and a larger percentage of F4/80$^{+}$ cells (a macrophage marker) than Flt3-derived DCs (data not shown). Together, these results indicate that the Flt3 culture system produces DCs that are more distinct from macrophages.

ERK signaling regulates additional molecules important for host defenses, such as NOS2 and Arg1 (32). Blockade of TLR2-induced ERK signaling by U0126 inhibited *Arg1* expression in both macrophages and DCs (Fig. 5A), following the pattern seen for IL-10

expression. In contrast, *Nos2* expression was promoted by ERK signaling in DCs but inhibited in macrophages (Fig. 5B), mimicking the pattern seen with IL-12/23p40. IL-10 is a negative regulator of IL-12 expression, and Arg1 acts as a negative regulator of NOS2 activity (through mechanisms such as competition for substrate L-arginine [33], uncoupling NOS2 from its cofactor or substrate resulting in nitric oxide scavenger superoxide production, and increasing sensitization of NOS2 to its endogenous inhibitor asymmetric dimethyl-L-arginine [34]). High IL-10 and Arg1 expression in macrophages is associated with decreased MHC-II expression, leading to diminished $CD4^+$ T cell responses (35) and an immunosuppressive macrophage phenotype. IL-12 and NOS2 expression is associated with an inflammatory macrophage phenotype (7, 10). TLR2-Tpl2-ERK regulatory differences between macrophages and DCs may underlie differences between these cells in antigen presentation, inflammatory responses, and tissue damage (10, 11, 36).

When we assessed whether other immune mediators showed inverse regulation by Tpl2-ERK, as seen for IL-12/23p40 and *Nos2*, V-Plex analysis revealed that a number of molecules, including IL-6 and TNF-$\alpha$, were reduced by genetic depletion of *Tpl2* in both macrophages and DCs (Fig. 7A and B), similar to the pattern seen for IL-10. In contrast, ERK signaling had an inverse regulatory impact in macrophages versus DCs for IL-27p28 and IP-10 (Fig. 7C to F), similar to the pattern seen for IL-12/23p40. IL-27p28, a IL-12 family cytokine subunit that heterodimerizes with Epstein-Barr virus induced gene 3 (EBI3) to create IL-27, has both pro- and anti-inflammatory roles (17). IP-10 acts as a chemoattractant for activated T cells (22) and is directly regulated by IL-27p28 (37). These results demonstrate the existence of a set of immune regulatory molecules (IL-12/23p40, NOS2, IL-27p28, and IP-10) that are inhibited by ERK signaling in macrophages but enhanced by ERK signaling in DCs, whereas others are positively regulated by ERK signaling in both cell types (e.g., IL-10, Arg1, TNF-$\alpha$, and IL-6).

Transcriptional regulation of IL-12 involves many transcription factors, including negative regulation by c/EBP$\beta$, cJun, IRF4, and STAT3 and positive regulation by cRel, STAT1, PU.1, cFos, and IRF1 (7). Expression of *cJun*, *cRel*, and *Irf4* was dependent on ERK signaling in both macrophages and DCs, while expression of *c/ebp$\beta$*, *Pu.1*, *Stat1*, and *Stat3* were largely unaffected by ERK signaling in either cell type (Fig. S2). In contrast, ERK signaling had an inverse regulatory impact on the expression of *cFos* and *Irf1* in macrophages versus DCs. U0126 treatment produced a 2-fold increase in P3C-induced expression of *cFos and Irf1* in macrophages as opposed to a modest decrease in DCs (Fig. 6A and B). IRF1 enhances TLR-dependent IL-12 production (17, 18), promotes expression of NOS2, a molecule that can also be produced synergistically following release of certain proinflammatory mediators (38), and induces transcription of *Il12p40*, *Nos2*, *Il27p28* (17, 18), and *Ip10* (23). Consistent with this model, we observed a decreased expression of *Il12p40* and *Il27p28* in macrophages from *Irf1*$^{-/-}$ mice (Fig. S3). cFos is a known positive regulator of IL-12 and NOS2 downstream of TLR2, TLR4, TLR5, and TLR9 (27, 39) through regulation of *cFos* expression and differential binding of NF-$\kappa$B to IL-12 and IL-10 promoters (7, 40), but its role in regulating IL-27p28 and IP-10 is less well elucidated and needs further study. Together, these data suggest that IRF1 and cFos may be important transcription factors downstream of TLR2-Tpl2-ERK in macrophages and DCs for regulation of IL-12/23p40 and other coregulated molecules.

