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Macrophage responses to lipopolysaccharide are modulated by a feedback loop involving prostaglandin E₂, dual specificity phosphatase 1 and tristetraprolin

Tina Tang, Thomas E. Scambler, Tim Smallie, Helen E. Cunliffe, Ewan A. Ross, Dalya R. Rosner, John D. O’Neil & Andrew R. Clark

In many different cell types, pro-inflammatory agonists induce the expression of cyclooxygenase 2 (COX-2), an enzyme that catalyzes rate-limiting steps in the conversion of arachidonic acid to a variety of lipid signaling molecules, including prostaglandin E₂ (PGE₂). PGE₂ has key roles in many early inflammatory events, such as the changes of vascular function that promote or facilitate leukocyte recruitment to sites of inflammation. Depending on context, it also exerts many important anti-inflammatory effects, for example increasing the expression of the anti-inflammatory cytokine interleukin 10 (IL-10), and decreasing that of the pro-inflammatory cytokine tumor necrosis factor (TNF). The tight control of both biosynthesis of, and cellular responses to, PGE₂ are critical for the precise orchestration of the initiation and resolution of inflammatory responses. Here we describe evidence of a negative feedback loop, in which PGE₂ augments the expression of dual specificity phosphatase 1, impairs the activity of mitogen-activated protein kinase p38, increases the activity of the mRNA-destabilizing factor tristetraprolin, and thereby inhibits the expression of COX-2. The same feedback mechanism contributes to PGE₂-mediated suppression of TNF release. Engagement of the DUSP1-TTP regulatory axis by PGE₂ is likely to contribute to the switch between initiation and resolution phases of inflammation.

The cyclooxygenase enzymes COX-1 and COX-2, encoded by the genes Ptgs1 and Ptgs2, catalyze rate-limiting steps in the synthesis of various prostanoid signaling molecules from the lipid precursor arachidonic acid. COX-1 is constitutively expressed by many cells. In contrast, COX-2 is expressed at low levels by the majority of cells, but transiently induced in response to growth factors, stresses or pro-inflammatory stimuli. Prostaglandin E₂ (PGE₂) is the major downstream product of COX-2-mediated arachidonic acid metabolism in many cells. PGE₂ increases blood flow, vascular permeability and nociception, thereby contributing to all four of the cardinal signs of inflammation: redness, swelling, heat and pain. The pro-inflammatory actions of PGE₂ underlie the clinical usefulness of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit both COX-1 and COX-2. However, constitutive COX-1-mediated prostaglandin synthesis in the gastric mucosa helps to maintain the integrity of this vulnerable tissue, which accounts for the increased incidence of gastric ulcers amongst patients using NSAIDs for prolonged periods. The rationale for the generation of COX-2-selective inhibitors was based on the assumption that COX-1 functions principally as a homeostatic enzyme, whereas COX-2 functions principally as a pro-inflammatory mediator. Selective inhibitors of COX-2 were predicted to exert anti-inflammatory effects whilst sparing gastric side effects.

As we are reminded by the costly failure of the COX-2-selective inhibitor Rofecoxib (Vioxx), biology is rarely so straightforward or convenient. COX-1-dependent synthesis of thromboxane by platelets promotes vasoconstriction and platelet aggregation. These prothrombotic actions are opposed by prostaglandin I₂ (prostacyclin).
The significantly increased risk of myocardial infarction and stroke in patients taking Vioxx eventually led to the withdrawal of this drug from the market. The basis of elevated cardiovascular risk is still not fully understood, but has been ascribed to an imbalance between COX-1-mediated pro-thrombotic and COX-2 mediated anti-thrombotic influences. Another confounding aspect of prostaglandin biology is that the actions of PGE2 are not invariably pro-inflammatory; nor are the effects of COX-2-selective inhibitors invariably anti-inflammatory. PGE2 has been shown to enhance expression of IL-10, inhibit the expression of TNF and other inflammatory mediators, and promote the differentiation of macrophages towards an alternatively-activated, anti-inflammatory M2 phenotype. In rheumatoid synovial explant cultures or peripheral blood-derived monocytes, NSAIDs increased the expression of TNF. Prior in vivo exposure to COX-2-selective inhibitors also primed human peripheral blood monocytes and mouse peritoneal macrophages for increased expression of TNF in response to an LPS challenge.

