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PROSTAGLANDIN E2 RESTRAINS MACROPHAGE MATURATION

Prostaglandin E2 restraints macrophage maturation via E prostanoid receptor 2/protein kinase A signaling

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Abstract

Prostaglandin E$_2$ (PGE$_2$) is a lipid mediator that acts by ligating four distinct G protein-coupled receptors, E prostanoid (EP) 1-4. Previous studies identified the importance of PGE$_2$ in regulating macrophage functions, but little is known about its effect on macrophage maturation. Macrophage maturation was studied in vitro in bone marrow cell cultures, and in vivo in a model of peritonitis. EP2 was the most abundant PGE$_2$ receptor expressed by bone marrow cells and its expression further increased during macrophage maturation. EP2/-/- macrophages exhibited enhanced in vitro maturation as compared to wild type cells, as evidenced by higher F4/80 expression. An EP2 antagonist also increased maturation. In the peritonitis model, EP2/-/- mice exhibited a higher percentage of F4/80$^{\text{high}}$/CD11b$^{\text{high}}$ cells and greater expression of macrophage colony-stimulating factor receptor (M-CSFR) in both the blood and the peritoneal cavity. Subcutaneous injection of the PGE$_2$ analog misoprostol decreased M-CSFR expression in bone marrow cells and reduced the number of peritoneal macrophages in wild type, but not EP2/-/-, mice. The suppressive effect of EP2 ligation on in vitro macrophage maturation was mimicked by a selective protein kinase A (PKA) agonist. Our findings reveal a novel role for PGE$_2$/EP2/PKA signaling in the suppression of macrophage maturation.
Introduction

Mononuclear phagocytes play a vital role in innate immune defense as well as disease states such as diabetes, atherosclerosis, and emphysema. The mononuclear phagocyte system encompasses both circulating peripheral blood monocytes and tissue macrophages. It is generally believed that monocytes and macrophages originate from a common myeloid precursor in the bone marrow. Tissue macrophages may derive from either local proliferation or from recruitment of peripheral monocytes. While local proliferation may play a role under steady-state conditions, repopulation of resident tissue macrophages in the setting of inflammation is more dependent on the recruitment of precursor cells from the blood. After emigrating from the blood to the tissues, monocytes continue the process of maturation and undergo the final differentiation steps necessary to become a macrophage. Macrophage maturation has also been studied in vitro, with a commonly employed protocol utilizing bone marrow cells cultured in the presence of macrophage colony-stimulating factor (M-CSF). This in vitro approach results in a high yield of relatively homogenous primary macrophages which are not conditioned by the tissue microenvironment.

Prostaglandin E_2 (PGE_2) is a lipid mediator derived from the metabolism of arachidonic acid by cyclooxygenase (COX). It is the most abundant prostanoid in most tissues, and is a well-known regulator of numerous physiologic and pathophysiologic processes including blood flow, parturition, fever, cancer and inflammation. PGE_2 exerts its actions through four different types of G protein-coupled receptors called E prostanoid (EP) receptors 1-4. PGE_2 has diverse effects on differentiation, migration, and activation of immune cells, including lymphocytes,
dendritic cells, and macrophages. Its effects on macrophage functions have been amply documented to be mainly suppressive\textsuperscript{12,13}, reflecting an increase in the levels of the intracellular second messenger cAMP generated upon ligation of EP2 and EP4 receptors\textsuperscript{14}. By contrast, there is little known about the role of PGE\textsubscript{2}/cAMP in macrophage maturation. Questions of how PGE\textsubscript{2} affects macrophage maturation, whether endogenously generated PGE\textsubscript{2} modulates this process, the role of specific EP receptors in such effects, and if PGE\textsubscript{2} influences macrophage maturation in vivo, remain unanswered.

In the present study, we have used pharmacologic and genetic approaches to determine the role of PGE\textsubscript{2}/EP2 signaling in the regulation of macrophage maturation in vitro and in vivo. We report that PGE\textsubscript{2}/EP2 signaling suppresses macrophage maturation, and have characterized key afferent and efferent mechanisms for this effect. As PGE\textsubscript{2} production is altered by both disease states as well as medications, and as maturation status influences macrophage functions, these findings have broad implications.
Methods

Animals

Mice harboring a targeted deletion of both alleles of the ptger2 encoding the EP2 receptor were originally generated by Dr. Richard Breyer (Vanderbilt University)\(^\text{15}\). These six-to-eight week old female EP2-deficient (EP2-/¬) mice, bred on a C57BL/6 background, and age-matched, female C57BL/6 wild type (WT) animals (EP2+/+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the University of Michigan Unit for Laboratory Animal Medicine. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals, with the approval of the University of Michigan Committee for the Use and Care of Animals.

