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Prostaglandin E2 inhibition of IL-27 production in murine dendritic cells: a novel mechanism which involves IRF1

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Abstract

IL-27, a multifunctional cytokine produced by antigen-presenting cells, antagonizes inflammation by affecting conventional dendritic cells (cDC), inducing IL-10, and promoting development of regulatory Tr1 cells. Although the mechanisms involved in IL-27 induction are well-studied, much less is known about the factors that negatively impact IL-27 expression. Prostaglandin E2 (PGE2), a major immunomodulatory prostanoid, acts as a pro-inflammatory agent in several models of inflammatory/autoimmune diseases, promoting primarily Th17 development and function. In this study, we report on a novel mechanism which promotes the pro-inflammatory function of PGE2. We showed previously that PGE2 inhibits IL-27 production in murine bone marrow derived DCs. Here, we show that, in addition to BMDCs, PGE2 inhibits IL-27 production in macrophages and in splenic cDC and we identify a novel pathway consisting of signaling through EP2/EP4→induction of cAMP→downregulation of IRF1 expression and binding to the *p28* ISRE site. The inhibitory effect of PGE2 on *p28* and *irf1* expression does not involve endogenous IFN β , STAT1 or STAT2, and inhibition of IL-27 does not appear to be mediated through PKA, EPAC, PI3K, or MAPKs. We observed similar inhibition of *il27p28* expression in vivo in splenic DC following administration of dimethyl PGE2 in conjunction with LPS. Based on the anti-inflammatory role of IL-27 in cDC and through the generation of Tr1 cells, we propose that the PGE2-induced inhibition of IL-27 in activated cDC represents an important additional mechanism for its in vivo pro-inflammatory functions.

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Author Contributions

K.M.H., J.-H.Y. and D.G. designed experiments; K.M.H., J.-H.Y., W.K., K.M.R. and P.-C.K. performed experiments. A.M.G. provided *Stat2*^{-/-} mice and advice on proposed experiments. K.M.H. and D.G. wrote the manuscript.

Disclosure of Conflicts of Interest

All authors declare no conflicts of interest.

Introduction

Interleukin-27 (IL-27), a cytokine that belongs to the IL-12 family, is a p28/EBI3 heterodimer which signals through the IL-27R α (WSX-1)/gp130 receptor (1, 2). The inclusion of IL-27 within the IL-12 cytokine family is based on the fact that the IL-27 subunits EBI3 and p28 are related to IL-12p40 and IL-12p35, respectively. The homology extends to the IL-27/IL-12 receptors with IL-27R α /WSX-1 being homologous to IL12R β 2 (3). Myeloid cells including dendritic cells (DC) are major IL-27 producers following activation through TLRs, CD40 and IFN type I and II receptors (3, 4). In a model using conditional IL27p28-deficient mice, CD11c⁺ DC were identified as the critical source of IL-27 (5). Expression of IL-27R α is limited to immune cells, especially T and NK cells (2), with IL-27R α increasing following T cell activation (6). Recently, cDCs were reported to express IL-27R α at higher levels than plasmacytoid DC (7).

Although initial studies suggested a pro-inflammatory role for IL-27, models of infectious and autoimmune diseases in *Il27ra* deficient mice identified IL-27 as a negative regulator of T cell functions (3, 8). Both cDC and CD4 T cells are targets for IL-27-mediated negative regulation. Mascanfroni et al (7) reported recently that IL-27 signaling in cDC limits Th1 and Th17 differentiation and function. In addition, IL-27 was also reported to induce the expansion and differentiation of Tr1 cells, one of the two major types of regulatory CD4 T cells (8, 9). The Foxp3⁺IL-10⁺IFN γ ⁺ Tr1 cells secrete high levels of IL-10, and both Tr1 differentiation and production of IL-10 are IL-27 dependent (10–13).

Although the induction of IL-27 production has been studied extensively, much less is known about mechanisms that limit IL-27 expression. Presently, only extracellular ATP acting on purinergic receptors and C5a acting through C5aR, were reported to inhibit IL-27 production (14, 15).

PGE₂, a major prostanoid derived from arachidonic acid, is produced by immune cells including cDC in response to pro-inflammatory stimuli and signals through four EP receptors (EP1-4) which vary in affinity, signal duration and signaling pathways (16–19). The role of PGE₂ in DC maturation and function has been studied extensively. In immature DC, PGE₂ accelerates maturation and promotes a pro-inflammatory phenotype (20–22). In terms of DC cytokines and chemokines, PGE₂ was reported to reduce IL-12, TNF α , CCL3 and CCL4 (23–25), while upregulating IL-23 and promoting Th17 function (26–30). The effects of PGE₂ on Th1 differentiation depend on the experimental system. In the presence of APCs, PGE₂ suppresses Th1 differentiation (23), whereas, when purified naïve CD4 T cells are differentiated in Th1 polarizing conditions, PGE₂ promotes Th1 differentiation (31). This apparent discrepancy can be explained by the fact that, in the first system, PGE₂ inhibits the APC release of IL-12, a required Th1 differentiation factor, whereas in the second system which includes exogenous IL-12, PGE₂ upregulates the expression of IL-12R β 2 in differentiating Th1 cells (31). The pro-inflammatory role of PGE₂ has been confirmed in vivo in models of contact hypersensitivity, inflammatory bowel disease, rheumatoid arthritis, and experimental autoimmune encephalomyelitis (EAE), and shown to

be mostly associated with increases in Th1/Th17 differentiation and/or function (18, 19, 32, 33).

Here we report on a novel pro-inflammatory function of PGE2, mediated through the inhibition of IL-27 production by cDCs. To date, little is known about the interaction between PGE2 and IL-27. Two publications, including one from our laboratory, reported previously that PGE2 inhibited LPS-induced IL-27p28 expression in BMDC (34) and THP-1 cells (35). In the present study we report that PGE2 inhibits IL-27 expression in bone marrow-derived DC (BMDC) and macrophages (BMDM), and in splenic DCs in vitro and in vivo. The inhibitory effect of PGE2 is mediated through EP2/EP4 receptors, cAMP induction, and inhibition of IRF1 expression and binding to the *p28* promoter ISRE site.

Materials and Methods

Mice

C57BL/6 (6–10 weeks old) and B6.129S2-*Irf1*^{tm1Mak} (*Irf1*^{-/-}) mice were purchased from Jackson Laboratory. 129S6/SvEv-*Stat1*^{tm1Rds} (*Stat1*^{-/-}) and wild type (WT) mice (129S6/SvEv) were from Taconic Farms. C57BL/6 *Stat2*^{-/-} and corresponding WT mice were provided by Dr. Ana Gamero (Temple University, Philadelphia, PA, USA). Mice were housed and maintained in accordance with protocols approved by IACUC at Temple University.