Cellular responses to PGE2 are mediated by four G-protein-coupled receptors named EP1–EP4, which are the products of the genes Ptger1–Ptger4. These receptors differ in their affinity for PGE2, and in the signal transduction pathways that they engage. Both EP2 and EP4 are Goαs-linked and activate adenylyl cyclase to elevate intracellular cAMP levels. EP4 has additionally been shown to signal via PI3K. EP1, which is coupled to Goq, signals via phospholipase C to induce calcium flux. EP3 exists in a number of distinct forms arising from differential splicing of the Ptger3 transcript, and appears to be promiscuous in terms of its signaling pathway engagement. There is current potential for cell-specific programing of responses to PGE2 via modulation of the expression of the four receptors or their variants. Anti-inflammatory actions of PGE2 have generally been ascribed to EP4 and/or EP2, however molecular mechanisms remain unclear. The increased expression of the anti-inflammatory cytokine IL-10 does not provide an explanation, as PGE2 can still inhibit macrophage expression of TNF in the absence of IL-10.

The inflammation-induced biosynthesis of PGE2 is regulated largely at the level of Ptgs2 mRNA. The typical transient pattern of expression of Ptgs2 mRNA is only partially explained by transcriptional activation involving nuclear factor κB (NF-κB) and other transcription factors. Efficient expression also requires the stabilization of Ptgs2 mRNA via the mitogen-activated protein kinase (MAPK) p38 signaling pathway, and conversely, MAPK p38 inhibitors accelerate the degradation of Ptgs2 mRNA. This post-transcriptional regulation is mediated by an adenosine/uridine-rich element (ARE) immediately 3′ to the Ptgs2 translation termination codon. When inserted into a stable reporter mRNA, the Ptgs2 ARE confers rapid decay that is mediated by shortening of the protective poly-(A) tail (deadenylation), and can be prevented by activation of MAPK p38.

The mouse Zfp36 gene encodes the ARE-binding protein tristetraprolin (TTP). In Zfp36−/− macrophages lacking TTP protein, Ptgs2 mRNA was highly stable and could not be destabilized by a MAPK p38 inhibitor. TTP binds to AREs in the 3′ untranslated regions of target transcripts and recruits a complex of deadenylase enzymes, which catalyzes shortening of the poly-(A) tail, usually as a prelude to the rapid destruction of the mRNA body. The mRNA-stabilizing activity of TTP is regulated by a phosphorylation switch. Pro-inflammatory agonists and cell stresses activate MAPK p38, which in turn phosphorylates and activates MK2 (MAPK-activated protein kinase 2). MK2 phosphorylates serines 52 and 178 of TTP, resulting in the recruitment of 14–3–3 adaptor proteins, impairment of the interaction between TTP and the deadenylase complex, and consequent stabilization of target mRNA. Protein phosphatase 2A (PP2A) catalyzes the removal of these two phosphate groups and the activation of TTP. Therefore a dynamic equilibrium exists between forms of TTP that are phosphorylated or unphosphorylated at serines 52 and 178, and this equilibrium favors the stabilization of TTP-regulated mRNAs under conditions of strong MAPK p38 activity. Coupling between MAPK p38 activity and the stability of pro-inflammatory mRNAs contributes to the precise orchestration of the on and off phases of inflammatory responses.

The activation of MAPK p38, and hence the phosphorylation state of TTP, is regulated by a negative feedback loop involving dual specificity phosphatase 1 (DUSP1). Pro-inflammatory stimuli induce MAPK p38-dependent expression of DUSP1, which then dephosphorylates and inactivates MAPK p38 to enforce the off-phase of the inflammatory response. Due to the failure of this feedback mechanism, Dusp1−/− mice and cells are prone to respond excessively to pro-inflammatory agonists, and over-express many inflammatory mediators, including COX-2. The over-expression of COX-2 by Dusp1−/− macrophages is not fully understood; both transcriptional and post-transcriptional mechanisms have been proposed. Here, we investigate the functional relationship between DUSP1, MAPK p38, TTP and COX-2. First, we use a number of genetically modified mouse strains to demonstrate that DUSP1 and MAPK p38 control the expression of COX-2 by regulating the phosphorylation of TTP at serines 52 and 178. Next we turn to the effect on macrophages of the major COX-2 product, PGE2. We show that PGE2 enhances the expression of DUSP1 and thereby downregulates the expression of COX-2, creating another auto-regulatory feedback mechanism. PGE2 also acts via the receptor EP4 to inhibit macrophage expression of TNF, in a manner that is at least partially dependent on both DUSP1 and the modulation of TTP's phosphorylation state. Despite being an authentic TTP target, IL-6 escapes negative regulation by PGE2. The influence of PGE2 on Dusp1 gene expression creates a potent mechanism for context-dependent and gene-specific modulation of inflammatory responses.