Reagents

Dulbecco’s modified Eagle’s medium without phenol red, RPMI 1640, and penicillin/streptomycin solution were purchased from Invitrogen (Carlsbad, CA). Aspirin was from Sigma-Aldrich (St. Louis, MO). AH-6809, misoprostol and PGE\(_2\) were from Cayman Chemicals (Ann Arbor, MI). Ono-AE3-208 was a kind gift from Ono Pharmaceuticals (Osaka, Japan). The PKA-specific cAMP analog, 6-Bnz-cAMP (N6-benzoyladenine-3\(^\text{′}\),5\(^\text{′}\)-cyclic monophosphate), and the Epac-1-specific cAMP analog, 8-pCPT-2\(^\text{′}\)-O-Me-cAMP (8-4-chlorophenylthio-2\(^\text{′}\)-O-methyladenosine-3\(^\text{′}\),5\(^\text{′}\)-cyclicmonophosphate), were obtained from Biolog Life Science Institute (Howard, CA). TRIzol was purchased from Invitrogen, and primers for quantitative real-time RT-PCR were obtained from Integrated DNA Technologies (Coralville, IA).
Cell harvest and culture

Isolation of bone marrow cells and peritoneal macrophages was performed as described previously. Blood leukocytes were collected from the inferior vena cava and transferred to 15 ml tubes. Clotting was prevented by addition of EDTA. Erythrolysis was performed with 0.8% ammonium chloride lysis buffer for 10 min at room temperature and stopped by addition of RPMI medium. Cells were washed with RPMI medium and suspended in PBS/2 mM EDTA/0.5% fetal calf serum (FCS) for flow cytometry staining. To study maturation of bone marrow derived macrophages, 2x10^6 bone marrow cells were cultured as described for 6 days in 35 mm diameter Petri dishes in 30% L929 cell supernatant in RPMI 1640 medium containing 20% FCS, L-glutamine, and penicillin/streptomycin. After 3 days cell culture was supplemented with new medium totaling 50% of original volume.

Quantitative PCR and RNA isolation

Cells were suspended in 1 ml of TRIzol and RNA was extracted as described previously. RNA was amplified by quantitative reverse transcription-PCR (qRT-PCR) performed with a SYBR Green PCR kit (Applied Biosystem, Warrington, UK) on an ABI Prism 7300 Thermocycler (Applied Biosystems, Carlsbad, CA). Relative gene expression was determined by the ΔCT method, and β-actin was used as reference gene. Primer efficiency tests were performed on all primers and ranged from 97% to 107%.
Flow cytometry

For staining for flow cytometric analysis, cells were resuspended in PBS/2 mM EDTA/0.5% FCS. Fc receptor-mediated and nonspecific antibody binding was blocked by addition of excess CD16/CD32 (BD Pharmingen, San Jose, CA). Staining was performed at 4°C in the dark for 15 min. During maturation experiments, when cells were collected each day for 6 days, samples were stabilized with 1% PFA and analyzed on the same day. The following monoclonal antibodies were used at appropriate dilutions for staining: CD11c, CD11b, GR-1 (all from BD Pharmingen), CD115 (Biolegend, San Diego, CA), and F4/80 (eBioscience, San Diego, CA). A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) was used for flow cytometric characterization of cell populations and data was analyzed with WinMDI and FlowJo software.

Peripheral blood count

Determination of peripheral blood white blood cell counts was performed by the University of Michigan Unit for Laboratory Animal Medicine’s Animal Diagnostic Laboratory using a Hemavet cell analyzer (Drew Scientific).

ELISA

The quantification of murine CCL2 protein (R&D Systems, Minneapolis, MN) from peritoneal lavage and PGE2 (Enzo, Ann Arbor, MI) from cell cultures was performed by commercially available ELISA kits following the instructions of the manufacturer.
**In vivo thioglycollate treatment**

Peritonitis was induced by intraperitoneal injection with 2 ml of 3% thioglycollate, as described previously \(^{16}\). This protocol is well-described to result in macrophage-predominant inflammation at days 3-4 post-injection \(^{18}\).

**In vivo misoprostol treatment**

To determine the effects of a stable PGE\(_2\) analog on peritonitis, mice were injected subcutaneously with 200 µl saline containing 50 µg of misoprostol in 0.5% DMSO two hours before and ten hours after intraperitoneal thioglycollate; control mice received 200 µl saline containing 0.5% DMSO alone.

