Blockade of Prostaglandin E2 Signaling Through EP1 and EP3 Receptors Attenuates Flt3L-Dependent Dendritic Cell Development from Hematopoietic Progenitor Cells

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Running Title: PGE₂ regulates Flt3 signaling in DC progenitor cells

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ABSTRACT

Dendritic cell (DC) homeostasis, like all mature blood cells, is maintained via hierarchal generation from hematopoietic precursors; however, little is known about the regulatory mechanisms governing DC generation. Here we show that prostaglandin E2 (PGE2) is required for optimal Flt3-ligand-mediated DC development and regulates expression of the Flt3 receptor on DC-committed progenitor cells. Inhibition of PGE2 biosynthesis reduces Flt3-mediated activation of STAT3 and expression of the anti-apoptotic protein Survivin, resulting in increased apoptosis of DC-committed progenitor cells. Reduced DC development caused by diminished PGE2 signaling is reversed by over expression of Flt3 or Survivin in DC progenitors and conversely is mimicked by STAT3 inhibition. PGE2 regulation of DC generation is specifically mediated through the EP1 and EP3 G-protein PGE2 receptors. These studies define a novel DC progenitor regulatory pathway where PGE2 signaling through EP1/EP3 receptors regulates Flt3 expression and downstream STAT3 activation and Survivin expression, required for optimal DC progenitor survival and DC development in vivo.
INTRODUCTION

Dendritic cells (DCs) are specialized antigen-presenting immune cells that induce adaptive immune responses and maintain self-tolerance and are attractive targets for therapeutic manipulation of the immune system\(^1,2\). Two main DC subsets have been identified based on their location, phenotype and function; antigen-presenting classical DC (cDC), and type I interferon-producing plasmacytoid DC (pDC)\(^3,4\), and are generated from Flt3-expressing hematopoietic progenitor cells in the bone marrow (BM)\(^5,6\). Recent studies identified a common DC, monocyte and macrophage precursor designated as macrophage DC progenitor cell (MDP)\(^7,8\) and a common DC progenitor (CDP) that is restricted to DC development\(^9\). MDP differentiate to CDP, which in turn give rise to cDC and pDC, but not monocyte, and finally to pre-cDC\(^10\), a committed precursor of cDCs\(^11,12\). Unlike MDP and CDP that differentiate within the BM, pre-cDCs traffic to secondary lymphoid organs where they further differentiate into cDC\(^4,13\). In addition, monocytes can also develop a DC phenotype under inflammatory conditions\(^14,15\).

Flt3 (also known as Flk2 and CD135) is a receptor tyrosine kinase that is broadly expressed on early BM hematopoietic progenitor cells (HPC), including DC progenitor cells\(^5,9,16\). Mice with a deficiency in Flt3 or Flt3 ligand (Flt3L) have reduced DC numbers\(^13,17\), whereas administration of Flt3L dramatically increases BM and peripheral DC\(^18\). While Flt3 signaling is crucial for DC generation from their progenitor cells at steady-state, the normal physiological mechanisms that control Flt3 expression and regulate Flt3L-dependent DC differentiation remain poorly understood.

Prostaglandin E\(_2\) (PGE\(_2\)) is the predominant metabolite of arachidonic acid metabolism produced through the sequential action of phospholipase A\(_2\), cyclooxygenases and prostaglandin
E synthase$^{19,20}$. There are two functionally distinct cyclooxygenase (COX) enzyme isoforms, COX1 and COX2 that are encoded by different genes$^{20}$. PGE$_2$ has been shown to modulate HPC differentiation, inhibiting CFU-GM$^{21-23}$ but promoting BFU-E and CFU-GEMM$^{24,25}$. The long-acting PGE$_2$ analog, 16,16-dimethyl-PGE$_2$ (dmPGE$_2$) was shown to increase hematopoietic stem cell (HSC) frequency and engraftment$^{26,27}$ and enhances HSC survival, proliferation and homing to the BM$^{27}$. Thus, PGE$_2$ regulates self-renewal and differentiation of HSC and HPC and its effects can differ depending on hematopoietic cell type and stage of differentiation.

The role of PGE$_2$ in DC maturation and migration under inflammatory conditions is well known. PGE$_2$ promotes the migration of monocyte-derived and Langerhans DCs, increases their expression of costimulatory molecules, and enhances their ability to stimulate T cells after exposure to proinflammatory cytokines and antigens$^{28-30}$. However, the role of PGE$_2$ in regulation of DC differentiation from their specific progenitor cells and maintenance of DC homeostasis at steady state has not been explored. We now report that PGE$_2$ is an important regulator of Flt3L-dependent DC development. PGE$_2$ regulates DC development by modulating Flt3 expression on DC-committed progenitor cells, resulting in increased STAT3-mediated Survivin expression that enhances survival of DC-committed progenitor cells. The effect of PGE$_2$ on DC progenitor cells is specifically mediated via signaling through the EP1 and EP3 G-protein coupled PGE$_2$ receptors and DC production was significantly lower in EP1, EP3 and EP1/EP3 knockout mice. Our results are the first to define a role of PGE$_2$ in normal Flt3L-dependent DC differentiation and describe a novel regulatory pathway for DC generation that can be pharmaceutically manipulated.
METHODS

Mice and human cord blood

C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) or Jackson Laboratories (Bar Harbor, Maine) and maintained in the Indiana University School of Medicine (IUSM) animal facility. EP1, EP3 and EP1/EP3 knockout mice were obtained from Dr. Richard M Breyer (Vanderbilt University, TN USA), all on the C57BL/6 background. All mouse experiments were approved by the Indiana University Institutional Animal Care and Use Committee. Human umbilical cord blood (UCB) was obtained from Wishard Hospital (Indianapolis, IN) with IRB approval.