Results

**Ptgs2 gene expression is negatively regulated by TTP.** A previous publication described high resolution mapping of TTP binding sites in the mouse macrophage transcriptome. Publicly accessible data from this study (http://ttt-atlas.univie.ac.at) revealed strong binding of TTP to the 3′ UTR of Ptgs2 mRNA, which was restricted to a region immediately downstream of the open reading frame, containing a cluster of six AUUUA motifs (Fig. 1a). This region mediated regulation of Ptgs2 mRNA stability by the MAPK p38 signaling pathway,
and was recognized by TTP \textit{in vitro}\textsuperscript{24,25}. Other putative TTP binding sites in the \textit{Ptgs2} 3′ UTR\textsuperscript{42} appear to be recognized by TTP poorly or not at all in mouse macrophages. RNA immunoprecipitation experiments confirmed binding of TTP to \textit{Ptgs2} mRNA in the mouse macrophage cell line RAW264.7 (Fig. 1b). We recently used homologous recombination to generate a novel mouse strain (known as \textit{Zfp36aa/aa}), in which serines 52 and 178 of endogenous TTP protein were substituted by non-phosphorylatable alanine residues\textsuperscript{43}. The mutant form of TTP could not be inactivated by MK2-mediated phosphorylation, and functioned as a constitutive mRNA destabilizing factor, decreasing the expression of several inflammatory mediators \textit{in vitro} and \textit{in vivo}\textsuperscript{43, 44}. \textit{Zfp36aa/aa} mice were strongly resistant to experimental endotoxemia and arthritis\textsuperscript{43, 44}. \textit{Zfp36aa/aa} bone marrow macrophages (BMMs) under-expressed \textit{Ptgs2} mRNA at a steady-state level (Fig. 1c), particularly at later time points. The decrease in



\textbf{Figure 1.} \textit{Ptgs2} gene expression is negatively regulated by tristetraprolin. (a) Number of TTP crosslinks (CL) is plotted against position on the \textit{Ptgs2} primary transcript. The primary transcript is illustrated below the graph, with coding exonic sequences as grey bars and non-coding exonic sequences as black bars. Positions of AUUUA motifs are represented above the transcript, and the sequence immediately 3′ to the stop codon is expanded below. Figure adapted from TTP-atlas data (http://ttp-atlas.univie.ac.at)\textsuperscript{35}. (b) RNA immunoprecipitation was performed on whole cell lysates of RAW264.7 cells, untreated or stimulated with LPS for 2 h, using a TTP antiserum or pre-immune control (PI). \textit{Gapdh}, \textit{Tnf} and \textit{Ptgs2} mRNAs were measured by quantitative PCR, and fold enrichment was calculated relative to the PI control. A representative of three similar experiments is shown. Error bars indicate SD of triplicate measurements. (c) \textit{Zfp36+/+} and \textit{Zfp36aa/aa} BMMs were stimulated with 10 ng/ml LPS for the times indicated, and \textit{Ptgs2} mRNA was measured by qPCR. The graph represents mean ± SEM of three independent BMM cultures of each genotype. \textbf{***} \textit{p} < 0.005; Holm-Sidak method for multiple comparison. (d) \textit{Zfp36+/+} and \textit{Zfp36aa/aa} BMMs were stimulated with 10 ng/ml LPS for 4 h then actinomycin D was added and \textit{Ptgs2} mRNA measured by qPCR at the intervals indicated. The graph shows mean ± SEM of three independent BMM cultures of each genotype. (e) \textit{Zfp36+/+} and \textit{Zfp36aa/aa} BMMs were stimulated with LPS for the times indicated and COX-2 protein was detected by western blotting. Representative of three repeats. (f) \textit{Zfp36+/+} and \textit{Zfp36aa/aa} BMMs were stimulated with LPS for the times indicated and \textit{PGE}$_2$ in the tissue culture supernatant was measured by ELISA. The graph represents mean ± SEM of three independent BMM cultures of each genotype. \textbf{***} \textit{p} < 0.005; Holm-Sidak method for multiple comparison.
steady-state Ptgs2 expression was accompanied by an increase in its rate of degradation (Fig. 1d). COX-2 protein was expressed at low levels in LPS-treated Zfp36aa/aa BMMs (Fig. 1e). The LPS-induced release of PGE$_2$ was significantly diminished in Zfp36aa/aa BMMs (Fig. 1f). Heterozygous Zfp36+/aa BMMs also under-expressed COX-2 protein (Supplemental Figure 1), consistent with our previous description of the non-phosphorylatable TTP mutant as a dominant inhibitor of inflammatory gene expression43. These findings confirm that Ptgs2 mRNA is an authentic, direct target of negative regulation by TTP.