**Data analysis**

All data are displayed as mean values ± SEM from 3 to 8 independent experiments, each employing a different mouse. Statistical differences among treatment groups were estimated by ANOVA with Tukey’s post hoc test for multiple comparisons, or by paired Student’s t-test, as appropriate, using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.
Results

EP2 is the most highly expressed PGE₂ receptor during *in vitro* macrophage maturation

As an *in vitro* model of macrophage maturation, freshly flushed bone marrow cells were cultured for up to 6 days in the presence of 30% L929 murine fibroblast supernatant, a source of M-CSF and other growth factors. Cell supernatant was collected every day of the culture and PGE₂ concentration was measured by ELISA. The PGE₂ level in cell supernatants exceeded that in cell-free medium at every day of culture, but its level did not change during macrophage maturation (Fig. 1A). Cells started to adhere to the bottom of cell culture plates around day 3, and expression of EP1-EP4 was determined by qRT-PCR analysis of both the adherent cell fraction on days 3-6 (Fig. 1B) and of pooled adherent plus non-adherent cells on days 1-6 (Fig. S1). EP2 was the most highly expressed of the receptors in adherent cells and its expression peaked at day 4, when mRNA levels exceeded those of EP1 and EP4 by 16-fold and that of EP3 by 64-fold (Fig. 1B). As adherence is itself a characteristic of mature macrophages, this kinetic pattern suggests the possible relevance of EP2 in the process of maturation. EP4, whose mRNA expression transiently disappeared at day 1 (Fig. S1) exhibited an increase in expression only after the increase in adherence ensued.

Lack of PGE₂ signaling through EP2 promotes macrophage maturation *in vitro*

Based on its abundance (Fig. 1B), EP2 is the receptor most likely to mediate any biological actions of PGE₂ during the process of macrophage maturation. Both pharmacologic and genetic approaches were utilized to determine the importance of the PGE₂/EP2 signaling axis in *in vitro* maturation. First, bone marrow cells were plated in the absence and presence of the EP2
antagonist AH6809; this agent was replenished at day 3 along with fresh medium. Macrophage maturation was assessed on the basis of surface expression of CD11b (myeloid lineage marker) and F4/80 (macrophage marker). As expected with this protocol, CD11b and F4/80 increased in a time-dependent fashion in untreated cells, consistent with macrophage maturation (Fig. 2A). By contrast, the neutrophil marker Gr-1 was expressed at day 1 but did not significantly change throughout 6 days of culture. Cells treated with the EP2 antagonist exhibited significantly higher expression of F4/80, while CD11b and GR-1 did not change (Fig. 2A). In contrast to the effect of the EP2 antagonist, an EP4 antagonist (Ono-AE3-208) added to parallel wells in the same experiments exhibited no effect on F4/80 expression (Fig. 2B), highlighting the specific importance of EP2 signaling in regulating macrophage maturation. Addition of the COX inhibitor aspirin, which blocks PGE\(_2\) production, increased F4/80 expression, while the addition of exogenous PGE\(_2\) decreased it (Fig. 2C). Although L929 supernatant itself contains PGE\(_2\), the effect of aspirin on F4/80 expression implicates the contributions of autocrine PGE\(_2\) derived from macrophage COX metabolism in restraining macrophage maturation. The importance of EP2 was validated using bone marrow cells from EP2-/- mice. The percentage of mature (CD11b\(^{pos}\)/F4/80\(^{pos}\)) macrophages at day 6 in culture was significantly higher in EP2-/- mice than in wild type controls (Fig. 2D). As compared to wild type cells, day 6 macrophages from EP2-/- mice likewise exhibited significantly increased expression of M-CSFR (also known as CD115) (Fig. 2E), a key transducer of macrophage maturation. These differences in F4/80 and M-CSFR expression between EP2-/- and wild type bone marrow-derived macrophages were not present at day 1 (data not shown), indicating that these changes are associated with maturation.
Together, these data demonstrate that endogenous and exogenous PGE₂/EP2 signaling suppress *in vitro* macrophage maturation.

