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MSK1 and MSK2 Inhibit Lipopolysaccharide-Induced Prostaglandin Production via an Interleukin-10 Feedback Loop

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Prostaglandin production is catalyzed by cyclooxygenase 2 (cox-2). We demonstrate here that MSK1 and MSK2 (MSK1/2) can exert control on the induction of cox-2 mRNA by Toll-like receptor (TLR) agonists. In the initial phase of cox-2 induction, MSK1/2 knockout macrophages confirmed a role for MSK in the positive regulation of transcription. However, at later time points both lipopolysaccharide (LPS)-induced prostaglandin and cox-2 protein levels were increased in MSK1/2 knockout. Further analysis found that while MSKs promoted cox-2 mRNA transcription, following longer LPS stimulation MSKs also promoted degradation of cox-2 mRNA. This was found to be the result of an interleukin 10 (IL-10) feedback mechanism, with endogenously produced IL-10 promoting cox-2 degradation. The ability of IL-10 to do this was dependent on the mRNA binding protein TTP through a p38/MK2-mediated mechanism. As MSKs regulate IL-10 production in response to LPS, MSK1/2 knockout results in reduced IL-10 secretion and therefore reduced feedback from IL-10 on cox-2 mRNA stability. Following LPS stimulation, this increased mRNA stability correlated to an elevated induction of both of cox-2 protein and prostaglandin secretion in MSK1/2 knockout macrophages relative to that in wild-type cells. This was not restricted to isolated macrophages, as a similar effect of MSK1/2 knockout was seen on plasma prostaglandin E_2 (PGE_2) levels following intraperitoneal injection of LPS.

Prostaglandins are a class of lipid-derived mediators that play an important role in regulation of the vascular and immune systems. Prostaglandins are synthesized from arachidonic acid, which is produced from phospholipids by the action of phospholipase A2. Arachidonic acid is next converted into prostaglandin H2 (PGH_2) via the action of cyclooxygenases. This is the rate-limiting step in prostaglandin synthesis, and PGH_2 is next quickly converted into thromboxane, prostacyclin, or prostaglandin D, E, or F via the action of specific prostaglandin synthases. Two main isoforms of cyclooxygenase exist in mammalian cells, cyclooxygenase 1 (cox-1; PTGS1) and cox-2 (PTGS2). cox-1 is constitutively expressed in most tissues, while cox-2 is strongly upregulated in response to inflammatory stimuli. Interest in cox-2 was prompted by the finding that cyclooxygenases were a target of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, which has subsequently resulted in the development of new, more specific cyclooxygenase inhibitors (reviewed in references 1–4).

Given this importance of prostaglandins, understanding how cox-2 levels are regulated is an interesting issue. The regulation of cox-2 mRNA transcription is complex, and several signaling pathways have been reported to be involved (reviewed in references 5 and 6). The cox-2 promoter contains binding sites for several transcription factors, including NF-κB, C/EBP, AP-1, and CREB, and the relative importance of these transcription factors may vary depending on the cell type and stimuli used. In macrophages, cox-2 mRNA levels are increased following stimulation with Toll-like receptor (TLR) agonists such as lipopolysaccharide (LPS) (7–10). This increase is prolonged, and the initial phase of induction is reported to require the proximal cyclic AMP response (CRE) site in the cox-2 promoter whereas the later phase requires C/EBP (11). Classically, CRE sites bind the transcription factor CREB or the related protein ATF1. In line with a role for CREB in cox-2 induction, inhibitors that block the phosphorylation of CREB have been shown to inhibit cox-2 mRNA induction (9). The CRE consensus sequence, however, is similar to that for the binding site for AP-1 proteins such as c-jun and c-fos. It has been also proposed that in macrophages the CRE site can be occupied by an AP-1 complex, as a cox-2 reporter could be repressed by a dominant negative c-jun construct (12). A role for c-jun in cox-2 induction has also been shown in other cell types (13, 14). A recent study reporting a more detailed characterization of the mouse cox-2 promoter has helped resolve these issues. In addition to the proximal CRE situated approximately 50 bp upstream of the transcription start site, there is also a 2nd distal CRE site 430 bp upstream of the start site, as well as an AP-1-like element 5′ to the proximal CRE site. Mutation of any of these sites in a cox-2 promoter reporter construct reduced the induction of the reporter by LPS in Raw264.7 cells, while chromatin immunoprecipitation (ChIP) analysis demonstrated that both CREB and c-jun were bound to the endogenous cox-2 promoter following LPS stimulation (15).

CREB is activated by phosphorylation on Ser133, which creates a binding site for the coactivators CBP and p300 (16–19). In response to LPS, CREB phosphorylation is dependent on the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 mito-

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TABLE 1 Primer sequences used for Q-PCR

Gene or RNA	Sequence	
	Sense	Antisense
cox-2 mRNA	AATATCAGGTCATTGGTGGAGAGG	TCAGACCAGGCACCAGACC
cox-2 transcript	TAAGCGAGGACCTGGGTTCAC	GGGTGTGATTTGTTTGGCATGG
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GAPDH gene	GCCTTCCGTGTTCCTACCC	TGCCTGCTTCACCACCTTC

gen-activated protein kinase (MAPK) pathways (9, 20, 21). ERK1/2 and p38 do not phosphorylate CREB directly but instead activate the downstream kinases MSK1 and MSK2. MSKs directly phosphorylate CREB on Ser133, and mouse knockouts in which both MSK1 and MSK2 are deleted have greatly reduced levels of CREB phosphorylation relative to wild-type cells in response to TLR agonists (9, 20, 21). MSKs are phosphorylated and activated by ERK1/2 or p38 on the same sites (22, 23). As a result, for stimuli such as LPS that activate both ERK1/2 and p38, inhibition of either the ERK1/2 or p38 MAPK cascades causes only a partial reduction in MSK activation. To completely block MSK activation in these circumstances requires the simultaneous inhibition of both the ERK1/2 and p38 MAPK cascades (22, 24).

Both the ERK1/2 and p38 MAPK cascades have been shown to regulate cox-2 mRNA transcription in response to TLR agonists (7–10, 25, 26). Interestingly, the combined inhibition of both the ERK1/2 and p38 MAPK cascades is required to completely block LPS-induced cox-2 mRNA transcription in Raw264.7 cells, a finding that would be consistent with a role for MSKs. Further to this, cox-2 mRNA transcription is also blocked by H89 and Ro 318220 (25), small-molecule kinase inhibitors that can inhibit MSK activity. A caveat with these compounds, however, is that they also inhibit a significant number of other kinases, including the ERK1/2-activated kinase RSK, and therefore it is difficult to make definitive conclusions based on their use alone (27).

We have previously generated mouse knockouts that lack both MSK1 and MSK2, and these mice are viable and fertile (28). The MSK1/2 knockout mice are, however, hypersensitive to LPS-induced endotoxic shock (21). In line with this, in response to LPS, MSK-deficient macrophages secrete elevated levels of TNF, interleukin 6 (IL-6), and IL-12 compared to wild-type cells. In response to LPS, the secretion of the anti-inflammatory cytokines IL-10 and IL-1ra, as well as induction of the phosphatase DUSP1 (mkp1), which limits p38 and c-Jun N-terminal kinase (JNK) activation, was significantly reduced by the knockout of MSK1 and MSK2 (21, 29, 30). The ability of MSKs to regulate IL-10 and dual-specificity phosphatase (DUSP) gene transcription in response to LPS in macrophages reflects a role for MSK-mediated CREB phosphorylation in the transcription of these genes (21, 31). Together these findings demonstrate a key role for MSKs in limiting the proinflammatory effects of TLR signaling.

As prostaglandins play important roles in immunity and given the evidence pointing to a role of MSKs in cox-2 mRNA transcription, we therefore looked at the effect of MSK1/2 knockout on the regulation of cox-2 expression and prostaglandin secretion in macrophages.

MATERIALS AND METHODS

Animals. MSK1/2 knockout, TTP knockout, IL-10 knockout, and CREB Ser133Ala knock-in mice have been described previously (28, 32–35). All experiments were carried out on mice that had been back-crossed with C57BL/6J mice for a minimum of 12 generations for IL-10 and MSK1/2 or 6 generations for CREB. Animals were maintained under specific-pathogen-free conditions in line with European Union and United Kingdom regulations. To prevent the development of inflammatory bowel disease in IL-10 knockouts, these mice (along with their littermate controls) were provided with drinking water supplemented with antibiotics from birth. All procedures were subject to local ethical review and carried out under a United Kingdom project license.