DUSP1 regulates Ptgs2 gene expression by modulating TTP phosphorylation. Steady state levels of Ptgs2 mRNA were elevated in LPS-treated Dusp1−/− BMMs compared with identically treated Dusp1+/+ controls (Fig. 2a), accompanied by an increase in Ptgs2 mRNA stability (Fig. 2b). The expression of COX-2 protein was increased in LPS-treated Dusp1−/− BMMs (Fig. 2c), as was the release of PGE$_2$ (Fig. 2d). We hypothesized that deletion of the Dusp1 gene increases the expression of Ptgs2 via enhanced phosphorylation and inactivation of TTP. To test this hypothesis, expression of Ptgs2 mRNA, COX-2 protein and PGE$_2$ was compared in wild type, Dusp1−/−, Zfp36aa/aa and Dusp1−/−:Zfp36aa/aa BMMs. At 1 h, Dusp1 gene disruption increased the expression of Ptgs2 mRNA, but targeted mutation of the Zfp36 locus was without effect (Fig. 3a). At 4 h, Ptgs2 mRNA continued to be over-expressed by Dusp1−/− BMMs, but in contrast was under-expressed by Zfp36aa/aa BMMs. Most importantly, Dusp1−/−:Zfp36aa/aa BMMs also under-expressed Ptgs2 mRNA at 4 h. A similar pattern was observed at the level of COX-2 protein (Fig. 3b) and PGE$_2$ biosynthesis (Fig. 3c). To investigate whether the same mechanism was relevant in vivo, mice of all four genotypes were challenged by intraperitoneal injection of LPS, and three hours later Ptgs2 mRNA expression was measured in spleen, an organ which plays a critical role in systemic responses to endotoxin (Fig. 3c). The LPS challenge strongly enhanced splenic Ptgs2 expression in mice of all four genotypes. Higher Ptgs2 expression was seen in Dusp1−/− than in wild type control mice, but significantly lower expression was seen in both Zfp36aa/aa and Dusp1−/−:Zfp36aa/aa mice. Therefore, both in vitro and in vivo, disruption of the Dusp1 gene and dysregulation of MAPK signaling enhances Ptgs2 gene expression in a manner dependent on the phosphorylation of TTP at serines 52 and 178.
PGE₂ modulates the phosphorylation of MAPK p38, the expression of DUSP1, COX-2, TNF and IL-6. We have performed two independent microarray experiments in primary mouse BMMs and one in primary human monocyte-derived macrophages, investigating LPS-induced changes of gene expression. The first mouse microarray experiment has been described 37 and deposited at the Gene Expression Omnibus (GSE68449), whilst the others are being prepared for submission. All three arrays have been extensively validated.

According to all three experiments, both mouse and human primary macrophages expressed mRNAs encoding the prostaglandin receptors EP2 and EP4, whereas mRNAs encoding the other two members of this receptor family, EP1 and EP3, were essentially undetectable (Fig. 4a,b). In macrophages of both species, expression of *Ptger4*/*PTGER4* mRNA was increased by LPS, whereas expression of *Ptger2*/*PTGER2* mRNA was decreased or unaffected. Expression of EP4 in BMMs was confirmed by flow cytometry (Fig. 4c), whilst EP2 protein could not be detected using available reagents. LPS induced a modest increase of EP4 protein levels, which was statistically significant at 2 h (Fig. 4c,d).