Inhibition of PGE2 synthesis

Mice were injected subcutaneously (s.c.) with Indomethacin (2.5 mg/kg/bid) (Sigma, St. Louis, MO) or Indomethacin plus PGE2 (1 mg/kg) for 6 days. Bone marrow and spleen were harvested and single cell suspensions prepared in Iscove’s modified Dulbecco’s medium (IMDM) (Lonza Biologics, Walkersville, MD) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (Hyclone, South Logan, UT). For in vitro experiments, lineage negative (Lin\(^{\text{neg}}\)) BM cells (CD5, B220, CD11b, Gr-1,7-4, and Ter-119)\(^{\text{neg}}\) or FACS sorted CDPs (Lin\(^{\text{neg}}\) c-Kit\(^{\text{int}}\) Flt3\(^{+}\) CD115\(^{+}\)) or CD34\(^{+}\) human UCB cells were treated with 1 uM Indomethacin, 10 nM SC560 (COX1 specific inhibitor), or 1 uM NS-398 (COX2 specific inhibitor) (Cayman Chemical, Ann Arbor, Michigan). Ethanol was used as a solvent for Indomethacin, SC560 and NS-398 at a final concentration of 0.01%.
**In vitro DC generation**

Mouse BM cells were enriched for the Lin<sup>neg</sup> cell population using the MACS lineage depletion kit (Miltenyi Biotech, Auburn, CA). Lin<sup>neg</sup> marrow cells or FACS sorted CDPs were cultured in 10% FBS and a predetermined optimal concentration of recombinant human Flt3 ligand (Flt3L) (100 ng/ml)<sup>9</sup> (Amgen, Seattle WA) supplemented IMDM for 9 days in the presence of Indomethacin, SC560, NS-398, PGE2 (0.1-1000 nM) or vehicle control. Medium was replaced every 3 days. For some studies, Lin<sup>neg</sup> BM cells were cultured for 5 days in supplemented complete medium (10% FBS) with 10 ng/ml recombinant mouse granulocyte-macrophage-colony stimulating factor (GM-CSF) to induce DC generation. For DC generation from human UCB, CD34<sup>+</sup> cells were purified from low density UCB using CD34 antibody conjugated-magnetic beads (Miltenyi Biotech) and cultured for 10 days in Flt3L (100 ng/ml), stem cell factor (50 ng/ml), IL-3 (10 ng/ml) and IL-6 (10 ng/ml) supplemented STEM Pro-CD34 (Invitrogen) medium as described<sup>31</sup> in the presence or absence of Indomethacin.

**Cell sorting and flow-cytometry analysis**

BM and spleen were harvested and single cell suspensions prepared in IMDM with 2% FBS. Total nucleated cell counts were obtained using a Hemavet-950 (Drew Scientific Inc., Waterbury, CT). Cells were incubated for 15 min with 0.5ug FcR block per million cells (BD Biosciences). To purify CDPs, BM cells were first immunomagnetically pre-enriched for Lin<sup>neg</sup> cells, then stained with fluorochrome conjugated anti-IL-7R, anti-c-Kit, anti-Flt3R and anti-CD115 and sorted on a FACS Aria (BD Bioscience). For phenotypic analysis of DC subsets, (CD3, CD19, NK1.1, Ter119)<sup>neg</sup> cells were analyzed for CD11c, MHCII, CD11b and B220
expression. All antibodies were purchased from BD Biosciences (San Jose, CA) or eBiosciences (San Diego, CA). Dead cells were excluded by staining the cells with LIVE/DEAD Fixable Violet Dead Cell Staining dye (Invitrogen Carlsbad, CA). For DC, pre-cDC and CDP analysis, at least 0.1, 0.25 and 1 x 10^6 million events were acquired, respectively.

**DC and DC progenitor cell analysis**

Total BM and spleen DC frequency was determined by gating on the CD3/CD19/NK1.1/Ter119neg CD11c^+ and MHC class II^+ cell population. The frequency of cDC and pDC was determined by gating on live CD3/CD19/NK1.1/Ter119neg CD11c^+ B220^- and CD11c^+ B220^+ cells, respectively. The percentage of CDP was determined by gating on Lin^neg IL-7R^- c-Kit^int CD115^+ Flt3^+ cells and pre-cDC by gating on [Ter119, CD19, CD3, NK1.1]neg MHC II^ CD11c^int/hi SIRPa^int Flt3^+ cells. Surface Flt3 expression on CDP and pre-cDC gated cell population was determined by flow-cytometry.

**DC progenitor proliferation and survival**

Indomethacin or vehicle treated mice were injected with bromodeoxyuridine (BrdU) (1 mg; i.p.). Mice were sacrificed 20 hours after BrdU injection and BM cells were harvested and stained with cell surface antibodies for CDP and pre-cDC. Cells were then fixed and permeabilized for intracellular BrdU staining according to the manufacturer’s instructions (BD Biosciences). For *in vitro* analysis of DC-committed progenitor cell proliferation, FACS sorted CDPs were labeled with CFDA (1uM) and cultured in Flt3L^- supplemented medium in the presence or absence of Indomethacin for 3 days. At the end of incubation, CDP proliferation was determined by
measuring intracellular CFDA intensity by flow cytometry\textsuperscript{13}. To examine the effect of blockade of prostaglandin synthesis on DC progenitor survival, FACS sorted CDP were cultured for 5 days in Flt3L supplemented complete medium in the presence or absence of Indomethacin and survival of Lin\textsuperscript{neg} CD11c\textsuperscript{+} MHCII\textsuperscript{+} Flt3\textsuperscript{+} pre-cDC was determined by Annexin-V staining. In addition, phospho-STAT3, activated caspase-3 and Survivin protein in pre-cDC and/or CDP were measured by flow-cytometry after intracellular staining.

**PGE\textsubscript{2} ELISA**

PGE\textsubscript{2} in the culture supernates of Flt3L stimulated Lin\textsuperscript{neg} BM cells was measured using a PGE\textsubscript{2} specific ELISA kit (Neogen Co., Lansing, MI) according to the manufacturer’s instructions.

**PGE\textsubscript{2} receptor analysis**

To determine the expression of PGE\textsubscript{2} receptors (EP1-4) on CDP and pre-cDC progenitors, Lin\textsuperscript{neg} BM cells were stained with EP receptor specific antibodies (all from Cayman Chemical) and EP receptor expression on gated cell populations determined by flow-cytometry. Analysis of EP receptors mRNA was performed by QRT-PCR using primer sequences as we previously described\textsuperscript{27}. To determine the EP receptor involved in DC development, Lin\textsuperscript{neg} BM cells were cultured for 9 days in Flt3L plus Indomethacin supplemented medium and treated with Butaprost (EP2 agonist), 17-phenyl-trinor prostaglandin E\textsubscript{2} (17 ptPGE\textsubscript{2}) (EP1, and EP3 agonist), each at 1 uM, Sulprostone (EP3 agonist) (50 nM), and 50 nM PGE\textsubscript{2}, all from Cayman Chemical, or L-902,688 (EP4 agonist) (1 uM), a kind gift from Merck-Frost, Canada, during culture. For some experiments, Lin\textsuperscript{neg} BM cells were cultured for 9 days in Flt3L supplemented medium with
Indomethacin, SC-51322 (EP1 antagonist), L798,106 (EP3 antagonist) or PGE$_2$ plus Indomethacin during culture.