Cell culture. To generate bone marrow-derived macrophages (BMDMs), bone marrow was flushed out from the femurs of one mouse in phosphate-buffered saline (PBS). Cells were pelleted by centrifugation and cultured on bacterial grade plastic for 7 days in BMDM medium (Dulbecco modified Eagle medium [DMEM] supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin, and 5 ng/ml recombinant macrophage colony-stimulating factor [mCSF]). Cells were then detached by scraping in Versene (Invitrogen) and replated on tissue culture plastic in BMDM medium. For inhibitor studies, cells were treated with either 500 nM INCB-018424 (ruxolitinib), 10 μM SB-747651A, 2 μM PD184352, 5 μM SB 203580, or 10 μM PF3644022 for 1 h before stimulation. Details of the selectivity of these inhibitors have been published previously (27, 36–38). Cells were stimulated with either 100 ng/ml LPS (Sigma), 2 μM CpG (ODN 1826), 10 μg/ml poly(I·C), or 100 ng/ml recombinant mouse IL-10. When required, actinomycin D (1 μg/ml) was used to inhibit transcription.

***In vivo* LPS injection.** Mice 6 to 8 weeks of age were injected intraperitoneally with LPS (1.8 mg/kg; Sigma). Mice were killed 3 h after LPS injection, and plasma samples were collected for PGE_2 analysis.

Immunoblotting. For immunoblotting, cells were lysed in SDS sample or Triton lysis buffer (25 mM HEPES, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% [vol/vol] glycerol, and 1% Triton X-100), run on 10% polyacrylamide gels, and transferred onto nitrocellulose membranes using standard techniques. Antibodies against cox-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Santa Cruz and Cell Signaling, respectively. The specificity of the cox-2 antibody was confirmed by small interfering RNA (siRNA)-mediated knockdown of cox-2 (data not shown). Blots were quantified using Alexa Fluor secondary antibodies and the Odyssey Imaging system from Li-Cor Biosciences.

Expression analysis. Total RNA was isolated using microRNeasy kits (Qiagen) and reverse transcribed using iScript (Bio-Rad) or qScript (VWR). mRNA levels were determined by quantitative PCR (Q-PCR) using Sybr green-based detection methods. Primers used are listed in Table 1. The GAPDH gene and/or 18S rRNA was used as a normalization control, and fold stimulations were calculated relative to wild-type unstimulated BMDMs as described previously (39).

Prostaglandin and IL-10 ELISAs. Release of prostaglandin E_2 was measured using a PGE_2 enzyme immunoassay (EIA) enzyme-linked immunosorbent assay (ELISA) kit from Cayman Chemicals, and release of prostaglandin D, prostaglandin F, and prostacyclin was measured using EIA ELISA kits from Assay Designs. IL-10 was measured using a Luminex-based assay (Bio-Rad).

RESULTS

Loss of MSK1 and -2 results in elevated production of prostaglandins in response to LPS. Previous work has suggested that MSKs, via CREB, would be required to promote cox-2 mRNA transcription in LPS-stimulated macrophages (9). cox-2 is the rate-limiting enzyme in prostaglandin synthesis, and thus a decrease in the induced levels of cox-2 protein in macrophages should lead to a decrease in prostaglandin secretion. We therefore hypothesized that knockout of MSK1 and MSK2 would decrease prostaglandin production by macrophages. To examine this, BMDMs were isolated from wild-type and MSK1/2 double-knockout mice and prostaglandin production was analyzed following stimulation with the TLR4 agonist LPS. cox-2 catalyzes the production of PGH_2, which is then converted by prostaglandin synthases to mature prostaglandins, including PGE_2, PGD_2 (which is further processed to give PGJ_2), PGI (which is further converted to PGF_1), and PGF_2. In wild-type BMDMs LPS stimulation was able to stimulate the release of PGE_2, PGJ_2, PGF_1, and PGF_2 into the culture media (Fig. 1A to D). Unexpectedly, higher levels of PGE_2, PGJ_2, PGF_1, and PDF_2 secretion were observed from MSK1/2 knockout cells relative to wild-type cells after 16 to 24 h of LPS stimulation. The effect of MSK1/2 knockout was not restricted to isolated macrophages and also occurred *in vivo*. In response to an intraperitoneal injection of LPS, MSK1/2 knockout mice had an elevated level of PGE_2 present in their serum compared to wild-type mice (Fig. 1E).

As these findings could indicate that MSK1/2 knockout increased rather than decreased TLR-induced cox-2 protein levels, we next examined the ability of LPS to induce cox-2 protein levels. Wild-type macrophages induced the expression of cox-2 protein in response to LPS, with the maximal levels seen at 8 h after stimulation (Fig. 2A). While MSK1/2 knockout did not affect the induction of cox-2 protein at 4 h, at later time points the MSK1/2 knockout cells expressed higher levels of cox-2 than wild-type controls (Fig. 2A). To determine if this effect is common to all TLRs, we also looked at the induction of cox-2 protein in response to the TLR9 agonist CpG and the TLR3 agonist poly(I·C). While CpG stimulation also increased the levels of cox-2 protein, in contrast to LPS, MSK1/2 knockout did not affect cox-2 protein levels relative to those in wild-type cells (Fig. 2B). Poly(I·C) did not induce measurable levels of cox-2 protein in these experiments (data not shown).

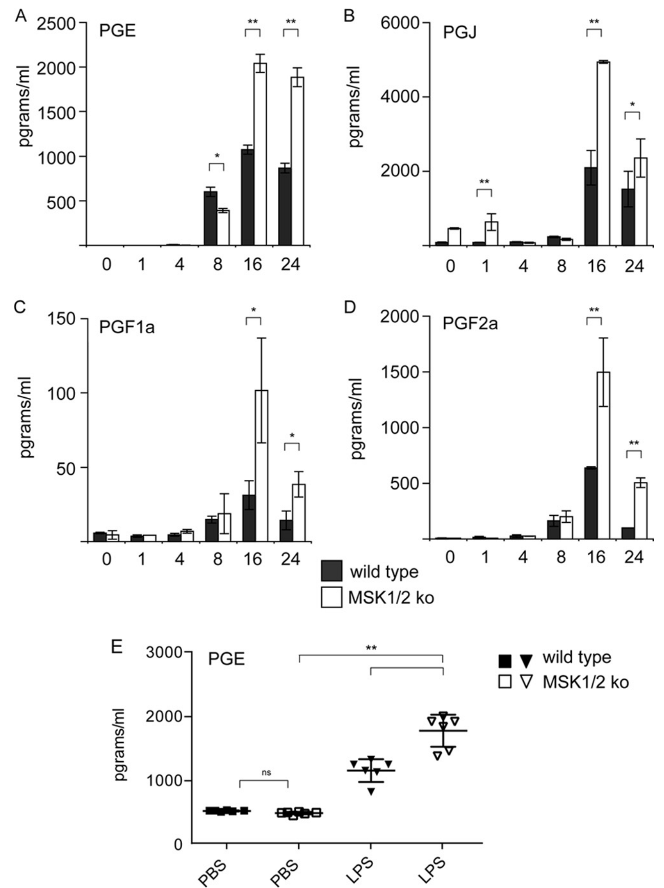

FIG 1 Prostaglandin secretion from LPS stimulated BMDMs. (A to D) Wild-type and MSK1/2 knockout (ko) BMDMs were prepared and stimulated with 100 ng/ml LPS for the indicated times. The levels of PGE (A), PGJ (B), PGF1a (C), and PGF2a (D) were determined by ELISA. Error bars represent the standard deviations of independent stimulations of BMDMs from 4 mice per genotype, and the results are representative of three independent experiments. (E) Wild-type or MSK1/2 knockout mice were given an intraperitoneal injection of 1.8 mg/kg LPS in 100 μl of PBS or a PBS control. After 3 h, blood samples were taken from the mice and the serum levels of PGE determined by ELISA. Open symbols represent the measurements on individual wild-type mice, and closed symbols represent the measurements on individual MSK1/2 knockout mice. Black bars represent the average and standard deviation for each group. Student's *t* tests were carried out on all results: *, $P < 0.05$; **, $P < 0.01$.