Although PGE₂ is commonly thought of as a pro-inflammatory signaling molecule², it is also known to exert anti-inflammatory effects in myeloid and other cells via activation of the cAMP pathway by EP2 and/or EP4⁶, 16, 17, 45. Expression of the *Dusp1* gene is regulated by cAMP via CREB binding sites in the proximal promoter ⁴⁶–⁵⁰. Induction of DUSP1 expression by PGE₂ was recently demonstrated in airway smooth muscle cells⁵¹. We therefore investigated whether PGE₂ could modulate the expression of DUSP1 and the activity of MAPK signaling pathways in BMMs. On its own, PGE₂ weakly increased the expression of *Dusp1* mRNA in BMMs (Fig. 4e), although we were not able to detect PGE₂-induced DUSP1 protein or changes in MAPK p38 phosphorylation. LPS rapidly and transiently increased *Dusp1* mRNA. Combined treatment with LPS and PGE₂ resulted in cooperative enhancement of *Dusp1* mRNA levels, particularly at the peak of expression, 1 h after the stimulus. Very similar cooperative regulation of *DUSP1* mRNA by LPS and PGE₂ was observed in primary human monocyte-derived macrophages (Fig. 4f).
Figure 4. Exogenous PGE2 modulates Dusp1 gene expression and MAPK p38 signaling. Expression of the mouse prostaglandin receptor genes Ptgter1–4 (a) or the corresponding human genes PTGER 1–4 (b) was measured by microarray as described in Fig. 3. Graphs represent RMA ± SEM from three independent mouse or human macrophage cultures. n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.005; ANOVA. (c) Representative flow cytometry of EP4 expression in BMMs treated with LPS for 0 or 4 h. (d) MFI of EP4 expression was measured at the indicated times after stimulation of BMMs with 10 ng/ml LPS. The graph represents mean ± SEM of three independent wild type BMM cultures. *p < 0.05; ANOVA. (e) BMMs were treated for the indicated times with LPS (10 ng/ml), PGE2 (1 nM) or both. Dusp1 mRNA was measured by qPCR, and plotted as fold increase compared to untreated controls. The graph shows mean fold increase ± SEM from three independent BMM cultures. **p < 0.01; ***p < 0.005; ANOVA. (f) Primary human monocyte-derived macrophages were treated, and DUSP1 mRNA was measured, as in (e). ***p < 0.005; ANOVA. (g) Dusp1+/+ and Dusp1−/− BMMs were treated with LPS (10 ng/ml) with or without PGE2 (1 nM) for the times indicated. DUSP1, phospho-p38, COX-2 and β-actin proteins were detected by western blotting. Representative of four experimental repeats.
In Dusp1+/+ BMMs the LPS-induced expression of DUSP1 protein was transient, but was sustained by addition of PGE₂ (Fig. 4g). The specificity of the antibody is confirmed by the failure to detect corresponding bands in Dusp1−/− BMMs. In three independent experiments PGE₂ decreased LPS-induced MAPK p38 phosphorylation by a factor of 0.48 ± 0.06 and increased DUSP1 levels by a factor of 1.80 ± 0.26 (mean fold changes ± SEM) at the 2 h time point. At the 4 h time point phosphorylated MAPK p38 was readily detected in BMMs treated with LPS alone but not in those treated with LPS + PGE₂. LPS-induced expression of COX-2 protein was also inhibited by addition of PGE₂. In Dusp1−/− BMMs, the activation of MAPK p38 in response to LPS was prolonged and the expression of COX-2 protein was enhanced. Neither of these responses was affected by addition of PGE₂. Hence the expression of COX-2 is regulated by a negative feedback loop that is mediated by its major catalytic product PGE₂ and dependent on the expression of DUSP1.

In both Dusp1+/+ and Dusp1−/− BMMs, exogenous PGE₂ inhibited the expression of TNF (Fig. 5a). These inhibitory effects were statistically significant at 4 and 8 h after the addition of LPS, and greater in magnitude in Dusp1+/+ than Dusp1−/− BMMs. Differential sensitivity of Dusp1+/+ and Dusp1−/− BMMs to anti-inflammatory effects of PGE₂ was confirmed in dose-response experiments (Fig. 5b). The anti-inflammatory effect was selective, since the expression of IL-6 was not decreased by PGE₂ in Dusp1+/+ macrophages (Fig. 5c). In Dusp1−/− BMMs, IL-6 expression was increased by the highest concentration of PGE₂. Zfp36a/a BMMs were relatively insensitive to inhibitory effects of PGE₂ on the release of TNF (Fig. 5d), confirming that PGE₂ modulates TNF expression in part by influencing the phosphorylation of TTP. A selective antagonist of EP2 did not influence the inhibition of TNF biosynthesis by PGE₂ (Fig. 5e, columns 4–7). A selective EP4 antagonist dose-dependently reversed the suppression of TNF expression by exogenous PGE₂ (Fig. 5e, columns 8–11) but did not, on its own, increase TNF production (Fig. 5e, column 12). This suggests that EP4 is the major mediator of the anti-inflammatory effects of PGE₂ in this context.