**Flt3 over expression in DC progenitor cells**

The retroviral MSCV-IRES-EGFP vector containing Flt3 was prepared as described previously$^{32}$. Twenty-four micrograms purified Flt3 in MSCV-IRES-EGFP or empty vector were transfected into Phoenix Eco cells using Lipofectamine LTX and plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Flt3 was over expressed in CDP enriched Lin$^{neg}$ BM cells by retroviral transduction as described previously$^{32}$.

**Statistical analysis**

All values are reported as mean ± SEM. Statistical significance was determined by paired or unpaired Student’s T-test or one-way ANOVA with Bonferroni’s post-hoc analysis, as appropriate.
RESULTS

Inhibition of PGE₂ biosynthesis reduces in vivo DC development

We have previously shown that PGE₂ differentially regulates myeloid and erythroid hematopoietic lineage differentiation²¹-²⁵ and is an important regulator of stem cell function²⁷. To investigate the role PGE₂ plays in regulation of DC production, we inhibited in vivo PGE₂ biosynthesis in mice by treatment with Indomethacin, a dual COX1 and COX2 inhibitor, for 6 days and quantitated DCs in BM and spleen by flow cytometry. The frequency and total number of BM DCs (CD3/CD19/NK1.1/Ter119 neg CD11c⁺ MHCII⁺) were 3.2±0.6 fold lower in Indomethacin-treated mice compared to vehicle-treated mice (Figure 1A). Mature T cells, NK cells and B cells showed no change after Indomethacin treatment; whereas, as we reported previously²², ³³, monocytes were increased after blockade of PGE₂ biosynthesis (Table 1). Spleen DC numbers were also significantly decreased after Indomethacin treatment although to a lesser degree than BM (1.8±0.2 fold) (Figure 1A). Analysis of DC subsets showed that the total number of cDCs (CD11c⁺ B220⁻) and pDCs (CD11c⁺ B220⁺) are significantly decreased in BM and spleen when PGE₂ biosynthesis is blocked (Figure 1B). The reduction in cDC was significantly greater than pDC in the BM (69±8% vs 29±3%; P<0.01), however, in the spleen, cDC and pDC reduction was equivalent (43.6±12.8% vs 35.5±13.1%; P= 0.64). Co-administration of PGE₂, with Indomethacin completely blocked the reduction in BM and spleen DCs (not shown) produced by Indomethacin treatment (Figure 1C), confirming a specific requirement for PGE₂ in DC development and suggesting that PGE₂ acts as a positive regulator of DC development. As previously reported¹⁸, in vivo administration of Flt3L in mice dramatically increased total DCs (cDC and pDC) in the BM. Similar to that observed for steady-state mice, blockade of PGE₂
synthesis during *in vivo* Flt3L treatment resulted in significantly reduced DC generation (Figure 1D).

Since DCs develop in the BM from DC lineage-committed HPC\textsuperscript{7, 9, 16} and we previously demonstrated that PGE\textsubscript{2} can differentially regulate HPC depending on lineage\textsuperscript{21-25, 34}, we examined whether the attenuated development of DCs observed upon inhibition of PGE\textsubscript{2} biosynthesis results from an effect on the DC-committed progenitor cell compartment. In mice treated with Indomethacin for 6 days, the early BM common DC progenitors, CDP (Lin\textsuperscript{neg} c-Kit\textsuperscript{int} Flt3\textsuperscript{+} CD115\textsuperscript{+}) (Figure 1E), and immediate cDC precursors, pre-cDC (CD3/CD19/NK1.1/Ter119\textsuperscript{neg} CD11c\textsuperscript{+} MHCII\textsuperscript{-} SIRP\textsubscript{\alpha}\textsuperscript{int} Flt3\textsuperscript{+}) (Figure 1F), were significantly reduced compared to control. In contrast, phenotypically-defined myeloid-committed progenitor cells, KL (Lin\textsuperscript{neg} c-Kit\textsuperscript{high} Sca-1\textsuperscript{-}) increased in Indomethacin-treated mice (Figure S1), which is consistent with the increase in CFU-GM we previously reported\textsuperscript{33}. The total number of MDPs per femur was not statistically different than control in Indomethacin treated mice, although they did trend lower (Figure S1B). These findings expand the scope of regulation of hematopoiesis by PGE\textsubscript{2} to include the DC-committed progenitor cell compartment.

**PGE\textsubscript{2} is a positive regulator of Flt3L-dependent DC generation from hematopoietic progenitor cells**

At steady-state, DC development is mainly dependent on Flt3 signaling\textsuperscript{13, 17, 18}. To evaluate the role PGE\textsubscript{2} plays in DC development from hematopoietic progenitor cells, we evaluated Flt3L-dependent generation of DC from BM cells *in vitro*. Lineage depleted mouse BM cells enriched for CDP were cultured for 9 days in Flt3L supplemented medium with
Indomethacin, with or without dmPGE2, and DC enumerated by flow cytometry using a previously defined phenotypic determination. Generation of CD11c+ B220− cDC and CD11c+ B220+ pDC was substantially reduced in the presence of Indomethacin (cDC: 48±3.1% and pDC: 27±1.4%) (Figure 2A). Addition of exogenous PGE2 completely reversed the Indomethacin mediated reduction in DC differentiation, indicating that PGE2 signaling is required for optimal DC generation. Consistent with the requirement of PGE2 for optimal DC generation, PGE2 levels were virtually absent in cultures grown in the presence of Indomethacin (Figure 2B). To test whether endogenous PGE2 biosynthesis by DC-committed progenitor cells directly regulates DC differentiation and to rule out effects mediated through accessory cells, including stromal cells, we evaluated the effects of PGE2 inhibition on DC generation from highly purified DC progenitors. Bone marrow CDP were FACS sorted (>98% purity) and cultured for 9 days in Flt3L-supplemented media with or without Indomethacin. Significantly fewer DC were generated from purified CDP in the presence of Indomethacin compared to control (Figure 2C) strongly suggesting autocrine secretion.

To determine the COX enzyme involved in PGE2 biosynthesis by DC-committed progenitor cells, we induced Flt3L-dependent DC differentiation in vitro in the presence of a selective COX1 (SC560) or COX2 (NS-398) inhibitor. The COX2 specific inhibitor NS-398 substantially reduced DC generation equivalent to that observed with Indomethacin, while the COX1 selective inhibitor, SC560, was substantially less effective (Figure 2D), suggesting that PGE2 production primarily via the COX2 isoform is required for optimal DC generation. Addition of exogenous PGE2 during Flt3L-induced DC development, enhanced DC generation particularly at 0.1 and 10 nM (Figure 2D) further supporting a role for PGE2 in DC development.
Similar to the mouse model, inhibition of PGE₂ synthesis impairs Flt3L, SCF, IL-3 and IL-6 induced CD₁a⁺ CD₁4⁻ DC progenitor cell generation and CD₁₁c⁺ CD₁4⁻ myeloid DC generation from human CD₃₄⁺ cord blood cells (Figure 2E), demonstrating that PGE₂ regulation of DC generation from HPC is conserved across species.