**EP2-/- mice exhibit increased macrophage maturation *in vivo***

Data presented in Fig. 2 D and E indicated that bone marrow-derived macrophages from EP2-/- mice are more mature (exhibit greater CD11b/F4/80 double positivity) than those from wild type controls. By contrast, no such difference between the two genotypes was observed in resident peritoneal macrophages (Fig. 3A). Moreover wild type and EP2-/- mice had similar number of CD11b⁺/F4/80⁺ cells in the bone marrow and number of monocytes in the blood (data not shown). Likewise, resident alveolar macrophages from EP2-/- and wild type mice demonstrated similar expression of F4/80 and the alveolar macrophage-specific maturation marker CD11c (data not shown). These observations suggested that, in contrast to the *in vitro* maturation model, under steady-state conditions *in vivo*, the degree of maturation in long-lived tissue macrophages was not affected by the lack of PGE₂/EP2 signaling. However, the importance of PGE₂/EP2 signaling in a dynamic model of macrophage maturation that occurs under *in vivo* inflammatory conditions remained to be defined. Mature peritoneal macrophages, like bone marrow-derived macrophages, can be defined as F4/80⁺/CD11b⁺ cells. This allows direct comparison of *in vitro* and *in vivo* data. Moreover, it has been shown that in the model of thioglycollate peritonitis, newly recruited and maturing macrophages originate from blood monocytes, whose precursor resides in the bone marrow. Thus, studying each of these compartments permitted us to follow macrophage maturation under dynamic rather than steady-state conditions.
Four days after intraperitoneal thioglycollate injection, approximately 90% of cells in the peritoneal cavity were mononuclear cells and no difference was observed between wild type and EP2-/− mice, as determined from microscopic inspection of modified Wright-Giemsa-stained cytopins (Fig. S2). Likewise, the percentage of F4/80<sup>pos</sup>/CD11b<sup>pos</sup> cells was not significantly different between the two genotypes (data not shown). Interestingly, however, as compared to wild type mice, cells in the peritoneal cavities of EP2-/− mice had significantly higher expression of F4/80, as assessed by mean fluorescent intensity (Fig. S3A). This difference in F4/80 expression between wild type and EP2-/− mice was explained by higher numbers of mature macrophages in the latter, reflected by gating on a F4/80<sup>high</sup>/CD11b<sup>high</sup> cell population (Fig. 3B). Additionally, EP2-/− mice had significantly more cells expressing M-CSFR compared to wild type (Fig. 3C). Defined by CD11b<sup>pos</sup>/CD115<sup>pos</sup> as described previously, a modest but significant increase in the percentage of blood monocytes in EP2-/− mice compared to wild type mice was also observed 4 days after thioglycollate injection (Fig. 3D). This difference in percentage of blood monocytes was validated by Coulter counter blood analysis (Fig. S3B). However, neither total peripheral blood monocyte nor total white blood cell numbers differed between the two genotype (Fig. S3C). In summary, EP2-/− mice in this in vivo model of peritonitis exhibited a greater number of mature macrophages in the peritoneal cavity as well as macrophage precursors in the blood in association with higher expression of M-CSFR on mononuclear cells, implicating the endogenous role of PGE<sub>2</sub> in the process of macrophage maturation.
**Misoprostol suppresses in vivo macrophage maturation**

Monocytes originate from the bone marrow, circulate in the blood, and, especially under inflammatory conditions, serve as a reservoir of myeloid precursor cells for renewal of tissue macrophage populations. To better understand the role of PGE2/EP2 signaling in the in vivo macrophage maturation process, misoprostol—a stable but receptor-nonselective PGE2 analog—was administered subcutaneously using a modification of a previously published protocol. Specifically, misoprostol was administered 2 h before and 10 h after intraperitoneal thioglycollate injection. Potential receptor selectivity of any actions of misoprostol were then evaluated by determining its actions in EP2-/-, as compared to wild type, mice. Bone marrow cells were isolated 24 h after thioglycollate injection and expression of M-CSFR and suppressor of cytokine signaling-1 (SOCS1) was analyzed by qRT-PCR. SOCS1 is a protein that negatively regulates MCSF-R signaling and is a critical controller of numerous macrophage functions as well as monocyte to macrophage differentiation. As seen in Fig. 4A, thioglycollate treatment increased transcripts for M-CSFR in bone marrow cells of wild type animals and misoprostol substantially blunted this increase. By contrast, misoprostol failed to attenuate the thioglycollate-induced increase in M-CSFR in EP2-/- mice. Conversely, SOCS1 expression in bone marrow cells was downregulated by thioglycollate but substantially upregulated by misoprostol treatment in wild type animals. No such upregulation was elicited by misoprostol in EP2-/- mice. These data suggest the importance of PGE2/EP2 in the suppression of expression as well as signaling of MSCF-R in bone marrow cells. The effects of misoprostol on peritoneal macrophage accumulation 4 days post-thioglycollate were also examined. The number of mononuclear cells in the peritoneal cavity of mice treated with misoprostol was significantly
decreased in wild type, but not in EP2-/mice (Fig. 4B). In fact, misoprostol treatment significantly increased the number of mononuclear cells in EP2-/mice. As misoprostol is a PGE$_2$ analog that can ligate all EP receptors, this finding presumably reflects the unmasking in the absence of EP2 of opposing actions mediated via other receptors. Notably, misoprostol failed to inhibit peritoneal production of CCL2 (MCP-1), the major chemokine mediating macrophage recruitment in the peritonitis model (Fig. 4C). The fact that MCSF levels in serum did not differ between the two genotypes (data not shown) suggests that the observed differences in macrophage maturation instead reflect the importance of the differences in M-CSFR expression on macrophages and macrophage precursors. These data suggest that misoprostol, via EP2, inhibits *in vivo* macrophage maturation, rather than recruitment signals.