While TLR2-driven ERK signaling regulates many molecules, a select subset of these molecules are enhanced by Tpl2-ERK signaling in DCs but inhibited by ERK signaling in macrophages. These different regulatory patterns may allow them to drive different functional responses in macrophages versus DCs in response to TLR2-Tpl2-ERK signaling, related to the distinct functions of these cell types in the immune system. Speculatively, while ERK-mediated suppression of IL-12 in macrophages may avert tissue damage associated with prolonged inflammation, ERK-mediated enhancement of IL-12 production in DCs may promote priming of Th1 responses and DC migration to draining lymph nodes (39, 41, 42). A greater understanding of the roles that TLR2-dependent ERK signaling plays in different immune cell types may inform the development of more effective cellular responses via host-directed therapy or selected

adjuvant activity for priming responses to infectious pathogens for whom TLR2 signaling is the major mechanism for innate cell recognition.

## MATERIALS AND METHODS

**Animals.** All experiments using animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University. C57BL/6J and *Irf1*$^{-/-}$ mice were acquired from Jackson Laboratories (Bar Harbor, ME, USA). *Tpl2*$^{-/-}$ mice were a generous gift from Philip Tsichlis (Tufts University, Boston, USA) and were generated and back-crossed onto the C57BL/6J genetic background as described previously (43).

**Cells and reagents.** In all components of this study, macrophages and DCs were obtained by culture of bone marrow harvested from mouse femurs and tibias. Bone marrow cells were incubated with ammonium-chloride-potassium (ACK) lysis buffer (Lonza, Walkersville, MD, USA) to lyse red blood cells. Cells were cultured in a humidified incubator at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) for macrophages or Roswell Park Memorial Institute medium (RPMI) (HyClone) for DCs, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 50 $\mu$M 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA), 1 mM sodium pyruvate (HyClone), 10 mM HEPES (HyClone), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (HyClone) (referred to as D10F or complete RPMI, respectively). Macrophage cultures were supplemented with 20% LADMAC cell-conditioned medium (made with a transformed cell line that secretes M-CSF [CSF-1]) (ATCC, Manassas, VA, USA). Macrophage medium was changed once on day 5. DC cultures included 2 $\mu$g/ml Fms-like tyrosine kinase 3 (Flt3) (Bio X Cell, West Lebanon, NH, USA). We chose to study Flt3-stimulated cultures in order to focus on cells with a DC phenotype distinct from macrophages (in contrast, granulocyte macrophage colony-stimulating factor [GM-CSF]-stimulated cultures produce DCs that display greater phenotypic overlap with macrophages) (30, 31). On day 9, cells were plated in 96-well plates in complete D10F without penicillin or streptomycin ($10^6$ cells/ml; 150 $\mu$l) and incubated with 10 ng/ml synthetic lipopeptide Pam3Cys-Ser-$Lys_4$ (P3C) (Invivogen, San Diego, CA, USA) for 2, 4, 6, and/or 24 h. In experiments utilizing U0126, macrophages and DCs were incubated with 10 $\mu$M U0126 (Calbiochem, Billerica, MA, USA) for 30 min prior to and during exposure to P3C. Each condition was performed in triplicate. U0126 stocks were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA); the maximum concentration of DMSO in cell incubations was 1%, and DMSO was included at the same concentration in control incubations without U0126.