While the numbers of DCs generated in the presence of Indomethacin are reduced, these DCs do up-regulate MHC-II, CD40 and CD86 upon LPS stimulation to a similar degree compared to DCs generated with Flt3L alone (Figure S2A), with no difference in IL-6 or IL-12 cytokine production (Table S1). Similarly, DCs generated from Indomethacin-treated C57BL/6 mice cultured with CFDA-SE labeled CD4⁺ T cells from BALB/c mice in a mixed lymphocyte reaction (MLR) assay induced a similar level of T cell proliferation compared to control (Figure S2B). These results indicate that while inhibition of PGE₂ signaling during DC development reduces total DC progenitor number and impairs optimal DC generation, those DCs that are produced appear to function normally at least in vitro.

DC differentiation from progenitor cells can also be induced by GM-CSF, although it is not required for in vivo DC development. To determine whether inhibiting PGE₂ biosynthesis exclusively reduces Flt3L-dependent DC development, or globally reduces DC differentiation, we induced mouse DC differentiation with GM-CSF in vitro in the presence or absence of Indomethacin. In contrast to Flt3L-induced DC generation, GM-CSF-mediated DC development was significantly higher after Indomethacin treatment compared to control (Figure 2F), indicating that PGE₂ differentially regulates Flt3L-dependent and GM-CSF-dependent DC development.
**PGE2 protects DC progenitors from apoptosis**

Attenuated DC generation as a result of reduced PGE2 signaling could result from a defect in DC-committed progenitor cell survival or as a consequence of effects on DC progenitor cell proliferation. To explore the functional consequences of inhibition of PGE2 biosynthesis on DC progenitor cell proliferation, we first examined the effect of Indomethacin treatment on BM CDP proliferation. Analysis of Flt3L-stimulated CDP proliferation *in vitro* was unaffected by Indomethacin (Figure 3A). To further explore the effects of PGE2 biosynthesis inhibition on DC progenitor cell proliferation *in vivo*, mice were treated with Indomethacin for 6 days and pulsed with BrdU for the last 20 hours prior to sacrifice. BrdU incorporation in CDPs was unchanged after Indomethacin treatment, while the total number of CDPs was decreased as we described (Figure 3B). These results demonstrate that reduced PGE2 signaling does not alter DC progenitor cell proliferation.

To investigate whether impaired DC development after inhibition of PGE2 biosynthesis resulted from an effect on DC progenitor cell survival, we first evaluated survival of pre-cDC generated from CDP enriched BM cells in the presence of Flt3L plus Indomethacin *in vitro*. Pre-cDC survival was significantly reduced, measured by both Annexin-V staining (Figure 3C; left panel) and expression of activated caspase-3 (Figure 3C; right panel). Analysis of active caspase-3 expression in BM CDP and pre-cDC after treatment of mice with Indomethacin also showed significantly higher levels of active caspase-3 in CDP and pre-cDC of Indomethacin treated mice, consistent with fewer viable CDP and pre-cDC (Figure 3D). These data suggest that PGE2 regulates DC production primarily by attenuating apoptosis thus promoting DC...
progenitor cell survival and are consistent with several studies that indicate that PGE2 protects many cell types of hematopoietic origin from apoptosis\textsuperscript{27, 36}.

**PGE\textsubscript{2} regulation of Flt3 expression enhances DC progenitor cell survival via STAT3-mediated elevation in Survivin**

STAT3 is a known transcription factor downstream of Flt3 signaling that is required for DC development\textsuperscript{37, 38}. PGE\textsubscript{2} has been shown to regulate STAT3 phosphorylation and protect cardiomyocytes from doxorubicin-induced apoptosis\textsuperscript{39, 40}. We therefore examined the effect of inhibiting PGE\textsubscript{2} biosynthesis on STAT3 phosphorylation in pre-cDCs generated from Lin\textsuperscript{neg} BM cells \textit{in vitro}. Inhibition of PGE\textsubscript{2} signaling during Flt3L-induced DC differentiation resulted in significantly reduced phospho-STAT3 levels in pre-cDC (Figure 4A). The STAT3 inhibitor Stattic mimicked the reduction in DC generation seen with Indomethacin and the combination of Stattic plus Indomethacin did not result in further reduction in DC differentiation compared to Stattic alone (Figure 4B), suggesting that PGE\textsubscript{2}/Flt3 regulates the level of STAT3 activation during DC differentiation. Identical reduction in DC generation from highly purified CDPs was observed in the presence of Indomethacin and Stattic alone and in combination, suggesting that both Indomethacin and Stattic are acting on the same DC progenitor cell population (Figure S3).

STAT3 modulates transcription of the anti-apoptotic protein Survivin\textsuperscript{41}, and we recently reported that PGE\textsubscript{2} increases Survivin transcription and decreases apoptosis in HSC\textsuperscript{27}. To determine whether PGE\textsubscript{2}/Flt3 signaling regulates DC progenitor survival through a STAT3-mediated increase in Survivin expression, Survivin protein was measured in pre-cDC generated from CDP during \textit{in vitro} culture with Flt3L with or without Indomethacin. Intracellular flow
cytometry demonstrated that Survivin was significantly lower in pre-cDC when PGE₂ biosynthesis was inhibited (Figure 4C). Retroviral over-expression of Survivin in CDP-enriched Lin<sup>neg</sup> BM cells demonstrated that the Indomethacin-mediated defect in DC differentiation was completely reversed by ectopic Survivin (Figure 4D), confirming that PGE₂/Flt3-mediated Survivin expression is required for optimal DC generation.

Since both PGE₂ and Flt3 regulate phospho-STAT3 and Survivin expression, we tested whether PGE₂ regulates DC differentiation directly downstream of its EP receptors or indirectly by modulating Flt3 receptor signaling. We first examined DC generation from CDP enriched Lin<sup>neg</sup> BM cells cultured with or without Flt3L and/or Indomethacin. Robust DC differentiation was observed in the presence of Flt3L, which was significantly reduced when PGE₂ synthesis was inhibited. DC generation was not observed in the absence of Flt3L regardless of the presence or absence of Indomethacin (Figure 4E). This suggests that PGE₂ by itself is not sufficient for DC differentiation but regulates DC development in conjunction with Flt3 receptor signaling.