**IL-10 is a potent inhibitor of TNF**

The inhibitory effect of exogenous PGE₂ is strongly time-dependent. If LPS-induced secretion of PGE₂ exerts negative feedback via EP4 to limit the expression of inflammatory mediators, the inhibition of COX-2 function and endogenous PGE₂ synthesis would be expected to increase TNF release. To test this, we first confirmed that LPS-induced release of PGE₂ was effectively eliminated by a selective inhibitor of COX-2 enzymatic activity, NS398 (Fig. 6a). The effect of NS398 on LPS-induced release of TNF was then tested in Dusp1−/− and Dusp1+/+ BMMs. Neither genotype of BMM displayed any change of TNF expression in the presence of a concentration of NS398 sufficient to inhibit LPS-induced PGE₂ release (Fig. 6b). Differences of timing could explain why the expression of TNF is sensitive to exogenously added but insensitive to endogenously produced PGE₂. The accumulation of PGE₂ is a gradual process requiring both steps, such that PGE₂ levels in cell culture medium become elevated only after 4 h (Figs 1f and 2d). In contrast both deletion of the Ptgs2 gene alongside -Dusp1/TTP axis dominates the expression of PGE₂ mRNA both in vitro (Fig. 3a, 1 hour) and in vivo (Fig. 3d), the cumulative expression of COX-2 protein (Fig. 3b) and the production of PGE₂ (Fig. 3c). We could find no evidence that the targeted gene of TTP had any impact on NF-κB activity or transcription of the target genes that we examined (Fig. 3). In contrast both deletion of the Dusp1 gene and targeted mutation of the Zfp36 gene clearly influenced the stability of Ptg2 mRNA (Figs 1d and 2b), as well as that of other TTP targets. These findings place the Ptg2 gene alongside Tnf, Cxcl1, Cxcl2, Ifnb1, Il1b and several others, as targets of the DUSP1-TTP regulatory axis, which tightly couples mRNA stability to the activity of the MAPK p38 signaling pathway. Increased expression of the Ptg2 gene may contribute to the innate immune pathology of both Dusp1−/− and Zfp36−/− mice whilst decreased expression of Ptg2 may play a role in the resistance of Zfp36a/a to experimental endotoxemia and arthritis.
The second half of our study focuses on possible consequences of altered PGE_2 release by LPS-activated macrophages. Both mouse and human primary macrophages expressed the adenyl cyclase-coupled PGE_2 receptors EP2 and EP4 and displayed cooperative regulation of Dusp1/DUSP1 gene expression by LPS and exogenous PGE_2. In wild type murine macrophages, exogenous PGE_2 accelerated the decline of MAPK p38 activity and reduced the expression of COX-2 protein. In Dusp1−/− macrophages neither of these effects was seen (Fig. 4).

Our findings reveal a negative feedback loop that is illustrated schematically in Fig. 7. PGE_2 down-regulates the expression of COX-2 by increasing the expression of DUSP1, decreasing the activity of MAPK p38 and enhancing the function of TTP. This contrasts with the MAPK p38-dependent positive feedback regulation of COX-2 expression by PGE_2 that has been described by others. We found no evidence for activation of MAPK p38 or induction of COX-2 expression by PGE_2 in primary murine macrophages. The reason for the discrepancy is not clear.
Exogenous PGE₂ strongly inhibited the LPS-induced expression of TNF by primary mouse macrophages (Fig. 5). The PGE₂ receptor EP4 contributed to this anti-inflammatory effect, whereas EP1 or EP3 appeared not to be expressed, and we could not find evidence for involvement of EP2. We are currently investigating whether LPS-induced up-regulation of EP4 sensitizes macrophages to anti-inflammatory effects of PGE₂. Importantly, Dusp1 gene disruption and targeted mutation of the Zfp36 (TTP) gene had opposite effects on the expression of TNF, increasing or decreasing it, respectively (Fig. 5b and d, left panels). However, both genetic modifications rendered macrophages similarly insensitive to PGE₂-mediated suppression of TNF synthesis (Fig. 5b and d, right panels). These results establish a detailed, novel molecular mechanism for anti-inflammatory effects of PGE₂.