MSKs exert both positive and negative effects on cox-2 mRNA levels. The above results demonstrate that MSK1/2 knockout results in an increased induction of cox-2 protein and prostaglandin secretion relative to those in wild-type cells following LPS stimulation (Fig. 1 and 2). This is in contrast to the predicted role for MSKs in promoting cox-2 mRNA transcription via the phosphorylation of CREB (9). We therefore examined the roles of CREB and MSKs in regulating cox-2 mRNA induction. MSKs are activated downstream of both the ERK1/2 and p38α MAPK cascades in response to the TLR4 agonist LPS in bone marrow-derived macrophages (21). To determine the effects of ERK1/2 and p38 on cox-2 mRNA transcription, wild-type BMDMs were treated with the MKK1/2 inhibitor PD184352 (which blocks the activation of ERK1/2) or the p38α/β inhibitor SB203580 before stimulation with LPS. The transcription of cox-2 mRNA was reduced by SB203580 alone but unaffected by PD184352 (Fig. 3A). However, a combination of both PD184352 and SB203580 was required to completely block cox-2 mRNA induction, a requirement similar to what has previously been found to be necessary to completely block MSK1 activation in response to LPS (21). To further examine this, cells were treated with SB-747651A, a recently described inhibitor of MSKs (37). Consistent with its ability to inhibit MSK activity, SB-747651A was able to block the LPS-induced phosphorylation of CREB and ATF1 in BMDMs (Fig. 3B). SB-747651A did not affect the activation of the upstream ERK1/2 or p38α MAPKs or their ability to phosphorylate MSK1 on Thr581, a site critical for MSK activation (Fig. 3B). SB-747651A inhibited the induction of cox-2 mRNA following 1 h of LPS stimulation (Fig. 3C); however, interestingly, SB-747651A caused an increase in cox-2 mRNA following 8 h of LPS stimulation (Fig. 3C). To confirm these results, we also examined cox-2 mRNA induction in MSK1/2 knockout cells. Stimulation with LPS resulted in a decrease in cox-2 mRNA levels in MSK1/2

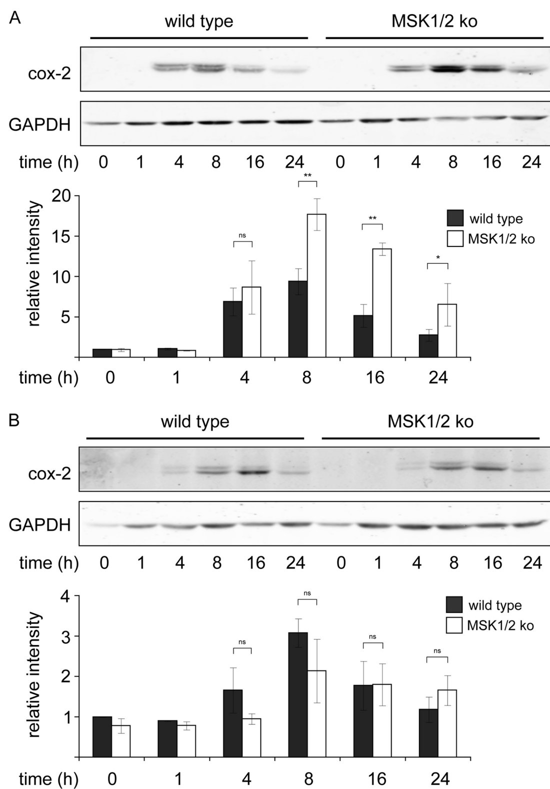

FIG 2 MSK knockout results in elevated levels of cox-2 protein following LPS stimulation. Wild-type and MSK1/2 knockout (ko) BMDMs were prepared and stimulated with 100 ng/ml LPS (A) or 2 μM CpG (B) for the indicated times. Cells were lysed and the levels of cox-2 and GAPDH determined by immunoblotting. Example blots are shown in the upper panel. Graphs represent the relative intensities of the cox-2 bands using GAPDH levels to correct for loading. Quantification was carried out as described in Materials and Methods. Error bars represent the standard deviation on blots from 3 independent preparations of BMDMs per genotype. Student's *t* tests were carried out on all results: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

knockouts relative to those in wild-type cells following 1 or 4 h of LPS stimulation (Fig. 4A). In agreement with the SB-747651A results, cox-2 mRNA levels were actually higher in the MSK1/2 knockout cells than in wild-type controls at 8 to 24 h of LPS stimulation. cox-2 mRNA transcription was also induced by the TLR9 agonist CpG or TLR3 agonist poly(I·C) in BMDMs. At early time points, MSK1/2 knockout reduced the induction of cox-2 mRNA in response to either CpG or poly(I·C); however, by 12 h the levels of cox-2 mRNA were similar in wild-type and knockout cells (Fig. 4B and C).

A role for CREB in regulating cox-2 mRNA transcription has been proposed (9, 15). As MSKs can phosphorylate CREB on Ser133, this provides a potential mechanism by which MSK1 and MSK2 could regulate cox-2 mRNA transcription. To determine if CREB phosphorylation was required for cox-2 mRNA transcription in this system, BMDMs were isolated from mice with a Ser-to-Ala mutation of Ser133 in the endogenous CREB gene. In the CREB Ser133Ala knock-in macrophages, cox-2 mRNA induction following 1 h of LPS stimulation was lower than in wild-type cells but was higher in the knock-in by 8 h of stimulation (Fig. 5A). These results show a trend similar to that found with the MSK1/2 knockout cells (Fig. 4A). The induction of cox-2 mRNA by CpG and poly(I·C) was also lower in the CREB Ser133Ala cells at early time points but normal at later time points (Fig. 5B and C), which again mirrors what was seen in the MSK1/2 knockout cells.

MSKs promote cox-2 mRNA degradation via an IL-10-mediated feedback loop. The above data indicate that MSKs can negatively regulate cox-2 induction in BMDMs in response to LPS but that this effect is less apparent with CpG or poly(I·C). MSKs regulate the production of IL-10 in BMDMs in response to LPS (21). Previous reports have indicated that IL-10 can inhibit the induction of cox-2 mRNA in macrophages (40, 41), suggesting a mechanism by which MSKs could negatively regulate cox-2 induction. In agreement with this, CpG and poly(I·C) stimulation resulted in less IL-10 secretion than LPS (Fig. 6A). In addition to this, increased cox-2 mRNA induction was observed in IL-10 knockout macrophages in response to LPS but was not apparent in response to CpG or poly(I·C) stimulation (Fig. 6B). We therefore investigated the possibility that MSKs regulate cox-2 mRNA levels via an IL-10 feedback loop. IL-10 alone did not increase cox-2 mRNA levels in macrophages (Fig. 7A); however, costimulation with IL-10 and LPS for 8 h, compared to stimulation with LPS alone, showed that IL-10 was able to repress the LPS-induced cox-2 mRNA levels (Fig. 7A) and resulted in decreased PGE_2 release. Analysis of IL-10 knockout BMDMs gave analogous results, with a large increase in the IL-10 knockout cells at later time points (Fig. 7B). To further determine if the increased cox-2 mRNA levels seen in MSK1/2 knockout relative to wild-type cells after prolonged LPS stimulation were due to MSK1/2-dependent IL-10 regulation, an IL-10 neutralizing antibody was used (Fig. 7C). Consistent with the earlier data, MSK1/2 knockout BMDMs had higher levels of cox-2 mRNA relative to wild-type cells after 16 h of LPS stimulation, and this was unaffected by the addition of an isotype control antibody. Stimulation with LPS in the presence of the IL-10 neutralizing antibody resulted in an increase in cox-2 mRNA in both wild-type and MSK1/2 knockout cells compared to results with LPS alone. Significantly, however, the overall levels of cox-2 mRNA in MSK1/2 knockout cells in the presence of the IL-10 neutralizing antibody were slightly lower than those in wild-type cells treated with the IL-10 antibody. This would be consistent with MSK negatively regulating cox-2 mRNA levels via an IL-10 feedback loop. Comparison of IL-10/MSK1/2 triple-knockout BMDMs with IL-10 knockout cells after 8 h of LPS stimulation gave results that were similar to those found with the use of the IL-10 neutralizing antibody (Fig. 7D). The trend for a decrease in cox-2 mRNA levels in the MSK1/2 knockouts when IL-10 is deleted or inhibited is in line with the positive regulatory role that MSKs play in initial cox-2 mRNA transcription in response to LPS (Fig. 3 and 4). The functional implication of IL-10 regulation of cox-2 mRNA via MSKs was further examined by the measurement of PGE_2 secretion by wild-type and MSK1/2 knockout BMDMs in response to exogenous IL-10 in combination with LPS for 16 h. This showed that costimulation, compared to stimulation with LPS alone, resulted in decreased PGE_2 release in the MSK1/2 knockout cells (Fig. 7E).