**Western blots.** Antibodies for Western blotting were obtained from Cell Signaling Technology (Danvers, MA, USA) and included rabbit anti-phospho ERK1/2 (D13.14.4E), anti-ERK1/2 (137F5), anti-I$\kappa$B$\alpha$ (polyclonal), anti-cFos (9F6), anti-IRF1 (D5E4), anti-$\beta$-actin (D6A8), and horseradish peroxidase-linked goat anti-rabbit IgG (catalog number 7074). Macrophages or DCs were plated, incubated with or without U0126 for 30 min, incubated with or without P3C (in the continuing absence or presence of U0126) for indicated periods, washed in cold phosphate-buffered saline (PBS), and then lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a protease and protein phosphatase inhibitor cocktail (Pierce). The lysates were quantified using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Waltham, MA, USA). Equal protein samples were boiled in reducing sample buffer (Bio-Rad), loaded on SDS-PAGE gels (Bio-Rad), and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were incubated with blocking buffer (5% nonfat dry milk in PBS-0.1% Tween 20) for 1 h at room temperature and then with primary antibodies in blocking buffer overnight at 4°C. Membranes were washed with PBS-0.1% Tween 20, incubated with secondary antibody in blocking buffer, washed, treated with enhanced chemiluminescence reagent (Pierce), and exposed to autoradiography film (Amersham GE Healthcare, Pittsburgh, PA, USA). Films were developed with an automated film processor (Konica Minolta, Wayne, NJ, USA). Photographs of the resulting bands were cropped, aligned, and converted to black and white images. Densitometric quantification was done using Image Studio Lite version 5.2 (Li-Cor Biosciences, Lincoln, NE, USA), and the analyte signal was divided by the $\beta$-actin signal for each lane to calculate normalized expression values. The relative expression was calculated by dividing normalized expression values (e.g., normalized expression of *Tpl2*$^{-/-}$ or wild-type cells at various time points) by the normalized expression of wild-type cells at time zero).

**Enzyme-linked immunosorbent assay.** Macrophages or DCs were plated, cultured overnight, incubated with or without U0126 for 30 min, and then incubated with or without P3C (10 ng/ml) for 24 h in the continuing presence or absence of U0126. Cell-free supernatants were collected and frozen at −80°C until analysis. Duoset IL-10 (DY417) and IL-12/23p40 (DY2398) ELISA kits were obtained from R&D Systems, and the manufacturer protocols were followed. ELISA plates were read using a model 680 microplate reader (Bio-Rad). Cytokine concentrations were determined from a seven-point standard curve from the same plates.

**Reverse transcriptase quantitative PCR.** Macrophages or DCs were plated, cultured overnight, incubated with or without U0126 for 30 min, and incubated with or without P3C (10 ng/ml) in the continuing presence or absence of U0126 for the indicated time points. Cells were lysed with 150 $\mu$l cell lysis buffer from the RNeasy Plus minikit (Qiagen, Valencia, CA, USA). RNA was prepared following the manufacturer's protocol and was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen). Equal amounts of cDNA per condition were amplified by real-time PCR using iQ SYBR green supermix (Bio-Rad) utilizing the primers shown in Table S1 in the supplemental material. Samples were amplified using a hot start at 95°C for 3 min, followed by 50 cycles (10 s at 95°C, 10 s at 59°C, and 30 s at 72°C) with a postamplification melting curve ramping from 65°C to 95°C in increments of 0.5°C

per 5 s. The abundance of each transcript was calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression in each sample using the formula $2^{-[C_{T(\text{target gene})}-C_{T(\text{GAPDH gene})}]}$.