Figure 6. Anti-inflammatory effects of PGE₂ are strongly time-limited. Dusp1+/+ and Dusp1−/− BMMs were stimulated with 10 ng/ml LPS in the presence of the indicated concentrations of NS398. PGE₂ (a) and TNF (b) were measured by ELISA. Graphs represent mean ± SEM from three independent BMM cultures of each genotype. (c) Wild type BMMs were stimulated with 10 ng/ml and harvested four hours later for measurement of TNF by ELISA. PGE₂ (1 nM) was added at different time points with respect to the addition of LPS at t = 0. The graph shows mean ± SEM from three independent cultures of BMMs. n.s., not statistically significant; ***p < 0.005; ANOVA.
IL-10 is a potent anti-inflammatory cytokine. IL-6 is not merely pro-inflammatory, but also has important functions in resolution and tissue remodeling. Both \( \text{Il6} \) and \( \text{Il10} \) genes are well-documented TTP targets, yet they escaped negative regulation by PGE2. In fact, both were up-regulated by PGE2 in macrophages lacking DUSP1 (Figs 5c and 2). Significantly, both genes are positively regulated by cAMP signaling and have been shown to be induced by PGE2. This may be an adaptation that permits their continued expression under conditions favoring the suppression of TNF. According to this model (illustrated for \( \text{Il6} \) in Fig. 7), PGE2 exerts opposing, direct positive and indirect negative effects on gene expression. It positively regulates expression at the transcriptional level via cAMP signaling, and negatively regulates expression post-transcriptionally via induction of DUSP1, inhibition of MAPK p38 and enhancement of TTP function. In wild type macrophages these effects are balanced. In the absence of DUSP1, the negative regulation is lost and the positive regulation unmasked.

Macrophage TNF biosynthesis was inhibited by exogenously added PGE2 but insensitive to endogenously produced PGE2, most likely due to a time delay in LPS-induced PGE2 biosynthesis (Fig. 6). This discrepancy highlights the artificial nature of in vitro experiments, in which relatively pure cell populations are exposed to a synchronous stimulus. In vivo, as cells migrate to a site of infection or tissue damage, they will encounter a milieu that is continually changing as the inflammatory response peaks and resolves. We suggest that the local biosynthesis of PGE2 helps to shape this program by modulating the responses of cells arriving at different stages. A monocyte or macrophage arriving early and encountering LPS in the absence of PGE2 will generate a response very different from that of a late-arriving cell that encounters LPS in the presence of high concentrations of PGE2. In this sense, PGE2 may provide temporal context for responses to pro-inflammatory stimuli, assisting in transitions between initiation and resolution phases. This mode of action of PGE2 may help to explain why cyclooxygenase inhibition sometimes does not have straightforwardly anti-inflammatory consequences, or can even be toxic to resolution.

Methods

Animals and experimental procedures. All mice were maintained at the Biomedical Services Unit of the University of Birmingham. Animal care and experimental procedures were performed according to Home Office guidelines under PPL 40/8003, and approved by the University of Birmingham Local Ethical Review Committee.

In vivo LPS challenge was performed by intraperitoneal injection of PBS or 5 mg/kg LPS. After three hours all mice were humanely killed. Spleens were recovered and homogenized in RLT buffer (Qiagen) prior to isolation of RNA as described below.

Reagents. LPS (\( \text{E. coli EH100} \)) was from Enzo Life Sciences. PGE2, PF04418948 and ONO-AE3-208 were from Cayman Chemical. Antibodies used in western blotting were from Santa Cruz (COX-2, sc-1745; DUSP1, sc-373841), Cell Signaling Technology (phosphorylated MAPK p38, #9211), Sigma Aldrich (tubulin, T9026 and \( \beta \)-actin, A1978). Flow cytometry antibodies against EP2 and EP4 were from Cayman Chemical (16684 and 16625). All other reagents were from Sigma Aldrich. The generation of \( \text{Dusp1}^{+/--} \), \( \text{Zfp36aa}^{+/--} \) and \( \text{Dusp1}^{+/--} \), \( \text{Zfp36aa}^{+/--} \) mouse strains was previously described. All strains were back-crossed to C57/BL6J for at least ten generations.