Steady-state mRNA levels are affected by changes in the rates of both mRNA transcription and degradation. As the primary unspliced transcript tends to be processed at a rate much higher than the rate of mRNA degradation, measurement of primary transcript levels can give a better indication of transcriptional rates than measurement of mRNA levels. We therefore examined the effect of MSK1/2 knockout on the cox-2 primary transcript. This

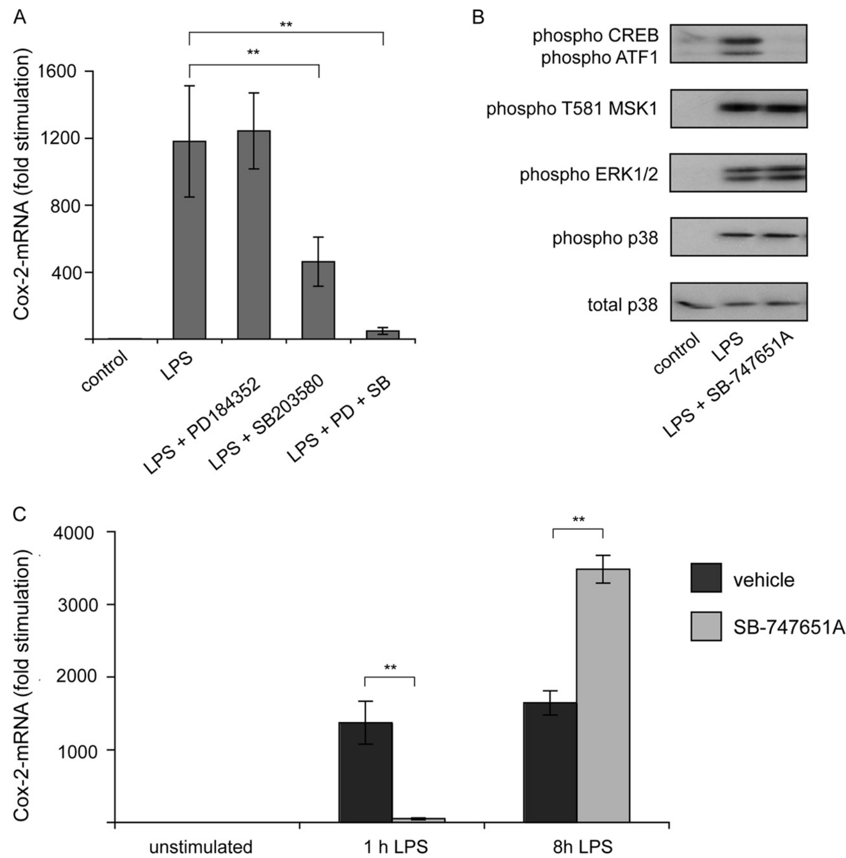

FIG 3 LPS-stimulated cox-2 mRNA transcription requires MAPK signaling pathways. (A) Wild-type BMDMs were preincubated for 1 h with 2 μM PD184352 or 5 μM SB203580 where indicated. Cells were then stimulated with 100 ng/ml LPS for 1 h, total RNA was isolated, and the levels of cox-2 mRNA were determined by quantitative PCR (Q-PCR). (B) BMDMs were preincubated with 10 μM SB-747651A for 30 min where indicated and then stimulated for 30 min with 100 ng/ml LPS or left unstimulated (control). Levels of phospho-CREB/ATF1, -MSK1, -ERK1/2, and -p38 as well as total p38 were determined by immunoblotting. (C) As for panel B except that cells were stimulated with for 1 h or 8 h with LPS, total RNA was isolated, and cox-2 mRNA levels determined by Q-PCR. Error bars represent the standard deviation on blots from 4 independent preparations of BMDMs per genotype. Student's *t* tests were carried out: **, $P < 0.01$.

revealed that at early time points, MSK1/2 knockout reduced the ability of LPS to increase the levels of the cox-2 mRNA primary transcription relative to those in wild-type cells (Fig. 8A). At late time points the increase in the primary cox-2 transcript in MSK1/2 knockout relative to that in wild-type cells was less than that seen for the cox-2 mRNA (Fig. 4A and 8A). This indicates that MSKs could control cox-2 mRNA levels, at least in part, via regulating mRNA stability. To examine this, BMDMs were stimulated with LPS for 6 h and then actinomycin D was added to block transcription. Following this, degradation of cox-2 mRNA was measured over time. As expected, in wild-type cells the cox-2 primary transcript levels decreased very rapidly relative to levels of cox-2 mRNA following addition of actinomycin D (Fig. 8B). Knockout of MSK1/2 increased the stability of the cox-2 mRNA compared to that in wild-type cells (Fig. 8C). Consistent with the hypothesis that MSKs regulate cox-2 mRNA stability via an IL-10 feedback loop, addition of exogenous IL-10 to the MSK1/2 knockout macrophages at the same time as actinomycin D decreased the stability of cox-2 mRNA to a level similar to that observed in wild-type cells (Fig. 8C). If MSKs regulate cox-2 mRNA stability via regulating the production of IL-10 and not via a direct effect, it would also be predicted that MSK1/2 knockout would have little effect on cox-2 mRNA stability at early time points following LPS stimulation, before significant amounts of IL-10 are secreted by the macrophage. In agreement with this, when BMDMs were stimulated for only 1 h before addition of actinomycin D, MSK1/2 knockout did not affect cox-2 mRNA stability (Fig. 8D).

If the model in which MSKs regulate cox-2 mRNA stability via IL-10 is correct, then IL-10 knockout cells should show effects similar to or greater than those in the MSK1/2 knockout macrophages. Similar to MSK1/2 knockout, IL-10 knockout resulted in increased stability of cox-2 mRNA following a 6-h LPS stimulation (Fig. 8E). IL-10 stimulates the activation of Jak1/Tyk2 kinases, which mediate many of the cellular effects of IL-10. INCB-018424 (ruxolitinib) is a selective Jak inhibitor (36), and we therefore used this compound to determine the roles of Jaks in regulating cox-2 mRNA stability. IL-10 knockout macrophages were stimulated for 6 h with LPS before the addition of actinomycin D, and then cox-2 mRNA stability was measured over time. Addition of exogenous IL-10 at the same time as actinomycin D decreased cox-2 mRNA stability, and this effect was impaired by the addition of INCB-018424 (Fig. 8F). We also examined the effect of IL-10 knockout on the induction of the cox-2 primary transcript by LPS. Interestingly, IL-10 knockout also caused an increase in the levels of cox-2 primary transcript following prolonged LPS stimulation relative to those in wild-type cells. This