Reagents

Prostaglandin E2, LPS (*Escherichia coli* O55:B5), peptidoglycan (*Staphylococcus aureus*), poly I:C and DNase were purchased from Sigma-Aldrich. Granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor and IFN γ were from Peprotech, Inc. Dimethyl-PGE2, butaprost, sulprostone, misoprostol, the specific activator of the exchange protein activated by cAMP (EPAC), 8-pCPT-2'-O-Me-cAMP (8-CPT), and the PI3K inhibitor LY294002 were purchased from Cayman Chemical. PKI (5–24) was purchased from Santa Cruz Biotechnology Inc. PKI (6–22), U0126, JNK inhibitor II, EP receptor antagonists PF-04418948 and ONO-AE3-208, and dibutyryl-cAMP were from Calbiochem. Capture and biotinylated anti-mouse IL-27p28 antibody and recombinant mouse IL-27p28 were from R&D Systems. Capture and biotinylated anti-mouse IFN- β antibodies were from BioLegend (ELISA detection limit of). Streptavidin-HRP was purchased from BioLegend. Tetramethylbenzidine substrate reagent set was from BD Biosciences. Anti-IRF1 and IgG control antibodies for ChIP were from Santa Cruz Biotechnology Inc. Primary anti-mouse antibodies for Western blot were from Cell Signaling Technology (IRF1), Abcam (GAPDH) and BD Biosciences (β -actin). IFN- β was purchased from PBL Assay Science. APC-conjugated anti-mouse CD11c and PE-Cy5-conjugated anti-mouse MHCII antibodies were purchased from eBioscience. FITC-conjugated anti-mouse CD80, CD86, and CD40 were purchased from BD Biosciences.

DC and macrophage culture

Primary DCs were derived from bone marrow cells as previously described (36). Macrophages were derived from bone marrow cells cultured in the presence of 10 ng/ml

macrophage colony-stimulating factor, and adherent cells were collected on day 7. Spleen DCs were isolated from spleens dissociated in the presence of 0.5 mg/ml liberase TL (Roche Diagnostics) and DNase, and magnetically sorted using CD11c MicroBeads per manufacturer's instructions (Miltenyi Biotec). Purity was determined by FACS analysis (>93% CD11c⁺).

qRT-PCR

Gene expression analysis of *il-10*, *Il12a*(p35), *Il12b*(p40), *Il23a*(p19), *ifnb*, *p28*, *irf1*, *β-actin* and *Gapdh* was performed using SYBR-green based qRT-PCR. The following primer sets were used: *Il10* sense 5'-CCTGGTAGAAGTGATGCCCC-3' and antisense 5'-TCCTTGATTCTGGGCCATG-3'; *Il12a*(p35) sense 5'-GAGGACTTGAAGATGTACAG-3' and antisense 5'-TTCTATCTGTGTGAGGAGGGC-3'; *Il12b*(p40) sense 5'-GACCCTGCCGATTGAACTGGC-3' and antisense 5'-CAACGTTGCATCCTAGGATCG-3'; *Il23a*(p19) sense 5'-TGCTGGATTGCAGAGCAGTAA-3' and antisense 5'-ATGCAGAGATTCCGAGAGA-3'; *ifnb* sense 5'-CCCTATGGAGATGACGGAGA-3' and antisense 5'-ACCCAGTGCTGGAGAAATTG-3', *p28* (in vitro experiments) sense 5'-TCTGGTACAAGCTGGTTCCTGG-3' and antisense 5'-TAGCCCTGAACCT CAGAGAGCA-3'; *p28* (in vivo studies) (37) forward 5'-ATCTCGATTGCCAGGAGTGA-3' and antisense 5'-GTGGTAGCGAGGAAGCAGAGT-3'; *irf1* sense 5'-CCCACAGAAGAG CATAGCAC-3' and antisense 5'-AGCAGTTCTTTGGGAATAGG-3'; *β-actin* sense 5'-AGCTTCTTTGCAGCTCCTTCGTTGC-3' and antisense 5'-ACCAGCGCAGCGATATCGTCA-3'; *Gapdh* sense 5'-GGAGCGAGACCCCACTAA-3' and antisense 5'-ACATACTCAGCACCGGCCTC-3'. Expression levels of each gene were calculated using relative standard curves and normalized to either *β-actin* or *Gapdh*.

Focused PCR-array

BMDCs were treated with LPS (1μg) with or without PGE2 (10⁻⁶M) for 90 min. mRNA was extracted and reverse transcribed using the RT² First Strand kit. Expression levels of transcription factors were determined using the RT² Profiler PCR Array Mouse Transcription Factors.

FACS analysis

Cells were treated as indicated and incubated at 4°C for 30 min with anti-mouse CD11c, CD80, CD86, CD40, MCHII or corresponding isotype controls. Cells were washed three times and collected using BD FACSCalibur. For all experiments, stained cell preparations were compared to isotype controls in order to identify and gate on positive cell populations. Data was analyzed using FlowJo (Ashland, OR) and GraphPad Prism 5.0.

Cytokine ELISA

Cytokine levels in cell culture supernatants were quantified by sandwich ELISA. Cells were plated in 12-well culture plates (1–2 × 10⁶/ml) and stimulated for 24hr. Detection limit was 5 pg/ml for IL-27 and 15 pg/ml for IFNβ.

Western blot analysis

BMDCs ($2-3 \times 10^6$) were serum starved for 4 hours prior to treatment with LPS and PGE2. Samples were lysed, denatured (95°C, 5 minutes) and loaded on 10% Bis-Tris gels. Following electrophoresis, protein was transferred to PVDF membranes (Bio-Rad) and probed overnight with primary antibodies. Membranes were washed, incubated with HRP goat anti-rabbit Ig (BD Biosciences) then detected using Immobilon Western chemiluminescent HRP substrate (Millipore). Membranes were then stripped and re-probed for β -actin.

Chromatin Immunoprecipitation Assay (ChIP)

BMDCs (1×10^7) were stimulated for 3–4hr then fixed with 1% formaldehyde for 15 minutes. Cells were treated with 125 mM glycine for 5 min to stop fixation, washed twice with PBS containing protease inhibitors, lysed, sonicated using a sonic Dismembrator (Fisher Scientific) and processed as previously described (38). Precipitated DNA and 10% input was subjected to qPCR with primers specific to the ISRE site within the p28 promoter: sense 5'-GCTGAAAGTACAAGTAGGACAGAA-3' and antisense 5'-AGCCATCTCCTGGGTAGG-3'. Results were analyzed using the $\Delta\Delta C_T$ method.

In vivo experiment

C57BL/6 mice (n= 4–8 per group) were injected intraperitoneally with vehicle (0.4% DMSO in PBS) or dmPGE2 (200 μ g/kg) followed by a second injection 4hr later with vehicle or dmPGE2 in addition to LPS (10mg/kg). CD11c⁺ cells were purified from spleens 4hr after the second set of injections. mRNA was extracted and subjected to qRT-PCR for p28 expression.

Statistical analysis

Results represent mean \pm SD. Comparisons between groups were performed using unpaired t-test or one way ANOVA followed by Bonferroni correction. Statistical significance was determined with $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Graphs were generated and statistical analysis performed using GraphPad Prism 5.0.

Results

PGE2 inhibits IL-27 production in CD11c+ DC and macrophages

The effect of PGE2 on IL-27 production was first tested in BMDCs stimulated with various concentrations of LPS (0.1, 0.5 and 1 μ g/ml). We observed a similar inhibition pattern with more than 50% inhibition of IL-27 by PGE2 10^{-6} to 10^{-8} M. In subsequent experiments we used LPS at a concentration of 1 μ g/ml unless otherwise noted. The PGE2 concentrations were chosen in agreement with the concentrations reported in vivo in inflammatory conditions (10^{-6} – 10^{-7} M) (39–41). PGE2 reduced IL-27 production in a dose-dependent manner in both LPS- and LPS+IFN γ stimulated BMDCs (Fig. 1A). A similar inhibitory pattern was observed in BMDCs stimulated with peptidoglycan or poly I:C (Fig. 1B–C), indicating that PGE2 affects both MyD88 and TRIF signaling pathways. The inhibition of IL-27 by PGE2 is not restricted to BMDCs. We observed a similar inhibitory pattern in

splenic DCs (Fig. 1D) and in BMDM stimulated with LPS, LPS+IFN γ , and poly I:C (Fig. 1E–F).