We next analyzed the effects of inhibition of PGE₂ biosynthesis on expression of Flt3 receptor on DC-committed progenitor cells. Flt3 expression was significantly decreased on BM CDP (40.0 ± 7.07%; p<0.01) and on pre-cDC (42.5 ± 3.72%; p<0.01) after treatment of mice with Indomethacin (Figure 4F), indicating that PGE₂ signaling regulates Flt3 receptor expression. Similarly, Flt3 mRNA was significantly reduced (35±4.8%; p<0.01) in FACS sorted pre-cDC after Indomethacin treatment. In contrast, Flt3 expression on Lin<sup>neg</sup> c-Kit<sup>high</sup> Sca-1<sup>-</sup> myeloid-committed progenitor cells was not decreased in Indomethacin-treated mice (Figure S4).

To confirm that the reduced Flt3 expression on DC progenitor cells is responsible for the reduction in DC generation after PGE₂ biosynthesis inhibition, we over expressed Flt3 in
CDP enriched Lin<sup>-neg</sup> BM cells and induced Flt3L-dependent DC development. Ectopic Flt3 expression resulted in enhanced DC generation as expected and completely reversed the defect in DC generation as a consequence of Indomethacin treatment (Figure 4G, left). Similarly, the reduction in phospho-STAT3 and Survivin in DC-lineage progenitors was reversed by Flt3 over expression (Figure 4G, middle and right respectively). These data indicate that reduced DC generation as a result of inhibition of PGE<sub>2</sub> biosynthesis/reduced PGE<sub>2</sub> signaling is due to a reduction in Flt3 receptor expression on DC progenitor cells rather than direct reduction of EP receptor regulation of STAT3 activation and Survivin expression.

**PGE<sub>2</sub> regulation of DC generation is mediated by EP1 and EP3 receptor signaling**

PGE<sub>2</sub> signals through four different G-protein coupled receptors, EP1-4, with unique, similar or opposing intracellular signaling pathways<sup>42</sup>. As a consequence, the effects of PGE<sub>2</sub> result from the net effect of signals generated at all 4 receptors. In order to identify the specific pathways involved in regulation of DC progenitor cell differentiation by PGE<sub>2</sub>, and to define a potential pharmaceutical target for modulation of DC generation, we investigated which EP receptors mediates DC development. All four EP receptors were found to be expressed on CDP and pre-cDC (data not shown) by flow cytometry analysis. In addition, mRNAs for all 4 receptors were detected in CDP and pre-cDC. To determine which EP receptor(s) regulates DC generation, DC cultures were treated with selective EP receptor agonists to reverse the inhibitory effects of Indomethacin. DC generation from Lin<sup>-neg</sup> BM cells was significantly reduced in the presence of Indomethacin as expected, but could be completely rescued by the addition of PGE<sub>2</sub> or by the dual EP1/EP3 agonist 17-phenyl trinor PGE<sub>2</sub> (17ptPGE<sub>2</sub>), and partially rescued by the
EP3 agonist Sulprostone (Figure 5A). In contrast, the EP2 specific agonist Butaprost and the EP4 specific agonist L-902,688, failed to rescue DC generation. We also evaluated the ability of EP receptor selective antagonists to inhibit DC development. Antagonism of EP1 receptor signaling with the EP1 specific antagonist SC51322 or EP3 receptor signaling with the EP3 specific antagonist L-798,106 resulted in significantly reduced DC differentiation both in vitro (Figure 5B, left) and in vivo (Figure 5B, right). Consistent with the effect of EP1 and EP3 receptor specific agonists to rescue DC generation when PGE2 biosynthesis/signaling is inhibited, 17-ptPGE2 and Sulprostone reversed the Indomethacin-mediated reduction in Survivin in pre-cDC generated from CDP (Figure 5C). These data demonstrate that PGE2 regulates DC differentiation through EP1 and EP3 receptor signaling.

To further validate the involvement of EP1/EP3 signaling in DC development, we evaluated total DC number in EP1 and EP3 receptor single knockout mice and EP1/EP3 double knockout mice. Total DC cell number was significantly lower in the BM and spleen of the EP1 and EP3 receptor single knockout mice compared to wild-type control mice (Figure 5D). In addition, double EP1/EP3 receptor knockout mice showed significantly more reduction in DC number in BM and spleen, mimicking results with Indomethacin, and confirming agonist/antagonist data demonstrating a role of both EP1 and EP3 receptors in DC-progenitor cell function.
DISCUSSION

While PGE₂ has been implicated in regulation of hematopoietic stem and progenitor cells, its role in steady-state DC development is largely unknown. We now demonstrate that PGE₂ is a positive physiological regulator of Flt3L-dependent DC development and is required for optimal DC-committed progenitor cell differentiation by regulating progenitor cell Flt3 expression and survival. Inhibition of PGE₂ biosynthesis and signaling reduces Flt3 expression on DC progenitors, resulting in suboptimal activation of the Flt3 signaling cascade, decreased activation of STAT3 and reduced production of the anti-apoptotic protein Survivin, causing DC progenitor apoptosis. While inhibition of PGE₂ signaling reduces DC production, it does not impair the immune function of those DC generated.

PGE₂ has distinct effects on different types of HPC, inhibiting granulocyte/monocyte, B cell, and T lymphoid precursors, while enhancing erythropoiesis. Pharmacological doses of PGE₂ have been shown to impair GM-CSF-mediated DC development from BM-derived hematopoietic progenitors and from monocytes. Steady-state DC development however, depends primarily on Flt3L not GM-CSF, thus the impairment of Flt3L-mediated DC development in the absence of endogenous PGE₂ synthesis/signaling suggests that physiological levels of PGE₂ are necessary for normal steady-state DC development. The variable effects of PGE₂ on different HPC may occur due to expression of different EP receptors and/or differential coupling of these receptors to intracellular signaling pathways. DC-committed progenitors originate in the BM from HSCs after multiple lineage-restricting differentiation processes. Since PGE₂ enhances HSC proliferation and survival, it is reasonable to speculate that its effect on DC differentiation is a consequence of its effects on HSCs. However, generation of
fewer DCs after Indomethacin treatment of highly purified CDP cultures, which lack HSC, indicates that PGE₂ regulation of DC differentiation is mediated by direct effects on DC-committed progenitor cells.

At least two cellular processes, proliferation and apoptosis, can control mature blood cell generation from HPC. In contrast to the effects of PGE₂ on myeloid, erythroid and multipotential progenitor cell proliferation \cite{22-25,34}, inhibition of PGE₂ biosynthesis did not affect BrdU incorporation in DC-committed progenitor cells, suggesting that PGE₂ does not primarily regulate DC-lineage progenitor cell proliferation. Since BrdU incorporation measures DNA replication not cell division, we confirmed this finding by \textit{in vitro} CFDA staining that measures cell division. Similar CFDA dilution in both vehicle and Indomethacin treated DC progenitor cells indicates that PGE₂ does not regulate DC progenitor cell proliferation; rather its primary effects are on progenitor cell survival. In support to this hypothesis, recent studies have also shown that Flt3 signaling can regulate progenitor cell survival without affecting proliferation \cite{47,48}.