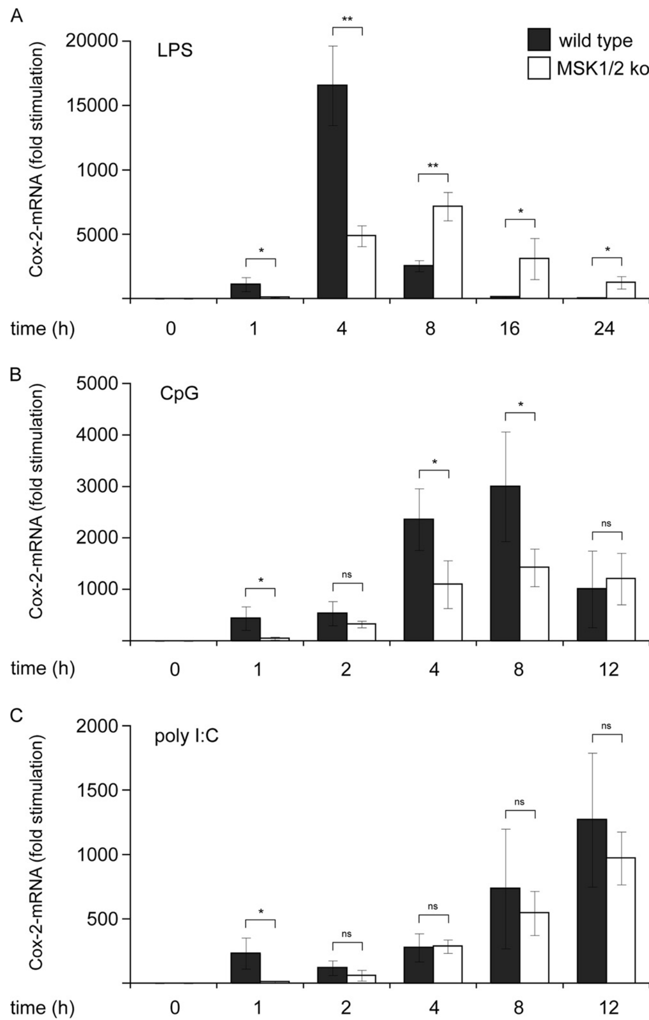

FIG 4 Effect of MSK knockout (ko) on cox-2 mRNA levels. Wild-type or MSK1/2 knockout BMDMs were prepared and stimulated with 100 ng/ml LPS (A), 2 μM CpG (B), or 10 μg/ml poly(I·C) (C) for the indicated times. Cells were then lysed, total RNA was isolated, and the levels of cox-2 mRNA were determined by quantitative PCR (Q-PCR). Fold induction of cox-2 mRNA levels was determined relative to the 0-h wild-type BMDMs using 18S rRNA as a reference. Error bars represent the standard deviation of independent preparations of BMDMs from 4 mice per genotype. Student's *t* tests were carried out on all results: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

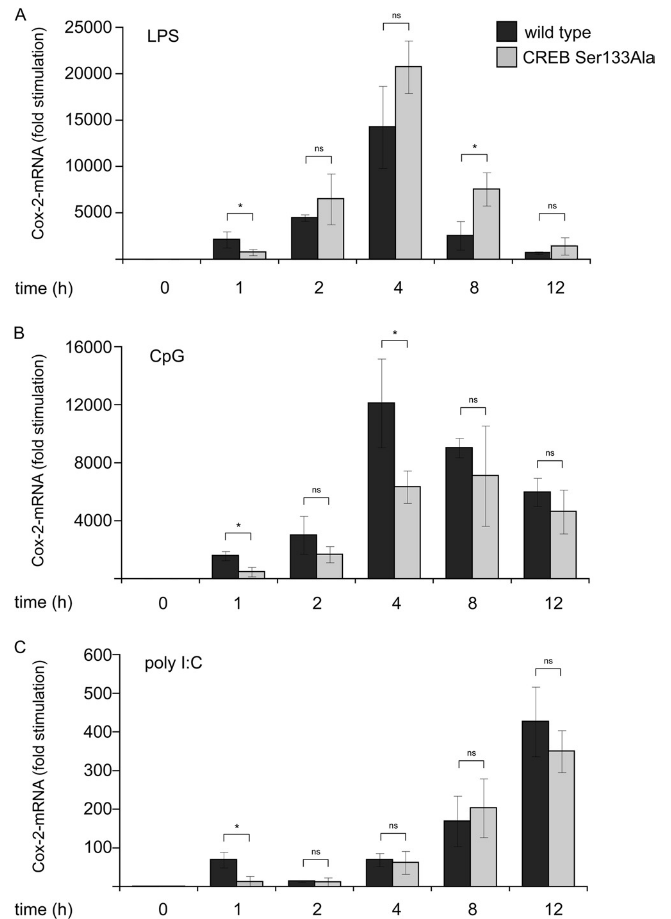

FIG 5 Effect of CREB Ser133Ala knock-in on cox-2 mRNA levels. Wild-type and CREB Ser133Ala knock-in BMDMs were prepared and stimulated with 100 ng/ml LPS (A), 2 μM CpG (B), or 10 μg/ml poly(I·C) (C) for the indicated times. Cells were then lysed, total RNA was isolated, and the levels of cox-2 mRNA were determined by quantitative PCR (Q-PCR). Fold induction of cox-2 mRNA levels was determined relative to the 0-h wild-type BMDMs using 18S rRNA as a reference. Error bars represent the standard deviation of independent preparations of BMDMs from 4 mice per genotype. Student's *t* tests were carried out on all results: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

suggests that IL-10 may also directly target the transcription of cox-2 mRNA in addition to regulating the mRNA stability (Fig. 8G). This effect was more pronounced than what was observed in the MSK1/2 knockouts (Fig. 8A) and may be explained by IL-10 production not being completely blocked by MSK1/2 knockout (Fig. 6) (21).

IL-10 regulates cox2 mRNA stability via p38α and TTP. The cox-2 mRNA 3′ untranslated region (UTR) contains AU-rich elements that have the potential to interact with mRNA binding proteins that control message stability (8, 42–44). One such mRNA binding protein is TTP (Zfp36). We therefore examined the effect of TTP knockout on cox-2 mRNA stability. In response to stimulation with LPS, followed by an actinomycin chase, the cox-2 mRNA was more stable in TTP knockout cells than in wild-type controls (Fig. 9A). Consistent with the results of previous experiments, addition of exogenous IL-10 at the same time as actinomycin D to wild-type cells reduced the stability of cox-2 mRNA. In contrast, IL-10 addition had no effect on cox-2 mRNA stability in the TTP knockout cells (Fig. 9A). The function of TTP can be inhibited by phosphorylation by MK2, a kinase activated downstream of p38α MAPK (45–47). We therefore examined whether IL-10 could still regulate cox-2 mRNA stability following inhibition of the p38α MAPK pathway. To do this, cox-2 mRNA levels were induced by stimulation of BMDMs with LPS for 5.5 h. An inhibitor of p38 or MK2 was then added, and 30 min after this, actinomycin D was added with or without exogenous IL-10. The levels of cox-2 mRNA remaining were then determined at 3 and 6 h following actinomycin D addition. To avoid possible complications from endogenously produced IL-10 during the initial LPS stimulation, these experiments were performed in IL-10 knockout BMDMs. Inhibition of p38 with SB203580 reduced the stability of the cox-2 mRNA (Fig. 9B), which would be consistent with a model in which a loss of p38 activity increases the turnover of cox-2 mRNA by TTP. In the presence of the p38 inhibitor, addi-

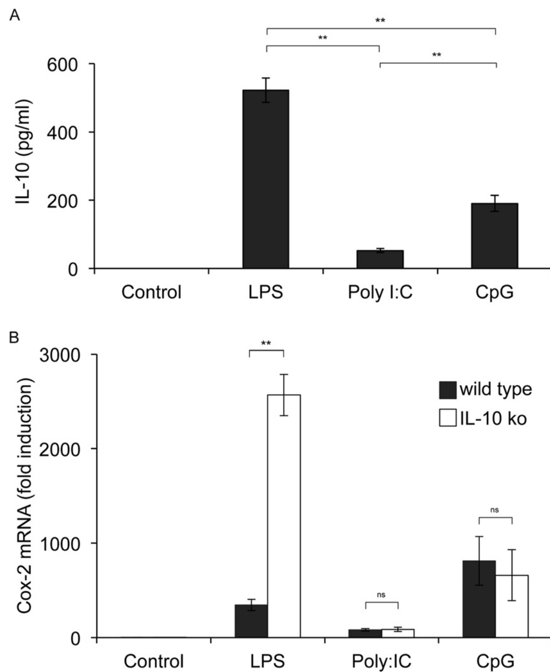

FIG 6 Regulation of TLR-induced IL-10 production by MSKs. (A) Wild-type BMDMs were isolated and stimulated with 100 ng/ml LPS, 2 μM CpG, or 10 μg/ml poly(I·C) for 8 h. IL-10 levels were then determined by a Luminex-based assay. (B) Wild-type and IL-10 knockout (ko) BMDMs were isolated and also stimulated with 100 ng/ml LPS, 2 μM CpG, or 10 μg/ml poly(I·C) for 8 h. cox-2 mRNA levels were determined by quantitative PCR (Q-PCR). Error bars represent the standard deviation of stimulations from 4 independent preparations of BMDMs per genotype. Student's t tests were carried out: ns, nonsignificant; **, $P < 0.01$.