Since various TLR ligands induce endogenous PGE₂, we tested the effect of endogenous PGE₂ on IL-27 production in BMDCs treated with LPS in the presence or absence of the COX2 inhibitor NS-398. Although there was some variability from experiment to experiment, the levels of IL-27 were increased in the presence of NS-398 concentrations which abolished the generation of endogenous PGE₂ (Fig. 1G). These results support a role for endogenous PGE₂ in the reduction of IL-27 production.

PGE₂ reduced mRNA expression levels of *p28*, but not *ebi3*, in both LPS- and LPS+IFN γ -treated DCs (Fig. 2A) and in LPS-treated BMDM (Fig. 2B), indicating that the reduction in IL-27 is transcriptional through inhibition of *p28* expression.

To determine if the effect of PGE₂ on IL-27 was associated with a general reduction in DC activation, we assessed the effects of PGE₂ on MHCII, co-stimulatory molecules and cytokine expression. PGE₂ had minimal effects on surface MHCII, CD80 and CD86 expression in DCs treated with LPS or LPS+IFN γ , with the only statistically significant decrease in the percentage of CD40⁺ cells (Fig. S1A) and CD40 MFI (230 with PGE₂ versus 390 without PGE₂). As expected in terms of cytokine expression, PGE₂ increased *il-10* and *il23a*, and had a pronounced inhibitory effect on *il12a* and *il12b* expression (Fig. S1B).

PGE₂ signals through EP2/EP4 and cAMP to reduce IL-27

To examine the role of EP receptors, BMDCs were treated with LPS in the presence of the receptor agonists butaprost (EP2), misoprostol (EP3, EP4>EP1), PGE₁OH (EP4>EP3) and sulprostone (EP3>EP1). Butaprost, misoprostol and PGE₁OH, but not sulprostone, inhibited IL-27 production (Fig. 3A), suggesting that EP1 and EP3 were not involved in the inhibition of IL-27. To confirm the role of EP2/EP4, DC were pretreated for 30 minutes with the selective receptor antagonists ONO-AE3-208 (EP4) and/or PF-04418948 (EP2), followed by treatment with LPS in the presence of PGE₂. Addition of either antagonist had slight effects on the inhibition of IL-27, whereas addition of both EP2 and EP4 antagonists completely reversed the inhibitory effect of PGE₂ (Fig. 3B), confirming the role of both EP2 and EP4.

Signaling through EP2 and EP4 is known to activate adenylate cyclase leading to increased levels of cAMP (42). To determine the involvement of cAMP in the inhibition of IL-27, we treated DCs with LPS in the presence of the stable, cell-permeable cAMP analog dibutyryl-cAMP (dbcAMP). We found that dbcAMP mimicked the effects of PGE₂ inhibiting IL-27 production in a dose-dependent manner (Fig. 3C). Furthermore, addition of the adenylate cyclase activator forskolin also inhibited IL-27 production (Fig. 3D). These results indicate that PGE₂ signals through cAMP to inhibit IL-27 production in LPS-stimulated DCs.

Increased intracellular cAMP levels lead to activation of a number of downstream signaling pathways, primarily through activation of EPAC and PKA (43, 44). To assess the involvement of EPAC, we treated DCs with LPS in the presence of the selective EPAC activator, 8-pCPT-2'-O-Me-cAMP (8-CPT). In contrast to PGE₂, 8-CPT had no effect on

LPS-induced production of IL-27 (Fig. 4A). To determine the role of PKA signaling, we pretreated DCs with the PKA inhibitors PKI (6–22), PKI (5–24), or H89 followed by LPS with or without PGE2. The PKA inhibitors did not affect IL-27 production in LPS-stimulated DCs, and did not reverse the inhibitory effect of PGE2 (Fig. 4B–C). These results strongly suggest that the inhibitory effect of PGE2 on IL-27, although cAMP-dependent, is not mediated through EPAC or PKA activation. We showed previously that PI3K activation mediated the inhibitory effect of PGE2 on CCL3/4 expression in DCs (45). However, PI3K does not appear to be involved in the inhibition of IL-27, since the PI3K inhibitor LY294002 alone or in combination with PKI (6–22) did not reverse the inhibitory effect of PGE2 (Fig. 4D). In terms of MAPK, none of the three major pathways (*i.e.* ERK1/2, JNK, and p38 MAPK) appear to be involved, since use of ERK and JNK inhibitors did not reverse the PGE2 inhibitory effect on *p28* expression (Fig. 4E), and we did not observe changes in p38 MAPK phosphorylation upon treatment with PGE2 (results not shown).

Inhibition of IL-27 by PGE2 is mediated through effects on IRF1

Molle and colleagues (37) reported that IL-27 $p28$ expression following TLR4 signaling is regulated at transcriptional level in two stages. The first stage (2–4 hours) is responsible for the initial *p28* expression and involves MyD88-dependent IRF1 and IRF3 activation. The second stage, responsible for sustained *p28* expression (6–12 hours), depends on IFNAR signaling leading to STAT1-dependent sustained IRF1 activation and formation of the ISGF3 (STAT1/STAT2/IRF9) complex. Prior studies identified a proximal ISRE site within both human and mouse *p28* promoters, and established that, in addition to the ISGF3 complex, both IRF1 and IRF3 act as essential *p28* transactivating factors by binding to the ISRE site (Fig. 5A) (37, 46–50).

To identify transcription factors (TF) affected by PGE2 in LPS-stimulated DCs, we used an RT² profiler PCR assay and compared early expression (90 minutes) of various TF in LPS-stimulated DCs treated with or without PGE2. Seven genes were differentially expressed (at levels higher than 2-fold difference), including *Irf1* which was downregulated by PGE2 (Suppl. Table 1). *Irf1* downregulation by PGE2 was confirmed by qRT-PCR (Fig. 5B), Western blot (Fig. 5C) and by flow cytometry (reduction from 71% IRF1⁺ cells in LPS-treated DCs to 53% following PGE2 treatment, data not shown). In addition, dbcAMP had a similar inhibitory effect as PGE2 on IRF1 mRNA and protein expression (Fig. 5B–C).

We further investigated the possible involvement of IRF1 in the PGE2 inhibition of IL-27 by comparing WT and *irf1*^{-/-} DCs. As expected from the reported role of IRF1 in IL-27 $p28$ expression, *irf1*^{-/-} DCs expressed much lower levels of *p28* mRNA than WT DCs. However, in contrast to WT DCs, the inhibitory effect of PGE2 was lost in *irf1*^{-/-} DCs (Fig. 5D). Next, we used ChIP assays to determine the effect of PGE2 on IRF1 binding to the ISRE site within the mouse *p28* promoter. Stimulation with LPS led to IRF1 binding to the ISRE site, and PGE2 and dbcAMP significantly reduced IRF1 binding (Fig. 5E). These results support IRF1 as a major mediator in the PGE2 inhibition of LPS-induced IL-27 expression. The fact that both PGE2 and dbcAMP have similar inhibitory effects on IL-27 production and on IRF1 binding to the *p28* ISRE site supports the role of cAMP as mediator of the inhibitory effect of PGE2 on IL-27 production.