**V-Plex.** Macrophages or DCs were plated, cultured overnight, and then exposed to P3C (10 ng/ml) for 24 h. Cell-free supernatants were collected and frozen at −80°C until analysis. V-Plex kits were obtained from Meso Scale Discovery (Rockville, MD, USA), and the manufacturer's protocols were followed (proinflammatory, number K15048D; cytokine, number K15267D; Th17, number K15246D). V-Plex plates were read using a QuickPlex SG 120 reader (Meso Scale Discovery). Molecule concentrations were determined from a seven-point standard curve from the same plates.

**Statistical tests.** Data were analyzed using Prism 7 (GraphPad, La Jolla, CA, USA). The unpaired Student's *t* test was used to assess significance for individual comparisons, and grouped two-way analysis of variance (ANOVA) was used to compare time course data points.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

**SUPPLEMENTAL FILE 2**, PDF file, 0.3 MB.

**SUPPLEMENTAL FILE 3**, PDF file, 0.1 MB.

**SUPPLEMENTAL FILE 4**, PDF file, 0.7 MB.

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## REFERENCES

1. Akira S, Takeda K. 2004. Toll-like receptor signaling. Nat Rev Immunol 4:499–511. https://doi.org/10.1038/nri1391.
2. Kawasaki T, Kawai T. 2014. Toll-like receptor signaling pathways. Front Immunol 5:461. https://doi.org/10.3389/fimmu.2014.00461.
3. Takeda K, Akira S. 2004. TLR signaling pathways. Semin Immunol 16:3–9. https://doi.org/10.1016/j.smim.2003.10.003.
4. Takeda K, Akira S. 2001. Roles of Toll-like receptors in innate immune responses. Genes Cells 6:733–742. https://doi.org/10.1046/j.1365-2443.2001.00458.x.
5. Richardson ET, Shukla S, Sweet DR, Wearsch PA, Tsichlis PN, Boom WH, Harding CV. 2015. Toll-like receptor 2-dependent extracellular signal-regulated kinase signaling in Mycobacterium tuberculosis-infected macrophages drives anti-inflammatory responses and inhibits Th1 polarization of responding T cells. Infect Immun 83:2242–2254. https://doi.org/10.1128/IAI.00135-15.
6. Bouhamdan M, Bauerfeld C, Talreja J, Beuret L, Charron J, Samavati L. 2015. MEK1 dependent and independent ERK activation regulates IL-10 and IL-12 production in bone marrow derived macrophages. Cell Signal 27:2068–2076. https://doi.org/10.1016/j.cellsig.2015.07.015.
7. Ma X, Yan W, Zheng H, Du Q, Zhang L, Ban Y, Li N, Wei F. 2015. Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells. F1000Res 4:1465. https://doi.org/10.12688/f1000research.7010.1.
8. Saraiva M, O'Garra A. 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol 10:170–181. https://doi.org/10.1038/nri2711.
9. Monje P, Hernández-Losa J, Lyons RJ, Castellone MD, Gutkind JS. 2005. Regulation of the transcriptional activity of c-Fos by ERK: a novel role for the prolyl isomerase PIN1. J Biol Chem 280:35081–35084. https://doi.org/10.1074/jbc.C500353200.
10. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969. https://doi.org/10.1038/nri2448.
11. Sica A, Mantovani A. 2012. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122:787–795. https://doi.org/10.1172/JCI59643.
12. Das S, Cho J, Lambertz I, Kelliher MA, Eliopoulos AG, Du K, Tsichlis PN. 2005. Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. J Biol Chem 280:23748–23757. https://doi.org/10.1074/jbc.M412837200.
13. Gantke T, Sriskantharajah S, Ley SC. 2011. Regulation and function of TPL-2, an IκB kinase-regulated MAP kinase kinase kinase. Cell Res 21:131–145. https://doi.org/10.1038/cr.2010.173.
14. Banerjee A, Gugasyan R, McMahon M, Gerondakis S. 2006. Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. Proc Natl Acad Sci U S A 103:3274–3279. https://doi.org/10.1073/pnas.0511113103.
15. Xu D, Matsumoto ML, McKenzie BS, Zarrin AA. 2018. TPL2 kinase action and control of inflammation. Pharmacol Res 129:188–193. https://doi.org/10.1016/j.phrs.2017.11.031.
16. Caparrós E, Munoz P, Sierra-Filardi E, Serrano-Gómez D, Puig-Kröger A, Rodríguez-Fernández JL, Mellado M, Sancho J, Zubiaur M, Corbí AL. 2006. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. Blood 107:3950–3958. https://doi.org/10.1182/blood-2005-03-1252.
17. Zhang J, Qian X, Ning H, Yang J, Xiong H, Liu J. 2010. Activation of IL-27 p28 gene transcription by interferon regulatory factor 8 in cooperation with interferon regulatory factor 1. J Biol Chem 285:21269–21281. https://doi.org/10.1074/jbc.M110.100818.
18. Molle C, Goldman M, Goriely S. 2010. Critical role of the IFN-stimulated gene factor 3 complex in TLR-mediated IL-27p28 gene expression revealing a two-step activation process. J Immunol 184:1784–1792. https://doi.org/10.4049/jimmunol.0902005.
19. Crabé S, Guay-Giroux A, Tormo AJ, Duluc D, Lissilaa R, Guilhot F, Mavoungou-Bigouagou U, Lefouili F, Cognet I, Ferlin W, Elson G, Jeannin