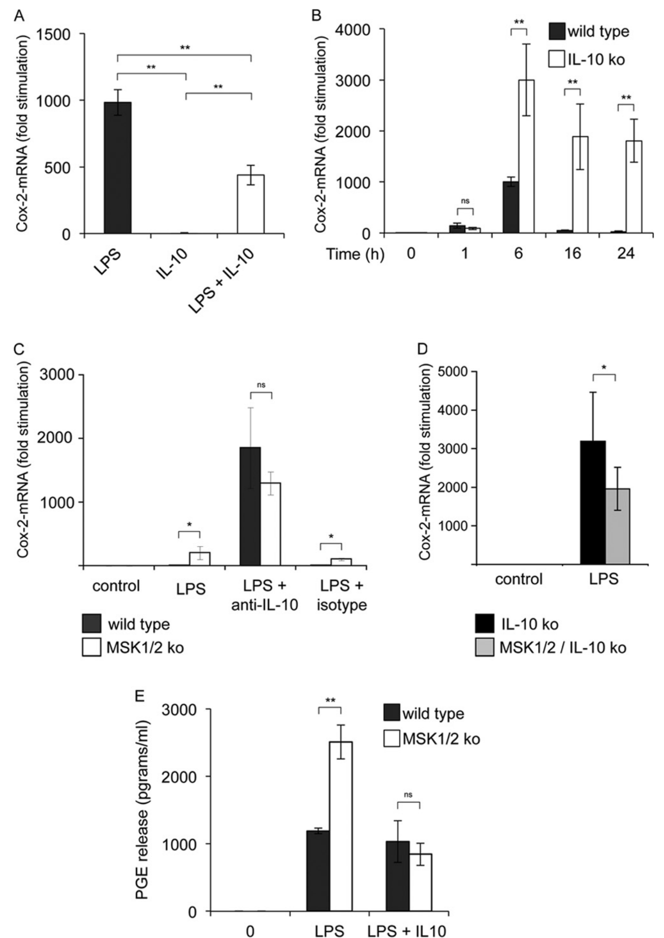

FIG 7 Role of IL-10 in cox-2 mRNA induction and PGE release. (A) BMDMs were stimulated with 100 ng/ml LPS or 100 ng/ml IL-10 as indicated for 8 h. Cells were then lysed, total RNA was isolated, and the levels of cox-2 mRNA were determined by quantitative PCR (Q-PCR). (B) Wild-type or IL-10 knockout (ko) BMDMs were stimulated with 100 ng/ml LPS for the indicated times, and cox-2 mRNA levels were determined by Q-PCR. (C) Wild-type or MSK1/2 knockout BMDMs were prepared and stimulated with 100 ng/ml LPS either on its own or in the presence of either an IL-10 neutralizing antibody or isotype control (both 1 μg/ml) for 16 h, and cox-2 mRNA levels were determined by Q-PCR. (D) IL-10 knockout (black bars) or IL-10/MSK1/2 triple-knockout (gray bars) BMDMs were stimulated with 100 ng/ml LPS for 8 h, and cox-2 mRNA levels were determined by Q-PCR. For each panel, fold stimulation for cox-2 mRNA was calculated relative to wild-type unstimulated (control) cells, using 18S rRNA as a reference. (E) Wild-type or MSK1/2 knockout BMDMs were prepared and stimulated with 100 ng/ml LPS either on its own or in the presence of endogenous IL-10 (100 ng/ml) for 16 h, and PGE secretion levels in the medium were determined by ELISA. Error bars represent the standard deviation of independent preparations of BMDMs from 4 mice per genotype. Student's t tests were carried out on all results: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

tion of exogenous IL-10 did not result in a further decrease in cox-2 mRNA stability (Fig. 9B). Identical results were also obtained using PF3644022 (Fig. 9C), a recently described selective inhibitor of MK2 and MK3 (38). These results would be in line with IL-10 regulating cox-2 mRNA stability via the inhibition of the p38α MAPK pathway. To test this, we examined the ability of IL-10 to regulate p38. If our hypothesis was correct, then IL-10 addition of exogenous IL-10 should repress LPS-induced p38 activation. In line with our hypothesis, addition of exogenous IL-10 repressed LPS-induced p38 activation. The ability of IL-10 to regulate p38 activity was blocked by pretreatment with the Jak inhibitor INCB-018424 (Fig. 9D).

DISCUSSION

We show here that knockout of MSK1 and -2 in macrophages increases cox-2 protein induction and prostaglandin secretion in response to LPS. Further investigation revealed that MSKs could exert both positive and negative effects on cox-2 induction; at early time points, MSKs act to promote cox-2 mRNA transcription, while at later time points, MSKs, via IL-10, promote the degradation of cox2 mRNA. Overall, this 2nd effect appears to be dominant, as evidenced by increased prostaglandin secretion by the MSK1/2 knockout compared to wild-type cells (summarized in Fig. 10).

The transcriptional regulation of cox-2 is complex and involves multiple factors. In agreement with results of previous studies, we show here that transcription of cox-2 mRNA in LPS-stimulated macrophages is regulated by both the p38 and ERK1/2 pathways (7, 8, 10, 11). During the initial phase of the LPS response, MSK1 and MSK2 help promote cox-2 mRNA transcription (Fig. 3 and 4). MSKs phosphorylate the transcription factor CREB on Ser133 (48), and, consistent with a role for CREB in cox-2 induction, previous studies have established the presence of a CREB binding site in the cox-2 promoter (9, 11, 12). We found that mutation of the MSK phosphorylation site, Ser133, in CREB to alanine reduced the induction of cox-2