Inhibition of IL-27 and IRF1 by PGE2 is not mediated through effects on STAT1 or STAT2

In DCs, *p28* expression has been shown to require the sequential recruitment of IRF1, IRF3 and ISGF3 to the endogenous promoter region (37). STAT1 and STAT2 are essential components of ISGF3 and participate in IRF1 expression (51–53). Therefore, we investigated whether the inhibitory effect of PGE2 on IL-27 is mediated through effects on STAT1 and/or STAT2. We first assessed the effects of PGE2 on STAT1 Tyr701 phosphorylation in LPS-treated DCs by Western blotting and flow cytometry. The results were inconsistent, with slight or no reductions in phosphorylated STAT1. Next, we addressed the role of STAT1/STAT2 by testing the effects of PGE2 in STAT1- and STAT2-deficient DCs. While WT DCs produced IL-27 in response to LPS and significantly increased IL-27 production upon LPS+IFN γ treatment, *Stat1*^{-/-} DCs produced much lower levels of IL-27 (more than 90% reduction) and lost the ability to respond to IFN γ . However, PGE2 retained its inhibitory activity for IL-27 (Fig. 6A). In terms of *irf1* expression, *Stat1*^{-/-} DCs were unable to increase *irf1* expression in response to IFN γ , but similar to the IL-27 data, PGE2 reduced *irf1* in both WT and *Stat1*^{-/-} DCs (Fig. 6B). These results strongly suggest that PGE2 inhibition of IL-27 and downregulation of *irf1* expression are STAT1-independent.

Stat2^{-/-} DCs maintained their capacity to respond to IFN γ , although IL-27 production was reduced for both LPS and LPS+IFN γ treatment as compared to WT DCs (Fig. 6C). PGE2 retained its inhibitory capacity in the *Stat2*^{-/-} DCs (Fig. 6C), and this was associated with a reduction in *irf1* expression (Fig. 6D), suggesting that, similar to STAT1, the effects of PGE2 on IL-27 and IRF1 are STAT2-independent

Reduction in endogenous IFN β by PGE2 plays a minor role in the inhibition of IL-27

Upon stimulation with LPS, DCs produce both IL-27 and IFN β . Subsequently, IFN β signaling through IFNAR results in the formation of the ISGF3 complex and sustained *p28* expression (37). PGE2 suppresses IFN β production in macrophages (54). In DC, LPS led to expression of both *ifnb* and *p28* with peaks at 1 and 3h, respectively. PGE2 completely reduced *p28* expression and IL-27 production, while *ifnb* expression and protein production were only partially reduced (Fig. 7A). To ascertain whether inhibition of IL-27 by PGE2 results from a reduction in DC-derived IFN β , we supplemented the cultures with exogenous IFN β . PGE2 retained most of its inhibitory activity (>80% for PGE 10⁻⁶M, and 50% for 10⁻⁸M) (Fig. 7B). We observed a similar pattern in *irf1* expression, with PGE2 still reducing *irf1* expression in DC treated with LPS in the presence of exogenous IFN β (Fig. 7C).

PGE2 reduces in vivo IL-27 expression in splenic DC

To determine whether PGE2 affects in vivo IL-27 production in splenic CD11c⁺ DC, we analyzed *p28* expression in mice inoculated with LPS and PGE2. The control group was injected intraperitoneally with vehicle, whereas experimental groups received dmPGE2 (stable PGE2 analog), LPS, or LPS+dmPGE2 (Fig. 8A). Splenic CD11c⁺ cells were collected four hours later and *p28* expression was analyzed by qRT-PCR. DCs from vehicle- and dmPGE2-treated mice did not express *p28* mRNA. LPS administration resulted in an increase in *p28* expression compared to the vehicle control, and co-administration of dmPGE2 reduced *p28* expression (Fig. 8B).

Discussion

Recently, the regulatory function of IL-27 in both innate and adaptive immunity and its crucial role in the generation of Tr1 cells, received a lot of attention (3, 8, 55). Although the mechanisms involved in the induction of IL-27 in cDCs and macrophages have been studied extensively, little is known about factors and mechanisms that negatively impact IL-27 expression. We were the first to report that PGE2 inhibited IL-27 production by BMDCs (56), and a later publication reported on a similar finding in THP-1 cells (35). Here we report that PGE2 inhibits IL-27 production and *p28* expression through a novel mechanism involving EP2/EP4-mediated cAMP increase, subsequent inhibition of *irf1* expression and reduced IRF1 binding to the *p28* promoter ISRE site. We also observed a similar reduction in *p28* expression in vivo in splenic CD11c⁺ DCs following inoculation of dmPGE2 in conjunction with LPS.

In vivo, PGE2 has been described as a pro-inflammatory agent in models of contact hypersensitivity, inflammatory bowel disease, rheumatoid arthritis and EAE, primarily through EP4 signaling leading to Th1/Th17 differentiation (27, 31, 34, 56–59). In vitro pro-inflammatory effects of PGE2 include DC and CD4 T cells. PGE2 contributes to DC maturation and promotes migration to draining lymph nodes (20, 36, 60). PGE2 also stimulates IL-23 production in DCs and upregulates IL-23R in T cells, promoting the generation of pathogenic Th17 cells (22, 26–29, 61). Although previous reports indicated that PGE2 favors Th2 at the expense of Th1 differentiation primarily through its inhibitory effect on IL-12 production in APCs (23, 62), PGE2 was reported to also facilitate Th1 differentiation in polarizing conditions which include addition of exogenous IL-12 through the upregulation of IL-12R β 2 and IFN γ R1 in the differentiating naïve T cells (31).

In contrast to the effects on Th1, Th2, and Th17 differentiation, the PGE2 effects on regulatory T cells are less studied, with reports suggesting differences between tumor versus non-tumor microenvironments. Tumor-derived COX2/PGE2 was shown to promote Foxp3⁺Treg and Foxp3⁻Tr1 cells in cancer (63–67). In contrast, PGE2 inhibits the differentiation of non-tumor murine Foxp3⁺Treg and of CD46-induced Tr1 cells (68, 69).

IL-27, expressed and secreted by activated cDCs, was identified as the essential Tr1 differentiation and growth factor, and shown recently to also affect cDC function (7, 70–72). In DCs, IL-27 reduced costimulatory molecules, IL-12, IL-6 and IL-23, and inhibited DC capacity to generate Th1/Th17 cells (7). In addition, adoptive transfer of IL-27-treated DC reduced EAE and suppressed recall responses of Th1 and Th17 cells (7). In CD4 T cells, IL-27 promoted both Tr1 differentiation and IL-10 production through STAT1/STAT3-mediated upregulation of c-Maf, AhR, and Egr2→Blimp1 (73, 74).

Previously, we reported that PGE2 inhibited IL-27 expression in BMDCs (34), and Zhu et al (35) reported similar results in THP-1 cells. Here we report that PGE2 inhibition of IL-27 is similar in BMDCs, BMDMs, and splenic DCs, and that it occurs upon activation of various TLRs. Both EP2 and EP4 receptors, previously identified in DCs and shown to mediate most PGE2 effects in immune cells, were involved (25, 75, 76). The role of cAMP was confirmed by similar inhibitory effects of PGE2, dbcAMP and forskolin. However, neither EPAC nor

PKA appeared to be involved. The lack of effect of PKA, PI3K, MAPK and JNK inhibitors and the lack of effect of PGE2 on p38 phosphorylation, suggests that none of these signaling pathways play an essential role in the PGE2-induced inhibition of IL-27.