Cell culture. Mice between 6 and 12 weeks of age were humanely culled, bone marrow flushed from femurs, and bone marrow-derived macrophages (BMMs) obtained by culture for 7 days in RPMI1640 containing 10% heat-inactivated FCS and 100 ng/ml M-CSF. Prior to experimentation, macrophage purity was assessed by flow cytometry. Routinely >95% of cells were F4/80+ at the end of the 7 day culture period. BMMs were harvested.
by scraping, seeded at a density of 10⁴/ml in appropriate culture vessels and rested overnight in the absence of M-CSF before being stimulated. Primary human macrophages were generated from peripheral blood monocytes of healthy donors as previously described, by culture for 5–7 days in RPMI1640 containing 10% heat-inactivated FCS and 100 ng/ml M-CSF. After this time, cells were harvested by scraping, seeded at a density of 10⁴/ml and rested overnight in the absence of M-CSF before being stimulated.

**Measurement of mRNA.** RNA was isolated from primary human or mouse macrophages using QIAshredder columns and RNeasy kits (Qiagen). cDNA was generated using iScript cDNA Synthesis kits (Bio-Rad). Gene expression was measured by quantitative PCR with a Roche Light-Cycler 480 Mark II, using custom-designed primers (Eurofins) and SYBR Premix Ex Taq (Takara). Relative expression was calculated using the \( \Delta\Delta\text{Ct} \) method with B2m or B2M mRNA for normalization.

For microarray analysis of gene expression in primary mouse macrophages, RNA was prepared as above and processed as described. For microarray analysis of gene expression in human monocyte-derived macrophages, RNA was prepared as above, cleaned and concentrated then applied to Affymetrix HuGene 1.0 ST arrays. Data analysis was essentially as described, using two-way mixed model ANOVA in Partek Genomics Suite version 6.6. One of the two mouse microarray experiments has been submitted to the Gene Expression Omnibus (GSE68449) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The second mouse BMM microarray and the human monocyte-derived macrophage microarray experiments are more fully described by manuscripts currently in preparation, and the data will be submitted to GEO in full. In the interim, these datasets are available from the author on reasonable request.

**RNA Immunoprecipitation.** 2 × 10⁷ RAW264.7 cells were left untreated or stimulated with 10 ng/ml LPS for 1 h, then harvested by scraping, washed twice with ice-cold PBS and lysed by repeated freeze-thawing in 1 ml of ice cold polystyrene lysis buffer (100 mM KCl, 10 mM HEPEs [pH 7.0], 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM DTT, 100 U/ml RNase inhibitor, protease and phosphatase inhibitor cocktails [Roche]). Immunoprecipitations and mRNA measurements were essentially as described, except that a pre-immune (PI) rabbit serum was used as the immunoprecipitation control, and quantitative PCR was used to derive fold enrichment of various mRNAs in the anti-TTP immunoprecipitates compared with the PI controls (calculated as 2⁻ΔΔCt).

**Measurement of proteins and PGE₂.** Intracellular proteins were detected by western blotting using reagents listed above. Secreted IL-6, TNF and PGE₂ were detected by sandwich ELISA using commercial kits, according to manufacturers' instructions. For detection of cell surface EP4, BMMs were fixed and permeabilized using Cytofix/Cytoperm solution according to manufacturer’s instructions (BD Biosciences) and subjected to flow cytometry using an APC-coupled antibody, Cya flow cytometer (Beckman Coulter) and Flojo software (TreeStar Inc).

**Statistics.** Statistical analysis was performed using GraphPad Prism 6.07. Pairwise comparisons were performed using Holm-Sidak's method, whilst multiple comparisons were performed using Dunnett's multiple comparison test. The same marks are used throughout; n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.005.

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**Author Contributions**

A.R.C. devised the study, interpreted experiments and wrote the manuscript. T.T., T.E.S., T.S., H.E.C., E.A.R., D.R.R. and J.O’N. designed, performed and interpreted experiments and contributed to the preparation of the manuscript.

**Additional Information**

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