PGE₂ is a lipid messenger regulating the expression of effector genes downstream of G-protein coupled receptors. PGE₂ signaling through EP receptors is known to directly promote survival of colon cancer cells and myocardium by up-regulation of STAT3 and Survivin \cite{40,49}. Since both Flt3 and PGE₂ signaling pathways can activate STAT3 and induce Survivin expression, it is possible that Flt3 signaling may synergize with PGE₂ signaling to modulate the survival of DC progenitors through optimal Survivin expression. PGE₂ signaling alone was not sufficient to protect DC progenitors from apoptosis and completely failed to generate DCs in the absence of Flt3L suggesting that PGE₂ regulates DC differentiation primarily by modifying Flt3 signaling.
Flt3 is expressed on DC-committed progenitor cells and is essential for DC development in lymphoid organs. The presence of Flt3 on these progenitor cells directly correlates with their steady state differentiation into DCs\textsuperscript{5,6}. Despite its importance in DC development, little is known about how Flt3 expression is regulated. Reduced Flt3 expression on DC-committed progenitors as a result of PGE\textsubscript{2} biosynthesis inhibition, and complete reversal of reduced DC generation in the absence of PGE\textsubscript{2} biosynthesis/signaling by over expression of Flt3 argue that PGE\textsubscript{2} regulates DC development indirectly by modulating Flt3 signaling. A recent study demonstrates the involvement of PU.1 in Flt3 gene expression on DC-committed progenitors and its role in DC development\textsuperscript{50}. PU.1 expression and activation is controlled by several intracellular signal transduction events, including induction of PI3K/AKT and Src signaling pathways\textsuperscript{51,52}. Since, these pathways are activated by EP1 and EP3 signaling\textsuperscript{53,54}, and our data identified that PGE\textsubscript{2} regulates Flt3L dependent DC development through the EP1 and EP3 receptors, it is possible that PGE\textsubscript{2} mediated induction of PI3K/AKT and Src signaling pathways regulates PU.1 expression in DC-committed progenitors that leads to increased expression of Flt3. However, in other progenitor lineages change in transcription factor activities in the absence of change in expression has been reported\textsuperscript{55}. Further study is needed to define the transcriptional events regulating PGE\textsubscript{2} induced Flt3 expression in DC progenitor cells.

Based upon these \textit{in vitro} and \textit{in vivo} findings, we propose a model where PGE\textsubscript{2} signaling through the EP1 and EP3 receptors regulates Flt3 expression on DC progenitors. Flt3 receptor signaling activates STAT3 and enhances Survivin expression in DC progenitors, which protects them from apoptosis, resulting in optimal DC generation, and that inhibition of PGE\textsubscript{2} signaling impairs DC generation (Figure 6). Due to its potent antigen presentation properties, DCs are
required for induction of immune responses at the onset of infection and quantitative and functional loss of DCs has been attributed to severity of infectious diseases\textsuperscript{56, 57}. In this context, our studies raise the possibility that NSAIDs inhibiting PGE\textsubscript{2} biosynthesis, particularly when taken over extended periods of time, may exacerbate disease as a consequence of reduced DC generation. In addition, several experimental and clinical studies support the use of DC vaccines\textsuperscript{58}; however, current DC vaccines are not optimal for the treatment of human diseases. One of the major hurdles for DC vaccine development is a generation of large numbers of DCs that can be used to deliver target antigens to generate anti-tumor or anti-infective immune responses. Understanding DC differentiation mechanisms would be an essential step to achieve this goal. Our study illustrates that physiological doses of PGE\textsubscript{2} are crucial for optimal DC generation and may prove useful for efficient DC generation for vaccine development. Furthermore, recent studies suggest a role for PGE\textsubscript{2} in self-renewal and expansion of HSC\textsuperscript{26, 27} and while the up-regulation of PGE\textsubscript{2} production after total body irradiation\textsuperscript{59} may be beneficial for HSC homing, engraftment and expansion in the short term, our studies suggest that prolonged elevated levels of PGE\textsubscript{2} may lead to altered hematopoietic progenitor function and DC generation. Further studies evaluating the regulatory role of PGE\textsubscript{2} in hematopoietic generation of immune cells post-transplant will identify how best to therapeutically modulate PGE\textsubscript{2} signaling during and after hematopoietic transplantation for maximal benefit.
ACKNOWLEDGEMENTS

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Authorship Contributions

P Singh designed the study, performed the experiments, interpreted data, and wrote the manuscript; J Hoggatt participated in designing the experiments, interpreting data and wrote the manuscript; P Hu participated in performance of research, collection, and analysis of data; JM Speth maintained the mice colony, performed experiments and critically read the manuscript; S Fukuda prepared Survivin and Flt3 expressing plasmids and critically read the manuscript; Richard M Breyer provided EP1 and EP3 and EP1/EP3 double knockout mice and participated in design of experiments; LM Pelus participated in designing the study, interpreted data and coordination and performance of the study, and wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.
REFERENCES


13. Waskow C, Liu K, Darrasse-Jeze G et al. The receptor tyrosine kinase Flt3 is required


25. Lu L, Pelus LM, Broxmeyer HE. Modulation of the expression of HLA-DR (Ia)
antigens and the proliferation of human erythroid (BFU-E) and multipotential (CFU-GEMM) progenitor cells by prostaglandin E. Exp Hematol. 1984; 12 (9) :741-748.


44. Shimozato T, Kincade PW. Prostaglandin E(2) and stem cell factor can deliver opposing signals to B lymphocyte precursors. *Cell Immunol.* 1999; 198 (1) :21-29.


52. Garrett-Sinha LA, Su GH, Rao S et al. PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction. *Immunity.* 1999; 10 (4) :399-408.