Upon analyzing mRNA expression of IL-27 subunits we found that PGE2 inhibited *p28*, but not *Ebi3*. Expression of mouse *p28* is partially dependent on c-Rel binding to the distal NF- κ B site. However, binding of IRF1 to the proximal ISRE site represents the essential event for both human and mouse *p28* expression (46–49). A temporal analysis of LPS-induced *p28* expression showed that the initial burst in *p28* transcription requires IRF1 and IRF3, with rapid nuclear translocation of IRF1 being MyD88-dependent (37). This is followed by TRIF/IRF3-mediated production of IFN β which then upregulates and sustains STAT1-dependent IRF1 expression, and activates the ISGF3 complex (STAT1/STAT2/IRF9). The latter events serve to sustain *p28* expression (37).

We investigated whether PGE2 inhibits IL-27 through effects on IFN β , IRF1, IRF3, STAT1 and/or STAT2. The effect of PGE2 does not appear to be mediated through reduction in IFN β . Although we observed PGE2 inhibition of endogenous IFN β , it was only partial (approx. 50%) as compared to >90% inhibition of IL-27. Moreover, the inhibitory effect of PGE2 was maintained in the presence of exogenous IFN β (at concentrations equivalent to, or higher than the endogenous levels observed in the absence of PGE2).

We identified IRF1 as the most important contributor to the effect of PGE2 on *p28* expression. In DCs stimulated with LPS and treated with PGE2, PGE2 inhibited *irf1* expression as early as two hours. A significant reduction in intracellular IRF1 protein was also observed by Western blot. In agreement with the crucial role of IRF1 in *p28* expression, LPS-treated *irf1*^{-/-} DC expressed low levels of *p28*. Moreover, PGE2 lost its suppressive activity for *p28* expression in *irf1*^{-/-} DCs, suggesting that the inhibitory effect was mediated primarily through reduction in IRF1. This was also supported by ChIP analysis, where PGE2 abolished LPS-induced IRF1 binding to the *p28* ISRE site. Although STAT1 represents a major factor in the induction of IRF1, it does not play a role in the inhibitory effect of PGE2. In *Stat1*^{-/-} DCs although IL-27 production was reduced, PGE2 still inhibited IL-27 and *irf1* expression. *Stat2*^{-/-} DCs expressed *irf1* at higher levels than WT DCs, presumably due to an increase in STAT1 dimers, but again PGE2 was able to inhibit both IL-27 production and *irf1* expression. These results suggest that the PGE2 inhibition of IRF1 and subsequently of *p28* expression is not mediated through STAT1 or STAT2.

Most of the effects of PGE2 on immune cells are mediated through induction of cAMP (77, 78). Not surprisingly, we found that, similar to PGE2, dbcAMP and forskolin inhibited IL-27 production in cDCs. What was surprising however, was the lack of involvement of either PKA or EPAC, the classical signaling molecules downstream of cAMP. We established a link between cAMP and IRF1 by showing that similar to PGE2, dbcAMP inhibits *irf1* expression, reduces the levels of intracellular IRF1 protein, and inhibits IRF1 binding to the ISRE site in the *p28* promoter. The involvement of cAMP in IRF1 inhibition has been previously reported for two other cAMP inducing agents, e.g. cholera toxin and *Bordetella pertussis*, without identifying the intermediary signaling molecules (79, 80). We were able to eliminate a number of possible intermediaries such as PKA, EPAC, PI3K,

MAPKs, STAT1 and STAT2. However, at the present time, the link between cAMP and IRF1 in the inhibition of IL-27 by PGE2 in cDC remains to be identified.

The relevance of our findings is reinforced by the fact that we observed similar inhibition of IL-27 in splenic DCs in vitro and in vivo following treatment with exogenous PGE2. In terms of the role of endogenous PGE2, the increase in IL-27 production in cDC stimulated with LPS in the presence of a selective COX2 inhibitor points to a role similar to the exogenous PGE2. This remains to be confirmed in vivo. Since the microsomal PGE2 synthase mPGES1 is the major contributor for the specific generation of inducible PGE2, mPGES1 deficient mice (58) are the most attractive target for investigating the role of endogenous PGE2 in modulating the IL-27 response. However, a more recent report cautions against the assumption that the genetic global deletion of mPGES1 would affect solely PGE2, since mPGES1^{-/-} DC exhibit preferential shunting towards PGD2 production (81). Therefore, the development and use of a conditional temporal mPGES1 KO might be a better option.

In conclusion, our results indicate that PGE2 inhibits IL-27 production in TLR-activated cDC by reducing *p28* expression through the EP2/EP4→cAMP→IRF1 pathway. The inhibition occurs also in the presence of type I and II IFNs, suggesting that the reduction in endogenous IFNβ is not a major contributing factor. In contrast, inhibition of IRF1 mRNA and protein expression and of IRF1 binding to the *p28* ISRE site plays a major role in the PGE2 inhibition of IL-27. In an inflammatory milieu, early release of PGE2 due to the activation of inducible COX2 and mPGES1 could be a determining factor in promoting acute inflammation. Among the variety of PGE2-related physiological effects, inhibition of IL-27 in APCs could contribute to a reduction in the differentiation and possibly activation of Tr1 cells, and act as an additional proinflammatory mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

8-CPT	8-pCPT-2'-O-Me-cAMP (EPAC activator)
BMDC	bone marrow-derived dendritic cells
BMDM	bone marrow-derived macrophages
ChIP	chromatin immunoprecipitation
COX2	cyclooxygenase 2
dbcAMP	dibutyryl-cAMP

EPAC	exchange protein activated by cAMP
IRF	interferon regulatory factor
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
PGE2	prostaglandin E2

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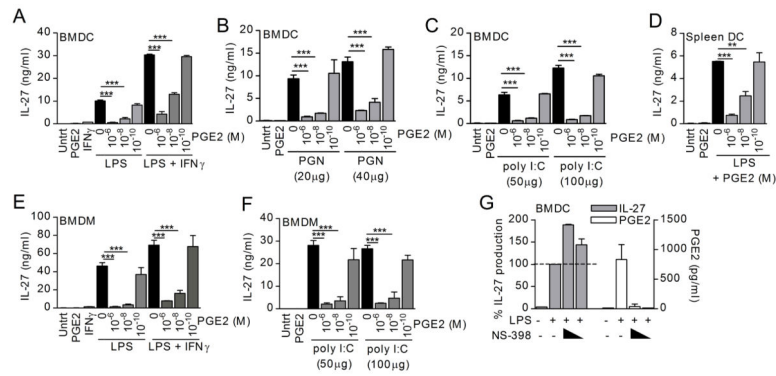


Figure 1. PGE2 inhibits IL-27 production in CD11c⁺ DC and macrophages

BMDC were stimulated with (A) LPS (TLR4; 100 ng/ml) or LPS+IFN γ (500 U/ml), (B) peptidoglycan (PGN, TLR2; 20 or 40 μ g/ml), or (C) poly I:C (TLR3; 50 or 100 μ g/ml) in the presence or absence of PGE2 for 24hr. One representative experiment of three is shown. (D) CD11c⁺ cells were isolated from the spleens of naïve C57BL/6 mice, plated for 1h, and stimulated with LPS (1 μ g/ml) and PGE2 for 24hr. BMDM were stimulated with (E) LPS (1 μ g/ml) or LPS+IFN γ (500U/ml), or (F) poly I:C (50 or 100 μ g/ml), in the presence or absence of PGE2 for 24h. One representative experiment of three is shown. Supernatants were collected and analyzed by ELISA for IL-27. (G) BMDC were pretreated with NS-398 (10⁻⁵M, 10⁻⁶M) for 30 min, then stimulated with LPS (1 μ g/ml). Supernatants were collected at 24hr and analyzed by ELISA for IL-27 or PGE2. Each sample was tested in duplicate and results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; ** $P < 0.01$ and *** $P < 0.001$.