**Roles of PKA and Epac in *in vitro* macrophage maturation**

PGE$_2$/EP2 signaling increases intracellular cAMP concentration, and cAMP-dependent protein kinase A (PKA) and guanine nucleotide exchange protein directly activated by cAMP (Epac) are two effectors of cAMP. To further investigate the downstream mechanisms by which PGE$_2$/EP2 signaling restrains *in vitro* macrophage maturation, the effects of cAMP analogs that selectively activate PKA or Epac were studied. Agonists and their concentrations utilized were based on our previous studies in macrophages, where we have also shown that Epac-1, but not Epac-2 was expressed in macrophages. Bone marrow cells were plated in 30% L929 cell supernatant in the absence or presence of PKA or Epac agonists, which were replenished with the addition of fresh medium at day 3 of culture. After 6 days of culture, PKA agonist-treated cultures demonstrated significantly fewer F4/80$^{pos}$/CD11b$^{pos}$ cells than did control cultures (Fig. 4D).
5A) and the effect of PKA agonist was similar in wild type and EP2-/− mice (data not shown). Interestingly, cells cultured with the Epac agonist manifested the opposite effect, showing an increased number of F4/80\textsuperscript{pos}/CD11b\textsuperscript{pos} cells compared to control (Fig. 5A). Moreover, PKA agonist decreased the number of adherent cells by ~80% (Fig. 5B). To exclude the possibility that the PKA agonist effect was simply due to a reduction in cell adherence, floating cells were also analyzed for the expression of CD11b and F4/80. As was the case for adherent cells, the floating cell fraction of cultures treated with PKA agonist also contained significantly fewer F4/80\textsuperscript{pos}/CD11b\textsuperscript{pos} cells (24.5%) than did control (39%) or Epac agonist-treated (34.5%) cultures (average values of 2 independent experiments), demonstrating that the PKA agonist indeed inhibited macrophage maturation. In addition, PKA agonist-treated cells exhibited a dramatic reduction in cell surface M-CSFR, while Epac agonist-treated cells exhibited a much more modest reduction (Fig. 5C). Thus, the effects of PGE\textsubscript{2}/EP2 signaling on macrophage maturation and M-CSFR expression are closely mimicked by the PKA agonist but not the Epac agonist. The fact that the suppression of macrophage maturation by PGE\textsubscript{2} is less robust than that by PKA agonist is likely explained by the opposing action of Epac – which is also activated by PGE\textsubscript{2}-
driven cAMP – on macrophage maturation.
Discussion

Local PGE$_2$ has been shown to modulate numerous functions of mature leukocytes $^{28}$. For example, in macrophages PGE$_2$ inhibits production of TNF-$\alpha$ $^{29}$ and IL-12 $^{30}$, decreases MHC II expression, which alters antigen presentation capacity $^{31}$, and suppresses ingestion and killing of microbes $^{12,32}$. These suppressive functions have mainly been linked to the ligation of EP2 and/or EP4 receptors $^{33}$. An inhibitory effect of PGE$_2$ on proliferation of myeloid progenitor cells in agar cultures was described in 1979 $^{34,35}$. A concentration of PGE$_2$ as low as $10^{-10}$ M was reported to inhibit macrophage colony proliferation on agar cultures $^{34}$. Subsequently, PGE$_2$ has been shown to suppress differentiation of dendritic cells via EP2 $^{36}$ or EP4 $^{37}$ receptors. In addition to these local actions, an endocrine effect of PGE$_2$ is suggested by the finding that PGE$_2$ either elaborated by keratinocytes or released from subcutaneously implanted time-release pellets influenced the characteristics of bone marrow-derived cells $^{38}$. Here we demonstrate that both endogenous and exogenous PGE$_2$ inhibits maturation of macrophage precursors in the bone marrow. This occurs via an EP2/cAMP/PKA pathway and is able to limit the accumulation of mature tissue macrophages recruited in response to an in vivo inflammatory stimulus.

Maturation is crucial for effective macrophage-mediated functions such as immune surveillance. Although the function of F4/80 remains obscure decades after its first description $^{39}$, it is generally considered the best available murine mononuclear phagocyte marker $^{40}$. Since F4/80 is highly expressed by resident peritoneal macrophages and is upregulated during in vitro differentiation of bone marrow-derived macrophages, we were able to utilize it as a marker of
maturation both in vitro and in vivo. In the in vivo circumstance, mononuclear phagocyte maturation must be distinguished from recruitment. In a peritonitis model it has been demonstrated that CCL2 is the main chemokine driving monocyte recruitment, and mice lacking CCL2 or its receptor CCR2 manifested decreased numbers of F4/80-positive cells in the peritoneal cavity after thioglycollate-induced peritonitis. We observed no effect of in vivo misoprostol on CCL2 levels in the peritoneum after thioglycollate injection, and preliminary experiments (not shown) likewise noted that it had no effect on CCR2 expression in bone marrow cells. Therefore, it is likely that the reduced accumulation of mature macrophages with misoprostol treatment in the peritonitis model is independent of chemokine-mediated cell recruitment and instead reflects impaired maturation.