P, Gauchat J-F. 2009. The IL-27 p28 subunit binds cytokine-like factor 1 to form a cytokine regulating NK and T cell activities requiring IL-6R for signaling. J Immunol 183:7692–7702. https://doi.org/10.4049/jimmunol.0901464.
20. Stumhofer JS, Tait ED, Quinn WJ, III, Hosken N, Spudy B, Goenka R, Fielding CA, O'Hara AC, Chen Y, Jones ML, Saris CJ, Rose-John S, Cua DJ, Jones SA, Elloso MM, Grötzinger J, Cancro MP, Levin SD, Hunter CA. 2010. A Role for IL-27p28 as an antagonist of gp130-mediated signaling. Nat Immunol 11:1119–1126. https://doi.org/10.1038/ni.1957.
21. Tighe RM, Liang J, Liu N, Jung Y, Jiang D, Gunn MD, Noble PW. 2011. Recruited exudative macrophages selectively produce CXCL10 after noninfectious lung injury. Am J Respir Cell Mol Biol 45:781–788. https://doi.org/10.1165/rcmb.2010-0471OC.
22. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. 2002. IFN-$\gamma$-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector t cell generation and trafficking. J Immunol 168:3195–3204. https://doi.org/10.4049/jimmunol.168.7.3195.
23. Coward WR, Watts K, Feghali-Bostwick CA, Jenkins G, Pang L. 2010. Repression of IP-10 by interactions between histone deacetylation and hypermethylation in idiopathic pulmonary fibrosis. Mol Cell Biol 30:2874–2886. https://doi.org/10.1128/MCB.01527-09.
24. Zhang J, Qian X, Ning H, Eickhoff CS, Hoft DF, Liu J. 2011. Transcriptional suppression of IL-27 production by Mycobacterium tuberculosis-activated p38 MAPK via inhibition of AP-1 binding. J Immunol 186:5885–5895. https://doi.org/10.4049/jimmunol.1003447.
25. Boonstra A, Rajsbaum R, Holman M, Marques R, Asselin-Paturel C, Pereira JP, Bates EE, Akira S, Vieira P, Liu YJ, Trinchieri G, O'Garra A. 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 177:7551–7558. https://doi.org/10.4049/jimmunol.177.11.7551.
26. Zhou L, Nazarian AA, Smale ST. 2004. Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. Mol Cell Biol 24:2385–2396. https://doi.org/10.1128/mcb.24.6.2385-2396.2004.
27. Kaiser F, Cook D, Papoutsopoulou S, Rajsbaum R, Wu X, Yang H, Grant S, Ricciardi-Castagnoli P, Tsichlis PN, Ley SC, O'Garra A. 2009. TPL-2 negatively regulates interferon-$\beta$ production in macrophages and myeloid dendritic cells. J Exp Med 206:1863–1871. https://doi.org/10.1084/jem.20091059.
28. Watford WT, Hissong BD, Durant LR, Yamane H, Muul LM, Kanno Y, Tato CM, Ramos HL, Berger AE, Mielke L, Pesu M, Solomon B, Frucht DM, Paul WE, Sher A, Jankovic D, Tsichlis PN, O'Shea JJ. 2008. Tpl2 kinase regulates T cell interferon-y production and host resistance to *Toxoplasma gondii*. J Exp Med 205:2803–2812. https://doi.org/10.1084/jem.20081461.