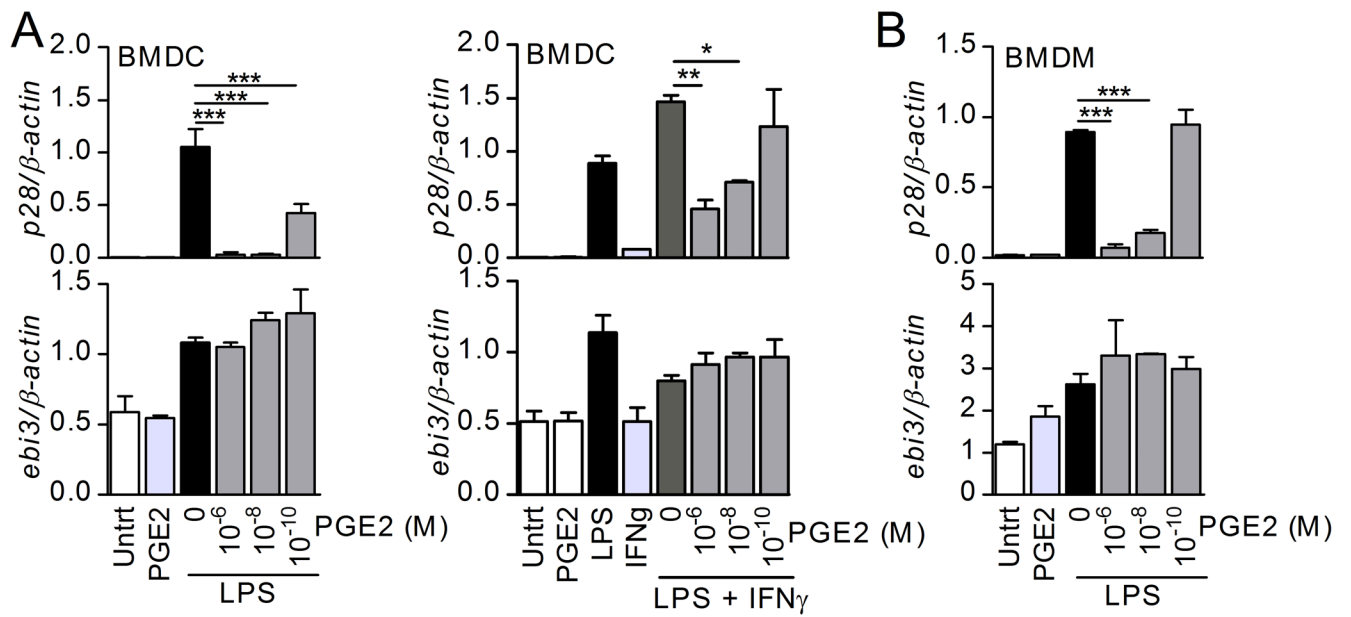


Figure 2. PGE2 inhibits IL-27p28 expression in DC and macrophages

BMDC (A) and BMDM (B) were stimulated with LPS or LPS+IFN γ in the presence or absence of PGE2 for 6hr. *p28* and *ebi3* expression was determined by qRT-PCR. One representative experiment of three is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