Regulation of macrophage maturation is believed to be mediated by colony stimulating factors (CSFs), which include M-CSF as well as granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF) and IL-3. The central importance of M-CSF in this regard is well-established. Expression of the high-affinity receptor M-CSFR, also known as CD115, increases during macrophage maturation, and in a variety of in vitro and in vivo models it has been shown that administration of M-CSF leads to increases in blood monocytes as well as peritoneal macrophages. In our study both bone marrow-derived macrophages as well as freshly recruited peritoneal macrophages from EP2/- mice exhibited an increased level of M-CSFR compared to cells from wild type animals. To our knowledge, however, this study is the first to report an increase in M-CSFR transcripts and a decrease in SOCS1 transcripts in bone marrow cells of mice subjected to thioglycollate peritonitis. Moreover, in vivo administration of misoprostol reduced M-CSFR and increased SOCS1 levels in bone marrow precursor cells.
Together, these data strongly suggest that down-regulation of M-CSFR represents one important mechanism by which EP2 signaling in response to either endogenous or exogenous PGE$_2$ suppresses macrophage maturation.

Interestingly, macrophages stimulated with M-CSF have been reported to increase PGE$_2$ production$^{44}$, suggesting the presence of an autocrine loop similar to that described in TLR signaling, in which PGE$_2$ is produced upon LPS stimulation and in turn, this prostanoid via EP2/cAMP/PKA modifies macrophage responses to LPS$^{45,46}$. Beyond its ability to regulate expression of M-CSFR, PGE$_2$ may also control the ability of this receptor to transmit signals$^{24}$, via its upregulation of SOCS1 in bone marrow precursors. Since M-CSF also activates a breadth of macrophage functions including phagocytosis, production of reactive oxygen species, chemotaxis, and microbial killing$^{43}$, PGE$_2$ regulation of M-CSFR very likely has functional implications beyond maturation. Indeed, our group has previously reported that alveolar macrophages from EP2-/- mice manifested an increased capacity for phagocytosis$^{32}$; the possible role of enhanced M-CSFR signaling in this phenomenon remains to be evaluated.

EP2 was the most abundantly expressed PGE$_2$ receptor on bone marrow cells and maturing macrophages, and both genetic and pharmacologic data in vitro and in vivo argue that this receptor, rather than EP4, mediates the suppressive actions of PGE$_2$ on macrophage maturation. The suppressive effect on macrophage maturation was mirrored by the addition of a selective PKA agonist. PKA has previously been implicated as mediating PGE$_2$-induced suppression of macrophage functions such as bacterial killing and cytokine synthesis$^{27,46}$. Bone marrow cells treated with a selective PKA agonist displayed significantly decreased macrophage
maturation and almost completely lacked M-CSFR on the cell surface. Interestingly, a selective Epac agonist increased macrophage maturation, albeit not by increasing M-CSFR. The mechanism by which Epac activation enhanced macrophage maturation remains to be explored. However, an action that is independent of M-CSF is not without precedent, since even mice lacking M-CSF, G-CSF, and GM-CSF generate macrophages and mount an inflammatory response in thioglycollate-induced peritonitis. In this context, it is worth noting as well that the expression of Epac was reported to increase during monocyte to macrophage maturation and that Epac became functional only in fully matured macrophages.

In summary, we have shown using both pharmacologic and genetic approaches that PGE$_2$, through EP2 and PKA, suppresses macrophage maturation. This is a reflection of actions exerted on bone marrow precursors which include reduced expression of M-CSFR, and results in reduced numbers of blood monocytes and recruited tissue macrophages in an in vivo peritonitis model. This form of regulation is pertinent to the actions of both endogenous as well as exogenous PGE$_2$. PGE$_2$ levels are often elevated in disease states such as infection, inflammation, and cancer. Furthermore, EP2 expression can also be altered in disease states. Finally, commonly used medications including nonsteroidal anti-inflammatory drugs as well as glucocorticoids are well known to inhibit PGE$_2$ biosynthesis. Thus, the dynamics of macrophage maturation would be expected to be modulated in association with disease states or treatments in which PGE$_2$ production or responses are altered.
Acknowledgments

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Authorship contributions

Z.Z. designed the research, performed experiments, analyzed data, and wrote the paper; K.O. performed experiments and analyzed data C.H.S. and D.M.A. designed the research and analyzed data; and M.P.-G. designed research, supervised the work, analyzed data, and wrote the paper.