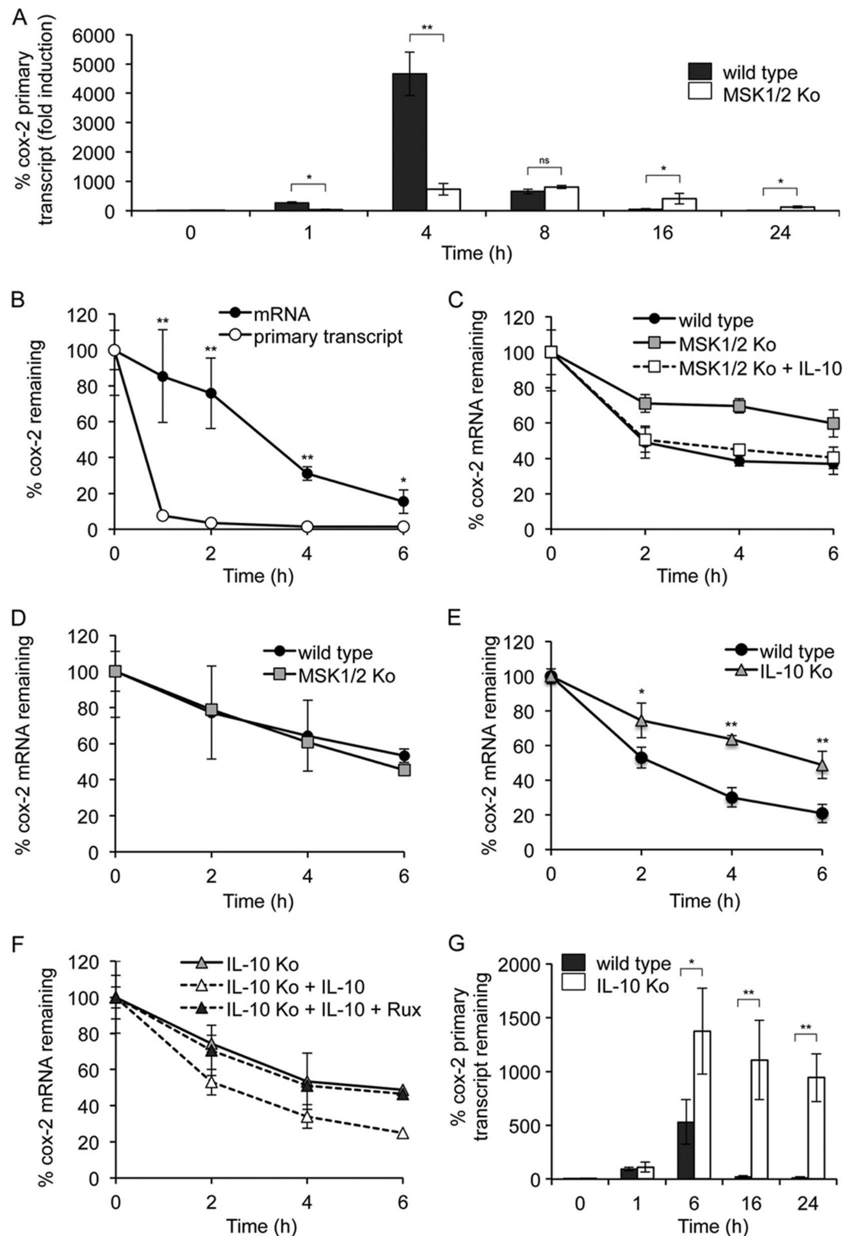

FIG 8 MSKs regulate cox-2 mRNA stability. (A) Wild-type or MSK1/2 knockout (Ko) BMDMs were prepared and stimulated with 100 ng/ml LPS for the indicated times. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 primary transcript were determined by quantitative PCR (Q-PCR). (B) Wild-type BMDMs were stimulated with 100 ng/ml LPS for 6 h before 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 primary transcript and mRNA were determined by Q-PCR. (C) Wild-type or MSK1/2 knockout BMDMs were stimulated for 6 h with LPS. Then, 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Where indicated, 100 ng/ml of IL-10 was added at the same time as actinomycin D. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by Q-PCR. (D) As for panel C except that BMDMs were stimulated for only 1 h with LPS before actinomycin D was added. (E) Wild-type or IL-10 knockout BMDMs were stimulated for 6 h with LPS before 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by Q-PCR. (F) IL-10 knockout BMDMs were stimulated for 6 h with LPS before 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Where indicated, 0.5 μM INCB-018424 (Rux) was added at the same time as actinomycin D. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by Q-PCR. (G) Wild-type or IL-10 knockout BMDMs were stimulated with 100 ng/ml LPS for the indicated times. Total RNA was then isolated, and the levels of the primary transcript for cox-2 were determined by Q-PCR. Error bars in each panel represent the standard deviation of independent stimulations on BMDMs from 4 mice per genotype. Student's *t* tests were carried out on all results: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

mRNA levels in response to TLR agonists (Fig. 5). The effect of the CREB knock-in is less dramatic than that of the MSK1/2 knockout, suggesting that MSK may regulate cox-2 via additional substrates. Of note, ATF1 is a related transcription factor to CREB and is also phosphorylated by MSKs on a site analogous to Ser133 in CREB (28). As ATF1 also binds CRE sites, it is therefore possible that MSKs additionally regulate cox-2 via phosphorylation of ATF1. MSKs also phosphorylate histone

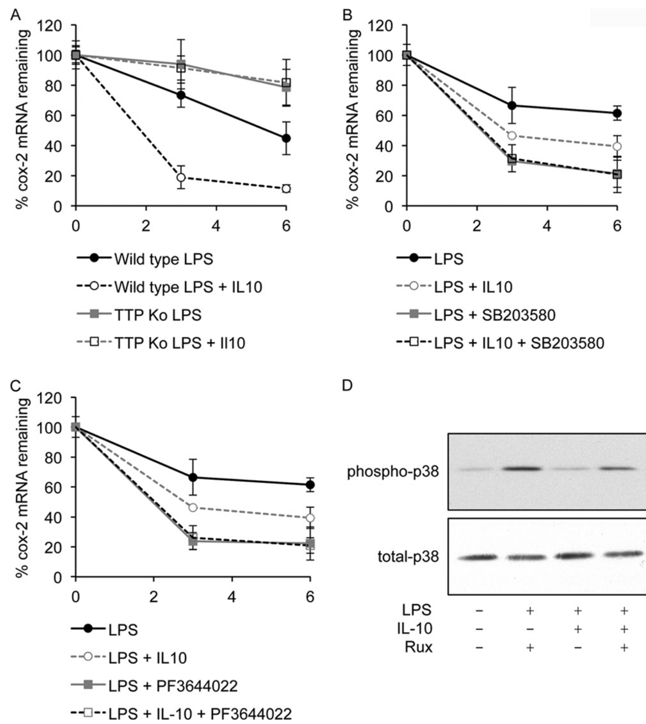

FIG 9 IL-10 regulates cox-2 mRNA stability via p38 and TTP. (A) Wild-type or TTP knockout (Ko) BMDMs were stimulated for 6 h with LPS. Then, 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Where indicated, 100 ng/ml of IL-10 was added at the same time as actinomycin D. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by quantitative PCR (Q-PCR). (B) IL-10 knockout BMDMs were stimulated for 6 h with LPS before 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Where indicated, 5 μM SB203580 was added 30 min prior to actinomycin D. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by Q-PCR. (C) IL-10 knockout BMDMs were stimulated for 6 h with LPS before 1 μg/ml actinomycin D was added and the cells were incubated for the indicated times after actinomycin addition. Where indicated, 2 μM PF3644022 was added 30 min prior to actinomycin D. Cells were then lysed, total RNA was isolated, and the levels of the cox-2 mRNA were determined by Q-PCR. Error bars represent the standard deviation on blots from 4 independent preparations of BMDMs per genotype. (D) Wild-type BMDMs were pretreated with 0.5 μM INCB-018424 (Rux) for 30 min and then stimulated with 100 ng/ml LPS or 100 ng/ml IL-10 as indicated for 6 h. Cells were lysed, and total and phospho-p38 MAPK levels were determined by immunoblotting.

H3 on Ser10 (49), which may contribute to chromatin remodelling at transcriptionally active promoters. Consistent with this possibility, recently MSKs have been shown to phosphorylate histone H3 at the cox-2 promoter in fibroblasts (50).

An unexpected finding in this study was the increase in cox-2 mRNA levels at later time points in the MSK1/2 knockouts relative to those in wild-type cells following LPS stimulation. This translated to an increase in cox-2 protein and prostaglandin secretion in LPS-stimulated MSK1/2 knockouts compared to those in wild-type cells. Several findings point to this being dependent on a feedback mechanism requiring IL-10. First, the inhibitory effect of MSK knockout on cox-2 mRNA levels was much more pronounced following LPS stimulation than CpG. LPS results in more IL-10 secretion than CpG by BMDMs (Fig. 6). In addition, similar effects were seen in LPS-stimulated IL-10 knockout cells: like MSK1/2 knockouts, IL-10 knockout also resulted in both elevated levels of cox-2 mRNA following LPS stimulation and increased stability of cox-2 mRNA, whereas increased cox-2 mRNA levels were not observed in response to CpG (Fig. 6 and 7). Finally, combined knockout of both MSKs and IL-10 did not have an additional effect on cox-2 mRNA stability relative to IL-10 knockout alone (Fig. 8). The effect of IL-10 knockout was more pronounced than that of MSK1/2 knockout; this likely reflects the fact that MSK1/2 knockout reduces but does not completely abolish IL-10 secretion (21). The incomplete block of LPS-induced IL-10 induction by MSK knockout is in part due to the ability of LPS to sustain IL-10 induction via an IFN-β-mediated feedback loop (21, 31).