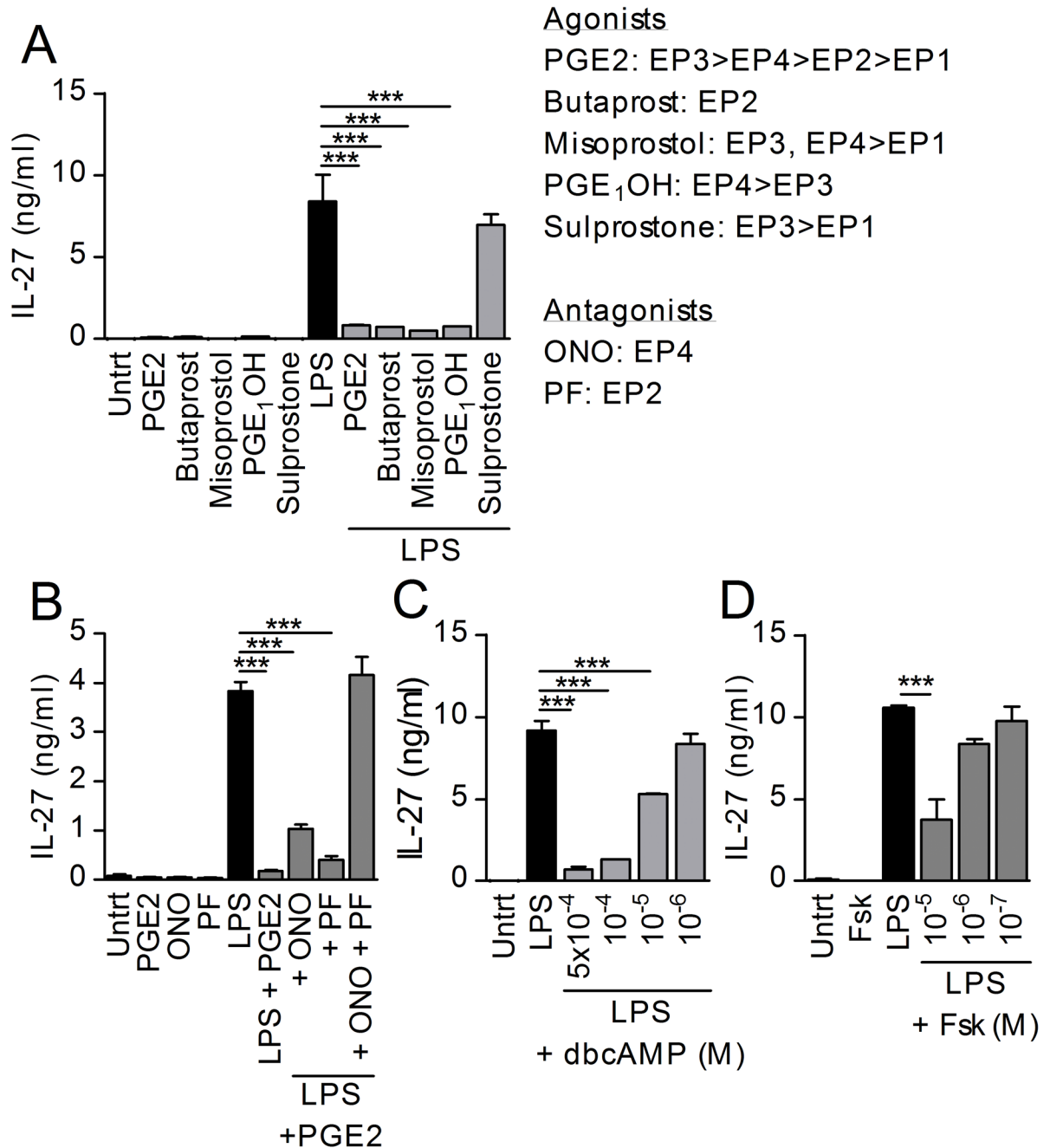


Figure 3. PGE₂ signals through EP₂/EP₄ and cAMP to inhibit IL-27 production

(A) BMDC were stimulated with LPS in the presence or absence of PGE₂, butaprost, misoprostol, PGE₁OH and sulprostone (10^{-5} M). (B) BMDC were pretreated for 30 min with selective receptor antagonists, ONO-AE3-208 (EP₄) and/or PF-04418948 (EP₂) (10^{-6} M), followed by stimulation with LPS in the presence of 10^{-7} M PGE₂. (C) BMDC were stimulated with LPS and dbcAMP. (D) BMDC were stimulated with LPS and forskolin. Supernatants were collected at 24hr and analyzed by ELISA for IL-27. One representative

experiment of three is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; *** $P < 0.001$.

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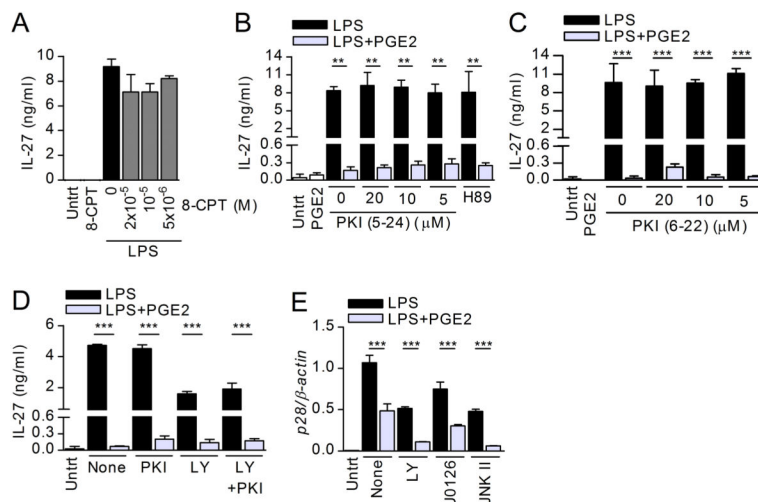


Figure 4. Inhibition of IL-27 by PGE2 is not mediated through EPAC, PKA, PI3K, or MAPK pathways

(A) BMDCs were stimulated with LPS in the absence or presence of the selective EPAC activator 8-CPT for 24hr. One of two independent experiments is shown. BMDCs were pretreated for 3hr with the PKA inhibitors PKI (5–24) or H89 (B), or PKI (6–22) (C) followed by LPS or LPS+PGE2 (10^{-7} M) for 24hr. One of two independent experiments is shown. (D) BMDCs were pretreated for 30 min with the PKA inhibitor PKI (6–22), the PI3K inhibitor LY294002 (LY) or both, followed by LPS or LPS+PGE2 for 24hr. Supernatants were collected and subjected to IL-27 ELISA. One of two independent experiments is shown. (E) BMDCs were pretreated for 1h with the PI3K inhibitor LY294002 (LY), ERK1/2 inhibitor U0126, or JNK inhibitor II (JNK II), followed by stimulation with LPS or LPS+PGE2 for 3hr. *p28* expression was determined by qRT-PCR. One of three independent experiments is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; ** $P < 0.01$ and *** $P < 0.001$.

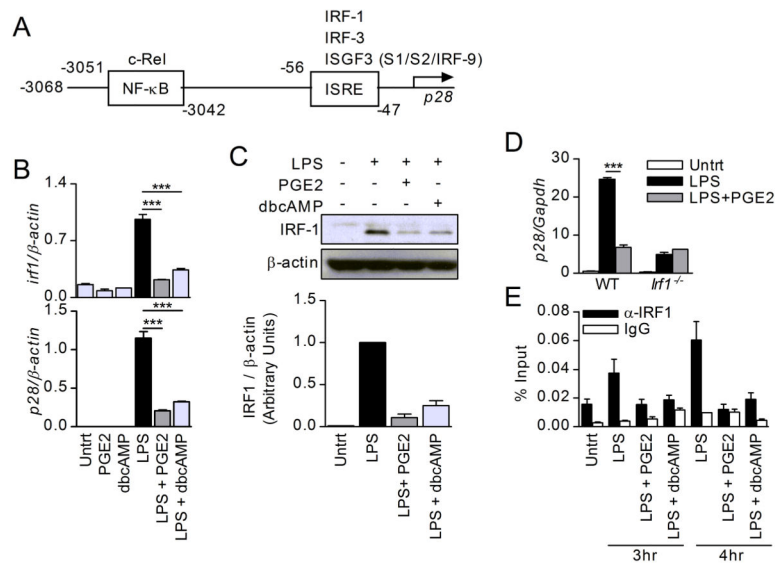


Figure 5. Effects of PGE2 on IRF1

(A) Schematic of the murine *p28* promoter with NF- κ B and ISRE sites. (B) BMDC were stimulated with LPS in the presence of either PGE2 (10^{-6} M) or dbcAMP (10^{-4} M) for 2hr. *Irf1* and *p28* expression was determined by qRT-PCR. One of two independent experiments is shown. (C) BMDC were treated with LPS with or without PGE2 or dbcAMP for 3hr. Total cell lysates were collected and analyzed by Western blot for IRF1 and β -actin. One Western blot scan from three independent experiments is shown, with cumulative densitometry analysis below. (D) BMDC from WT and *Irf1*^{-/-} were stimulated with LPS with or without PGE2 for 3hr and analyzed by qRT-PCR for *p28*. One representative experiment of two is shown. (E) BMDC were stimulated with LPS in the presence or absence of PGE2 or dbcAMP for the indicated times. Cells were fixed, lysed, sonicated and subjected to ChIP utilizing anti-IRF1 (black bars) and control IgG (white bars). Precipitated DNA was purified and analyzed by qPCR using primers specific for the ISRE site within the *p28* promoter. One of three independent experiments is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; *** $P < 0.001$.