Conflict of interest disclosure

The authors declare no competing financial interests.
References


48. Bryn T, Mahic M, Enserink JM, Schwede F, Aandahl EM, Tasken K. The cyclic AMP-Epac1-Rap1 pathway is dissociated from regulation of effector functions in monocytes but


Figure Legends

**Figure 1. PGE$_2$ and its receptors during macrophage maturation.** (A) PGE$_2$ is present during macrophage differentiation. Freshly flushed bone marrow cells were cultured for up to 6 days in the presence of 30% L929 supernatant; after 3 days, cultures were replenished with new medium totaling 50% of original volume. Day 0 indicates 30% L929 supernatant alone in the absence of cells. Each day of culture, an aliquot of cell supernatant was aspirated and analyzed for PGE$_2$ concentration by ELISA. Data are expressed as the mean ± SEM from 6 to 8 experiments, each utilizing cells from a single mouse. (B) EP2 is most highly expressed among EP receptors during macrophage maturation. At each of days 3 to 6 of cell culture (as described in panel A), adherent cells were collected and analyzed for the expression of EP receptors with qRT-PCR. Data presented in all panels are expressed as the mean ± SEM from 3 to 4 experiments, each utilizing cells from a single mouse. * p<0.05, # indicates significant (p<0.05) difference from all other conditions.

**Figure 2. EP2 antagonism/deficiency promotes macrophage maturation.** (A) EP2 antagonism promotes macrophage maturation. Freshly flushed bone marrow cells were cultured for 6 days with 30% L929 supernatant in the absence or presence of EP2 antagonist (AH6809) at 10 µM; after 3 days culture was replenished with new medium containing AH6809 totaling 50% of original volume. Each day of the culture, cells were collected, stained and analyzed for the markers indicated. Mean fluorescent intensity (MFI) is expressed as the mean ± SEM from 3 experiments, each employing a different mouse. * p<0.05, *** p<0.001. (B) EP4 antagonism does not promote macrophage maturation. Freshly flushed bone marrow cells were cultured...
with the EP2 antagonist (AH6809) at 10 µM, or with 1 µM of EP4 antagonist (ONO-AE3-208); control cells were cultured with DMSO. Cells were stained with F4/80- APC and CD11b-FITC and analyzed after 6 days of culture as described in panel (A). Control containing DMSO was set as 100%. Mean fluorescent intensity (MFI) is expressed as the mean ± SEM from 3 experiments, each employing a different mouse. * p<0.05. (C) Effects of aspirin and exogenous PGE2 on macrophage maturation. Freshly flushed bone marrow cells were cultured with aspirin at 200 µM, or with 1 µM PGE2; control cells were cultured with DMSO. Cells were stained with F4/80-APC and CD11b-FITC and analyzed as described in panel (A). Control containing DMSO was set at 100%. Mean fluorescent intensity (MFI) is expressed as the mean ± SEM from 3 experiments, each employing a different mouse. * p<0.05, *** p<0.001. (D) EP2 deficiency promotes macrophage maturation. Freshly flushed bone marrow cells isolated from EP2-/- and wild type mice were cultured in 30% L929 cell supernatant as described in panel (A). The dot plot shown is representative of 3 experiments, each employing cells from a different mouse. The percentage of CD11b<sup>pos</sup>/F4/80<sup>pos</sup> cells in both genotypes was compared using paired t-test. * p<0.05. (E) CD115 (M-CSFR) is upregulated in EP2-/- mice during in vitro macrophage maturation. After 6 days of culture, bone marrow-derived cells were collected and analyzed for the expression of CD115 with qRT-PCR. Data are expressed as the mean ± SEM from 3 experiments, each employing cells from a different mouse. * p<0.05

Figure 3. EP2-/- mice exhibit increased macrophage maturation in a model of thioglycollate peritonitis. (A) Maturation of peritoneal macrophages under steady state conditions does not differ between EP2-/- and wild type mice. Peritoneal cells were isolated by lavage from naïve wild type and EP2-/- mice, and stained for the markers indicated. The dot plot shown is
representative of 4 experiments, each employing cells from a different mouse. (B) and (C) EP2-/- mice have higher numbers of mature macrophages (CD11b^{high}/F4/80^{high}) and cells expressing CD115 in the peritoneal cavity in a model of peritonitis. EP2-/- and wild type mice were injected intraperitoneally with thioglycollate. Peritoneal cells were isolated by lavage 4 days later and stained with CD11b-FITC, F4/80-APC and CD115-PE as described in panel (A). The dot plots shown are representative of 3 to 4 experiments, each utilizing a separate mouse. The percentage of CD11b^{high}/F4/80^{high} cells (panel B) and CD11b^{pos}/CD115^{pos} cells (panel C) was compared between both genotypes using a paired t-test. * p<0.05. (D) EP2-/- mice have a higher number of blood monocytes during peritonitis. Blood leukocytes were obtained from EP2-/- and wild type mice 4 days after intraperitoneal injection of thioglycollate and stained as described in panels (B) and (C). Blood monocytes were identified as a low side scatter (SSC) cell population showing cell surface expression of CD11b and CD115. The dot plot shown is representative of 4 experiments, each utilizing a different mouse. Percentages of CD11b^{pos}/CD115^{pos} cells in both genotypes were compared using paired t-test. * p<0.05, n.s. not significant.