29. Sugimoto K, Ohata M, Miyoshi J, Ishizaki H, Tsuboi N, Masuda A, Yoshikai Y, Takamoto M, Sugane K, Matsuo S, Shimada Y, Matsuguchi T. 2004. A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation. J Clin Invest 114:857–866. https://doi.org/10.1172/JCI20014.
30. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol 179:7577–7584. https://doi.org/10.4049/jimmunol.179.11.7577.
31. Na YR, Jung D, Gu GJ, Seok SH. 2016. GM-CSF grown bone marrow derived cells are composed of phenotypically different dendritic cells and macrophages. Mol Cells 39:734–741. https://doi.org/10.14348/molcells.2016.0160.
32. Morris SMJ, Kepka-Lenhart D, Chen L. 1998. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol 275:E740–E747. https://doi.org/10.1152/ajpendo.1998.275.5.E740.
33. Nishimura H, Rosenblum WI, Nelson GH, Boynton S. 1991. Agents that modify EDRF formation alter antiplatelet properties of brain arteriolar endothelium in vivo. Am J Physiol 261:H15–H21. https://doi.org/10.1152/ajpheart.1991.261.1.H15.
34. Berka V, Wu G, Yeh HC, Palmer G, Tsai AL. 2004. Three different oxygen-induced radical species in endothelial nitric oxide synthase oxygenase domain under regulation by L-arginine and tetrahydrobiopterin. J Biol Chem 279:32243–32251. https://doi.org/10.1074/jbc.M404044200.
35. Narita Y, Kitamura H, Wakita D, Sumida K, Masuko K, Terada S, Nakano K, Nishimura T. 2013. The key role of IL-6–arginase cascade for inducing dendritic cell–dependent CD4+ T cell dysfunction in tumor-bearing mice. J Immunol 190:812–820. https://doi.org/10.4049/jimmunol.1103797.
36. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 164:6166–6173. https://doi.org/10.4049/jimmunol.164.12.6166.
37. Dong S, Zhang X, He Y, Xu F, Li D, Xu W, Wang H, Yin Y, Cao J. 2013. Synergy of IL-27 and TNF-$\alpha$ in regulating CXCL10 expression in lung fibroblasts. Am J Respir Cell Mol Biol 48:518–530. https://doi.org/10.1165/rcmb.2012-0340OC.
38. Martin E, Nathan C, Xie QW. 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J Exp Med 180:977–984. https://doi.org/10.1084/jem.180.3.977.
39. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol 171:4984–4989. https://doi.org/10.4049/jimmunol.171.10.4984.
40. Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, Maliszewski C, Akira S, Pulendran B. 2004. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol 172:4733–4743. https://doi.org/10.4049/jimmunol.172.8.4733.
41. Kapsenberg ML. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 3:984–993. https://doi.org/10.1038/nri1246.
42. Martin-Fontecha A, Sebastiani S, Höpken UE, Uguccioni M, Lipp M, Lanzavecchia A, Sallusto F. 2003. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med 198:615–621. https://doi.org/10.1084/jem.20030448.
43. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103:1071–1083. https://doi.org/10.1016/S0092-8674(00)00210-5.