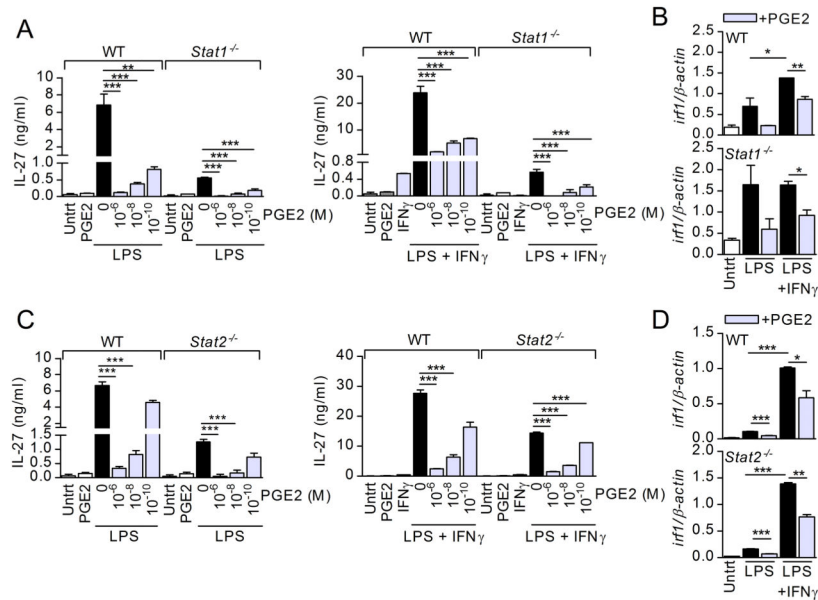


Figure 6. The inhibitory effect of PGE2 on IL-27 and *irf1* expression is not mediated through STAT1/STAT2

BMDC from WT 129S6 mice and *Stat1*^{-/-} mice (A) or WT C57BL/6 mice and *Stat2*^{-/-} mice (C) were stimulated with LPS or LPS+IFN γ in the presence or absence of PGE2 for 24hr. Supernatants were collected and subjected to IL-27 ELISA. ELISA data are representative of two independent experiments for *Stat1*^{-/-} data and three independent experiments for *Stat2*^{-/-} data. BMDC from WT 129S6 mice and *Stat1*^{-/-} mice (B) or WT C57BL/6 mice and *Stat2*^{-/-} mice (D) were stimulated with LPS or LPS+IFN γ in the presence or absence of PGE2 for 1h. *Irf1* expression was determined by qRT-PCR. One of two independent experiments is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction (A, C) or unpaired t-test (B, D); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

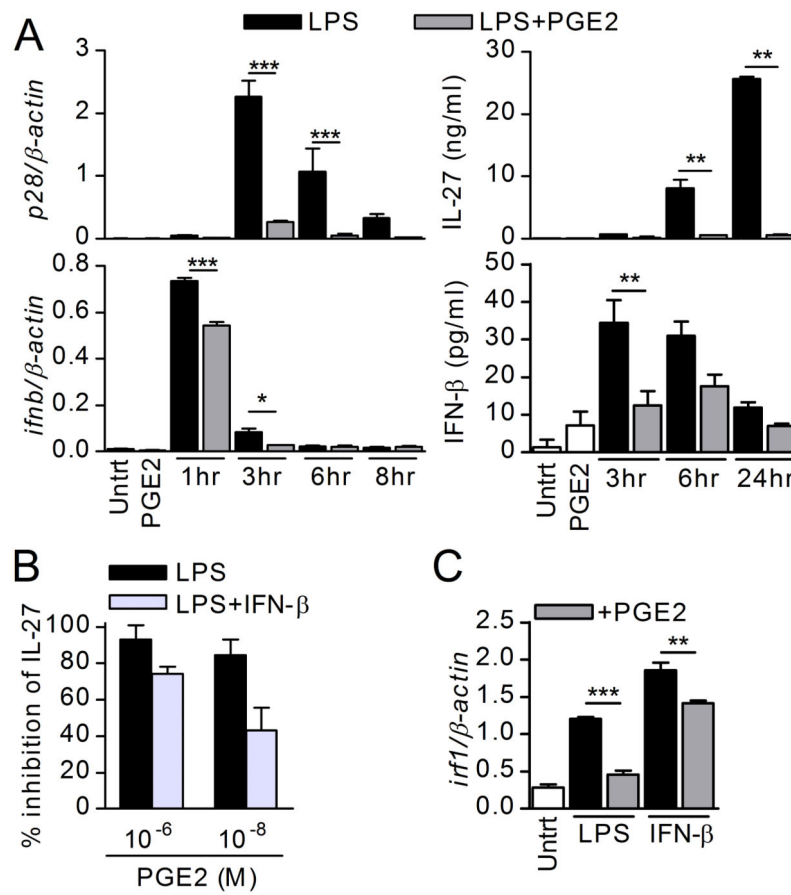


Figure 7. Inhibition of IL-27 by PGE2 is not mediated by the reduction in endogenous IFN β
 (A) BMDCs were stimulated with LPS with or without PGE2 for 1, 3, 6 and 8hr to analyze *p28* and *ifnb* expression levels by qRT-PCR or for 3, 6 and 24hr to analyze IL-27 and IFN β protein levels by ELISA. One of two independent experiments is shown. (B) BMDCs were treated with either LPS or LPS+IFN- β (1000U/ml) in the presence or absence of PGE2 (10⁻⁶M, 10⁻⁸M) for 24h. Supernatants were analyzed by IL-27 ELISA. Results are presented as percent inhibition compared to no PGE2 controls. One of four independent experiments is shown. (C) BMDCs were treated with either LPS or IFN β in the presence or absence of PGE2 for 1h. *Irf1* expression was determined by qRT-PCR. One of two independent experiments is shown. Results represent means \pm SD. Statistics were calculated using one way ANOVA followed by Bonferroni correction; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

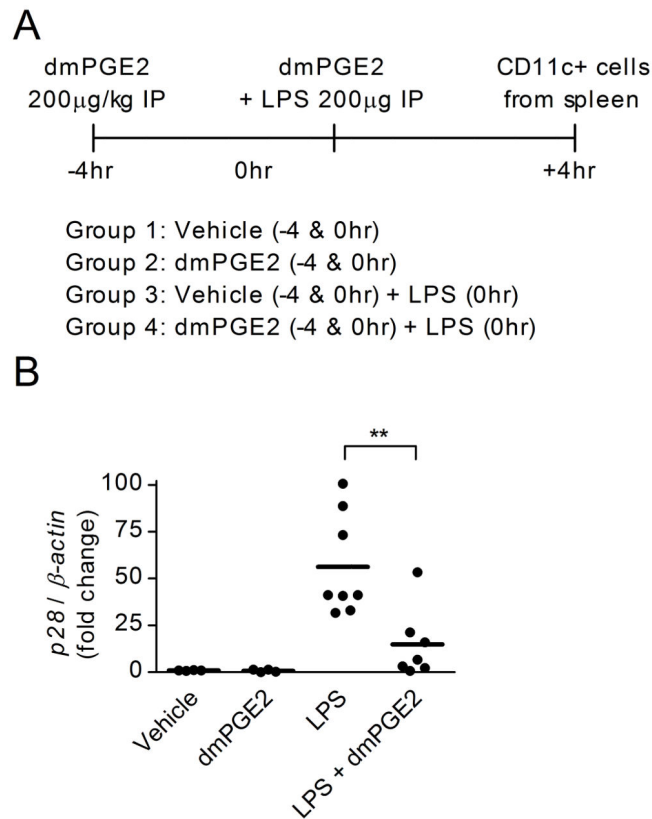


Figure 8. In vivo effects of PGE2 on splenic DC *p28* expression

(A) Schematic of experimental timeline and groups. (B) C57BL/6 mice (n=4–8) were injected intraperitoneally with vehicle (0.4% DMSO in PBS) or dmPGE2 (0.2mg/kg) twice at 4hr interval. LPS (10mg/kg) was given intraperitoneally to groups 3 and 4 with the second vehicle or dmPGE2 inoculation. CD11c⁺ cells were purified from the spleen 4hr after the second set of injections. Expression of *p28* was analyzed by qRT-PCR. Graph represents cumulative data from three experiments. Statistics were calculated using one way ANOVA followed by Bonferroni correction; ***P*<0.01.