**Figure 4. In vivo effects of misoprostol on macrophage maturation during peritonitis.** (A) Misoprostol downregulates CD115 and upregulates SOCS1 in bone marrow cells of wild type, but not EP2-/- mice, in a model of thioglycollate peritonitis. 2 h before and 10 h after intraperitoneal thioglycollate injection mice were injected subcutaneously with 200 µl saline containing either 50 µg of the nonselective PGE2 analog misoprostol in 0.5% DMSO or DMSO alone. After 24 h wild type and EP2-/- mice were sacrificed and bone marrow cells were isolated and analyzed for the expression of CD115 and SOCS1 with qRT-PCR. Data shown are
expressed as the mean ± SEM from 6 experiments, each employing a different mouse. * p<0.05, n.s. not significant (B) Misoprostol attenuates macrophage accumulation in the peritoneal cavity in a model of thioglycollate peritonitis. Mice were treated as described in panel (A). After 4 days peritoneal cells of EP2-/- and wild type mice were lavaged and counted. The percent of mononuclear cells was determined from modified Wright-Giemsa-stained cytospins. Data are expressed as the mean ± SEM from 8 experiments, each employing a different mouse. (C) Misoprostol fails to attenuate CCL2 (MCP-1) production. Mice were treated as described in panel (A). After 24 h the peritoneal cavities of EP2-/- and wild type mice were lavaged with 1 ml of lavage buffer and supernatant was analyzed for MCP-1 using ELISA. Data are expressed as the mean ± SEM from 6 experiments, each employing a different mouse. thio, thioglycollate, miso, misoprostol

Figure 5. Role of PKA and Epac in *in vitro* macrophage maturation. (A) PKA inhibits, while Epac promotes, macrophage maturation. Freshly flushed bone marrow cells isolated from EP2-/- and wild type mice were cultured in 30% L929 cell supernatant in the presence of Epac or PKA (500 µM each). After 3 days cell culture was supplemented with new medium containing Epac or PKA totaling 50% of original volume and after 6 days, cells were collected and stained with CD11b-FITC and F4/80-APC. The dot plot shown is from a single experiment representative of 3 independent experiments, each employing a different mouse. Percentages of CD11b<sup>pos</sup>/CD115<sup>pos</sup> cells in different conditions were compared. Data are expressed as the mean ± SEM from 3 experiments, each utilizing a separate mouse.* p<0.05, ** p<0.01, *** p<0.001. (B) PKA agonist reduces numbers of macrophages during *in vitro* macrophage maturation. Cells were cultured as described in panel (A). After 6 days of culture floating cells
were washed away and adherent cells were counted by light microscopy. Data are expressed as
the mean ± SEM from 3 experiments, each employing a different mouse.* p<0.05, ** p<0.01,
*** p<0.001. (C) PKA agonist diminishes M-CSFR expression on the cell surface. Cells were
cultured as described in panels (A) and (B). After 6 days of culture adherent CD11b\textsuperscript{pos}/CD115\textsuperscript{pos}
cells were stained with CD115-PE. MFI is expressed as the mean ± SEM from 3 experiments,
each employing a different mouse. ** p<0.01, ***p<0.001. PKA agonist (6-Bnz-cAMP), Epac
agonist (8-pCPT-2'-O-Me-cAMP).
Fig. 1

(A) Bar chart showing PGE₂ (pg/ml) levels over different days. The x-axis represents days (0 to 6), and the y-axis represents the PGE₂ levels. The bars indicate the ΔCt (relative to β-actin).

(B) Bar charts for EP1, EP2, EP3, and EP4 showing ΔCt (relative to β-actin) over different days. The x-axis represents days (3 to 6), and the y-axis represents the ΔCt values. The charts include asterisks and hash marks to indicate statistical significance.
Fig. 2

A

B

C

D

E

Fig. 2
Fig. 3

A

wt EP2-/−

CD11b

F4/80

63% 66%

n.s.

wt EP2-/−

B

wt EP2-/−

CD11b

F4/80

11% 20%

*

wt EP2-/−

C

wt EP2-/−

CD11b

CD115

35% 58%

*

wt EP2-/−

D

wt EP2-/−

FSC

SSC

CD115

3.5% 4.7%

***

wt EP2-/−

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Fig. 4

A

CD115

Socs1

n.s.

n.s.

B

% mononuclear cells

C

MCP-1 (pg/mL)

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Fig. 5
Prostaglandin E$_2$ restrains macrophage maturation via E prostanoid receptor 2/protein kinase A signaling

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