TABLES

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC/femur b</th>
<th>Neutrophils c</th>
<th>Monocytes c</th>
<th>B Cells c</th>
<th>T + NK Cells c</th>
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<td>Vehicle</td>
<td>17.0 ± 0.9</td>
<td>6.2 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>3.7 ± 0.5</td>
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<td></td>
<td></td>
<td>(37.6 ± 1.0)</td>
<td>(10.0 ± 0.7)</td>
<td>(27.7 ± 0.3)</td>
<td>(27.6 ± 1.0)</td>
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<tr>
<td>Indomethacin</td>
<td>17.3 ± 1.3</td>
<td>6.3 ± 0.4</td>
<td>2.5 ± 0.2 *</td>
<td>3.4 ± 0.4</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(38.0 ± 1.1)</td>
<td>(14.6 ± 0.3)</td>
<td>(21.6 ± 2.4)</td>
<td>(25.6 ± 1.2)</td>
</tr>
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</table>

a. Groups of 5 mice were treated with vehicle (0.01% ethanol in PBS) or Indomethacin (2.5 mg/kg) twice daily for 6 days.

b. Bone marrow cells were harvested from vehicle control and Indomethacin-treated mice and WBC determined on individual mice using a Hemavet 950 analyzer. WBC are expressed as Mean±SEM total nucleated cells per femur x 10^6 based on counts from individual mice. Data are representative of 5 experiments.

c. Harvested bone marrow cells from treated mice were stained with antibodies for GR-1, Mac1, B220, CD3 and NK1.1 and analyzed by flow cytometry. Data are expressed Mean±SEM total cells per femur x 10^6 based on analysis of individual mice. Data are representative of 3 experiments.

d. Mean % of WBC±SEM

* P<0.05
FIGURE LEGENDS

**Figure 1. Inhibition of PGE2 biosynthesis reduces DC and DC-committed progenitor cells in mouse BM and spleen.**

(A) Left: Representative flow plots showing gating strategy to determine DC in BM (upper plots) and spleen (lower plots) of mice treated with Indomethacin. Right: DC frequency of nucleated cells and total DC per femur (upper graphs), and spleen (lower graphs) (X±SEM; N=6 mice in 2 experiments, each assayed individually). (B) Representative flow plots of cDC (CD11c+ B220−) and pDC (CD11c+ B220+) in BM. The bar graphs show (left) total cDC and pDC per femur, and (right) total cDC and pDC in spleen (X±SEM; N=3 mice per experiment, each assayed individually; 2 experiments). (C) Total DC per femur in mice after treatment with Indomethacin +/- dmPGE2 (X±SEM; N=4 mice per group, each assayed individually) (D) Total DC per femur in mice after treatment with Flt3L +/- dmPGE2 (X±SEM; N=4 mice per group, each assayed individually). (E) Representative dot plots of CDP frequency and average total CDP per femur in the BM after 6 days of Indomethacin treatment (X±SEM; N=3 mice/group in each of 2 experiments). (F) Representative dot plots of pre-cDC frequency and total pre-cDC per femur (X±SEM; N=3 mice/group in each of 2 experiments). V: Vehicle. Indo: Indomethacin. *P< 0.05.

**Figure 2. PGE2 positively regulates Flt3L dependent DC development from hematopoietic progenitor cells.**

(A) Total cDC (CD11c+ CD11b− B220−) and pDC (CD11c+ CD11b− B220+) generation from Lin− BM cells in Flt3L cultures in presence of Indomethacin or Indomethacin plus dmPGE2 (X±SEM; N=3 mice/group in each of 2 experiments). (B) Detection of PGE2 at indicated time
points during Flt3L mediated DC differentiation in culture from Figure 2A. (C) Total DC cell generation from FACS sorted CDP (10^3 cells/well) cultured for 9 days in Flt3L supplemented media in the presence/absence of Indomethacin (X±SEM, N=5 mice; 2 experiments). (D) Total DC generation from Lin^-neg^ BM cells (2x10^5 cells/well) cultured for 9 days with Flt3L and SC560 (COX1 inhibitor) or NS398 (COX2 inhibitor) or different dose of PGE2 (0.1-100 nM). (X±SEM, N=6 mice; 2 experiments). (E) Effect of Indomethacin treatment on CD34+ CD11a+ CD14^- DC precursor and CD11c^CD14^- myeloid DC generation from purified UCB CD34+ cells (5x10^5 cells/well) (X±SEM, N=3 cord bloods). (F) Total DC generation from mouse Lin^-neg^ BM cells cultured with GM-CSF +/- Indomethacin (X±SEM, N=5 mice). *P< 0.05.

Figure 3. Effect of PGE2 biosynthesis inhibition on DC progenitors proliferation and survival.

(A) CFDA dilution 3 days after in vitro culture of FACS sorted CDP in Flt3L supplemented medium with or without Indomethacin. (X±SEM; N= 4 mice). (B) Representative dot plots of BrdU incorporation in CDP of BrdU-treated mice after Indomethacin treatment. Left: Average frequency of BrdU^+^ CDP; Right: Total CDP per femur (X±SEM, 2 experiments; N=3 mice/group/experiment). (C) CDP enriched Lin^-neg^ BM cells were cultured for 5 days in Flt3L supplemented medium with or without Indomethacin and Annexin V (left panel) and active caspase-3 (right panel) expression was determined in pre-cDC gated cell population. (X±SEM; 2 experiments N=3 mice/ experiment). (D) Active caspase-3 expression in BM CDP and pre-cDC, and CDP and pre-cDC viability (insets) in mice treated for 6 days with Indomethacin (X±SEM from 5 mice per group assayed individually; expressed as percent of control). *P< 0.05.
Figure 4. PGE₂ regulation of Flt3 expression enhances DC progenitor cell survival through STAT3-mediated elevation of Survivin.

(A) Representative FACS plot of pre-cDC phospho-STAT3 expression generated from CDP cultured in Flt3L +/- Indomethacin and relative phospho-STAT3 expression in pre-CDC (X±SEM, N=3 mice pooled per experiment; 3 experiments). (B) Total DC generation from Lin<sup>neg</sup> BM cells (2x10⁵ cells/well) cultured for 9 days with Flt3L +/- Indomethacin and/or Stattic (X±SEM, 2 experiments; N=3 mice/group/experiment, *P<0.05 compared to vehicle, #P>0.05 compared to Indomethacin). (C) Survivin expression in pre-cDC generated in vitro from Flt3L cultured CDP +/-Indomethacin (X±SEM, N=3 mice pooled per experiment; 3 experiments). (D) Total DC generation from Lin<sup>neg</sup> BM cells non-transduced (NT), or transduced with Survivin over-expression or control vector (X±SEM, N=3 mice/group/experiment; 2 experiments; *P<0.05 compared to vehicle, † P<0.05 compared to vector control). (E) Total DC generation from Lin<sup>neg</sup> BM cells (2 x10⁵ cells/ well) cultured for 9 days +/- Flt3L and/or Indomethacin. (F) Mean fluorescence intensity (MFI) of Flt3 receptor on CDP and pre-cDC (X±SEM, N=4 mice). (G) Lin<sup>neg</sup> BM cells transduced with Flt3 MSCV-IRES-EGFP or control vector and cultured +/- Indomethacin. Left: DC generation after 9 days culture (X±SEM, N=6 mice assayed individually; 2 experiments); Middle: MFI of phospho-STAT3 expression; Right: Survivin expression in pre-cDC after 5 days culture (X±SEM, N=3 mice). *P< 0.05.