An inhibitory effect of IL-10 on cox-2 expression has been observed previously; however, the mechanism was not determined in these studies (40, 41, 51). cox-2 mRNA is known to contain multiple AU-rich elements in its 3′ UTR (8, 42–44), and these AU-rich elements can regulate mRNA stability via binding to specific mRNA binding proteins such as TTP and HuR. AU-rich elements have previously been shown to occur in multiple LPS-induced genes (52, 53). We find here that TTP regulates cox-2 mRNA stability in LPS-stimulated macrophages and that it is required for IL-10 to promote cox-2 mRNA degradation (Fig. 9). TTP has previously been described to regulate multiple genes, either via inhibiting translation or by promoting deadenylation or degradation of the mRNA (47, 54–56). In addition to this, the p38 MAPK pathway has been demonstrated to regulate the stability of several mRNAs downstream of TLR signaling, including cox-2. For example, treatment of multiple cell types with p38 inhibitors has been shown to result in the destabilization of cox-2 mRNA, and the transfection of reporter constructs has demonstrated a requirement for the AU-rich elements in the 3′ UTR for this effect (8, 57, 58). Roles for several AU binding proteins have additionally been suggested downstream of p38 (58–60). Significantly, the p38-activated kinase MK2 has been established to phosphorylate TTP and inhibit its ability to promote mRNA degradation in several systems (46, 61, 62). In BMDMs we have established that the cox-2 mRNA is also stabilized by p38 and MK2, as inhibition of these kinases greatly reduced the stability of the cox-2 mRNA (Fig. 9). In addition, we found that IL-10 was able to inhibit p38 activity (Fig. 9D). Cross talk between p38 and IL-10 has also been reported to occur in the regulation of TNF translation (63). The ability of IL-10 to inhibit p38 activity would fit with its roles in suppressing the inflammatory actions of macrophages given the important roles that p38 plays in regulating proinflammatory cytokine production. Interestingly, p38 itself is involved in regulating IL-10 production, both via activating MSKs (21) and via MK2 and the inhibition of TTP activity, as the IL-10 gene is also reported to be a TTP target gene (64, 65). How IL-10 inhibits p38 activity is not clearly established, but it could potentially involve inhibition of the upstream activators of p38 or activation of a p38-specific phosphatase. In cells, p38 is activated by the upstream kinases MKK3 and MKK6, which are in turn activated by a MAP3K (66, 67). In the TLR signaling pathway, Tak1 has been proposed as the predominant MAP3K required for p38 activation, although a recent study has found that in macrophages deletion of Tak1 does not abolish LPS-induced p38 activation (68). This would suggest that another MAP3K may be able to act downstream of

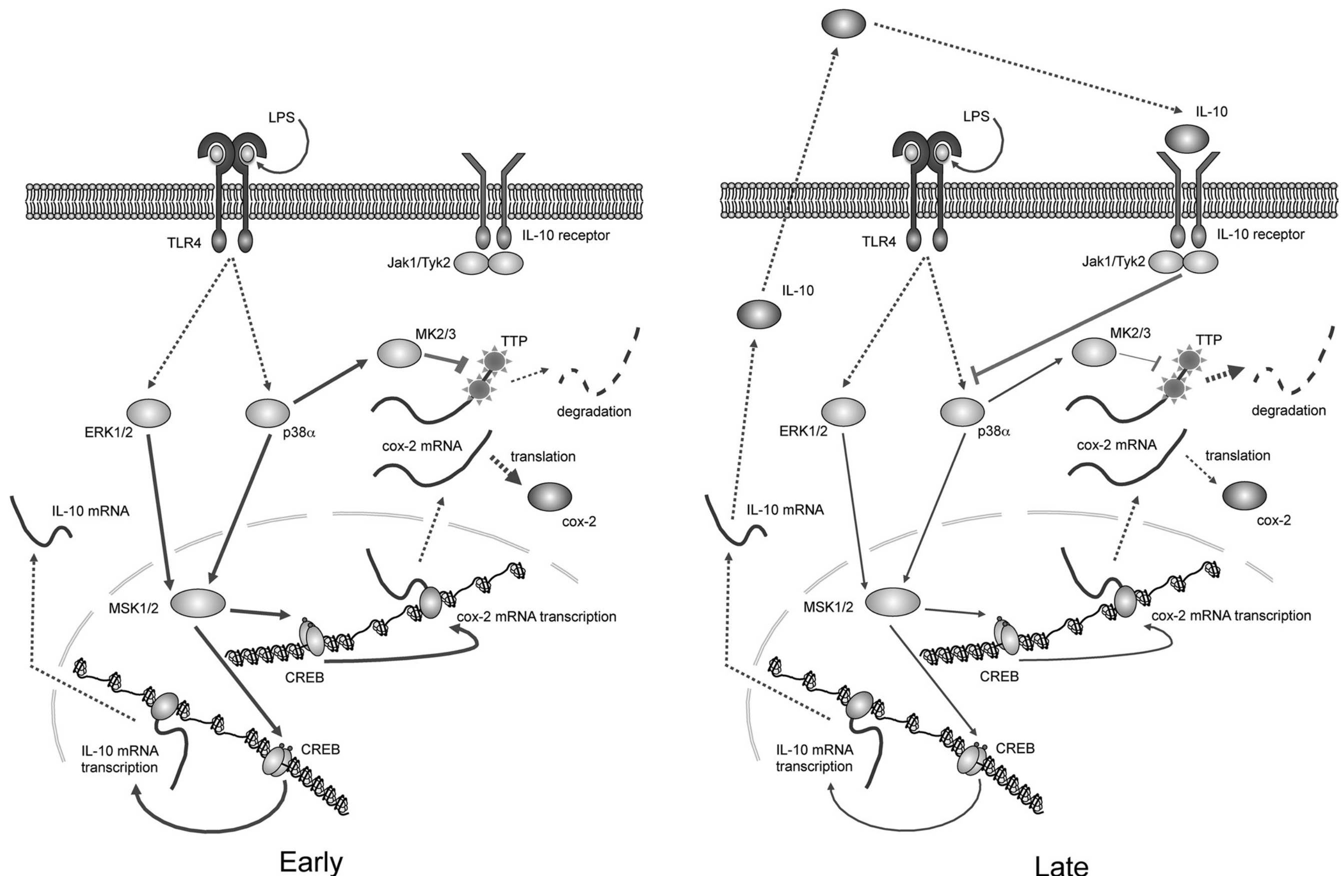

FIG 10 MSKs regulate cox-2 mRNA transcription via CREB and cox-2 mRNA stability via an IL-10 feedback mechanism. Upon early TLR activation, downstream of TLR4, MSK1/2 phosphorylate the transcription factor CREB, leading to cox-2 and IL-10 mRNA transcription. Concurrently activation of p38 MAPK and its subsequent activation of MK2 lead to inhibition of the zinc finger protein TTP, and thus less mRNA degradation. However, at later time points of TLR activation, IL-10 which has been secreted from the cell feeds back in an autocrine and paracrine manner stimulating the IL-10 receptor. This leads to downstream activation of the Jak pathway which inhibits p38 and MK2, releasing TTP from its inhibitory state. This, in turn, leads to increased TTP mediated turnover of cox-2 mRNA. In our MSK1/2 knockout macrophage system we see decreased IL-10 secretion leading to attenuation of this feedback mechanism resulting in reduced cox-2 mRNA turnover and higher levels of cox-2 protein and prostaglandin release than in wild-type macrophages.

TLR4, and roles for MEKK3 and ASK1 have been proposed in the TLR pathway (69, 70). Interestingly, using cells from ASK1 knockout mice, a requirement for ASK1 has been shown for TLR4-induced p38 but not JNK activation. As IL-10 has been shown to inhibit p38 but not JNK, this may suggest that ASK1 may be inhibited by IL-10. The regulation of ASK1 is complex and can be regulated by multiple phosphorylation events. A recent report has shown, however, that in endothelial cells, IFN-γ stimulation leads to the phosphorylation of ASK1 by Jak2, resulting in the recruitment of SOCS1 and the subsequent degradation of ASK1 (71). As both Jak1 and Jak2 can phosphorylate ASK1 *in vitro*, it is possible that in macrophages IL-10 inhibits p38 via a Jak-dependent inhibition of ASK1 (71). However, further work would be required to investigate this or other potential pathways to establish the precise mechanism by which IL-10 affects p38 activation.

In summary, we show here that while MSKs do promote the early stages of cox-2 mRNA transcription via the phosphorylation of CREB, they can also inhibit cox-2 expression via a negative feedback loop involving IL-10. For the LPS stimulation of macrophages, the IL-10 feedback effect appears dominant, and so MSK1/2 knockout results in an elevated production of prostaglandins in response to LPS.

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