Figure 5. EP1 and EP3 receptors regulate Flt3L mediated DC development.

(A) Lin<sup>neg</sup> BM cells cultured for 9 days in Flt3L supplemented media with Indomethacin alone, or Indomethacin plus selective EP receptor agonists and DC generation was measured by flow
cytometry. (X±SEM, N=6 mice assayed individually; from 2 experiments) (B) Left: *In vitro*, Flt3L dependent DC generation from Lin<sup>−</sup> BM cells in presence of EP1 and EP3 receptor antagonist (X±SEM, N=8 mice assayed individually; from 3 experiments). Right: Total DC number per femur in mice treated with Indomethacin, EP1 or EP3 antagonists *in vivo* (X±SEM, N=4 mice assayed individually). (C) Lin<sup>−</sup> BM cells cultured for 5 days in Flt3L supplemented media with Indomethacin alone or with selective EP receptor agonists. Survivin expression in pre-cDC gated cells was determined by flow cytometry (X±SEM, N=3 mice assayed individually). *P< 0.05 compared to vehicle; † P<0.05 compared to Indomethacin. (D) Total CD11c<sup>+</sup> MHCII<sup>+</sup> DCs in the BM and spleen of (A) EP1 and EP3 knockout mice and (B) EP1/EP3 double knockout mice. (X±SEM, N=3 mice assayed individually). *P< 0.05 compared to wild-type control.

**Figure 6. Model for the regulation of DC development by PGE<sub>2</sub>**.

In this model, we propose that PGE<sub>2</sub> signaling through the PGE<sub>2</sub> G-protein EP1 and EP3 receptors regulates Flt3 expression on DC-committed progenitors (CDP and pre-cDC) and modifies downstream phopho-STAT3 activation and Survivin expression. Elevated Survivin expression protects DC progenitors from caspase-3 mediated apoptotic death and results in optimal DC development.
A) Gated on [CD3 NK1.1 CD19 Ter119]^- cells

B) PGE2 (pg per 10^6 cells)

C) Total DC generated from CDP

D) DC generation (% of vehicle)

E) CD34+ CD11a+ CD14- DC Progenitor cells

F) GM-CSF induced DC generation (% of control)
Figure 3

A

CDP (day 0)

Day 4

Vehicle

Indo

CFDA

B

Gated on CDP

Vehicle

Indo

FSC

% BrdU+ CDP

Total CDP per femur X10^3

C

Vehicle

Indomethacin

Annexin V pre-cDC

Annexin V

% of control

Vehicle

Indo

Indomethacin

Caspase 3 in pre-cDC

% of control

Vehicle

Indo

D

Gated on CDP

Vehicle

Indo

Count

Caspase 3

Gated on pre-cDC

Vehicle

Indo

Count

Caspase 3

Active Caspase 3 in CDP

% of control

Vehicle

Indo

Active Caspase 3 in pre-cDC

% of control

Vehicle

Indo
Figure 4

A

![Graph showing Survivin expression in pre-cDC (% of control) with bars for Isotype, Vehicle, and Indomethacin.

B

![Bar graph showing Total DC generation X 10^4 for Vehicle (V), Indomethacin (Indo), Static, Stat, Static, +Indo.]

C

![Bar graph showing Survivin expression in pre-cDC (% of control) with bars for Vehicle (V) and Indomethacin (Indo).]

D

![Bar graph showing DC generation (% of control) for Vehicle (V), Indomethacin (Indo), Survivin (S).]

E

![Bar graph showing Total DC generation X 10^4 for Flt3L (−, +) and Indomethacin (−, +).]

F

![Graph showing MFI of Flt3 on CDP with bars for Vehicle (V) and Indomethacin (Indo).]

G

![Graph showing GFP+ DC generation and pSTAT3 in pre-cDC (MFI) for Vehicle (V) and Indomethacin (Indo).]
Figure 5

A

![Bar graph showing DC generation (% of control) with various treatments.]

B

![Bar graph showing DC generation (% of control) with various treatments and EP receptor antagonists.]

C

![Bar graph showing Survivin expression in pre-DC (% of control) with various treatments and EP receptor antagonists.]

D

![Bar graph showing DC in BM x 10^5 and DC in spleen x 10^5 with various treatments and EP receptor antagonists.]

Legend:

- V: Vehicle
- Indo: Indomethacin
- PGE\(_2\): Prostaglandin E\(_2\)
- Butaprost
- Sulprostone
- L-902,688
- 17 pPGE\(_2\)
- EP1 EP3
- SC51322
- NS
- ∆:
- *:
- †:
Figure 6

- **HSC** (Lin<sup>−</sup> Sca<sub>1</sub><sup>int</sup> c-Kit<sup>−</sup> IL-7R<sup>+</sup>)
- **CLP** (Lin<sup>−</sup> Sca<sub>1</sub><sup>−</sup> c-Kit<sup>−</sup> IL-7R<sup>+</sup>)
- **CMP** (Lin<sup>−</sup> Sca<sub>1</sub><sup>+</sup> CD34<sup>+</sup> c-Kit<sup>+</sup>)
- **MDP** (Lin<sup>−</sup> c-Kit<sup>−</sup> CD115<sup>+</sup> CXCR1<sup>+</sup> Flt3<sup>+</sup>)
- **Monocytes**

**Dendritic cells**
- **pDC** (CD11c<sup>−</sup> CD11b<sup>−</sup> B220<sup>+</sup>)
- **CDP** (Lin<sup>−</sup> c-Kit<sup>−</sup> CD115<sup>+</sup> Flt3<sup>+</sup>)
- **Pre-cDC** (CD11c<sup>+</sup> MHC-II<sup>−</sup> Flt3<sup>−</sup> SIRPα<sup>−</sup>)
- **cDC** (CD11c<sup>+</sup> MHC-II<sup>+</sup> B220<sup>−</sup>)

**Regulates Flt3 expression on CDP and pre-cDC**

**Increased DC development**

**For personal use only.**
Blockade of prostaglandin E2 signaling through EP1 and EP3 receptors attenuates Flt3L-dependent dendritic cell development from hematopoietic progenitor cells

Pratibha Singh, Jonathan Hoggatt, Peirong Hu, Jennifer M. Speth, Seiji Fukuda, Richard M. Breyer and Louis M. Pelus