

**PGE2 AND IL-27: NOVEL PROINFLAMMATORY MECHANISMS
INVOLVING DENDRITIC CELLS AND TYPE 1 REGULATORY T CELLS**

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ABSTRACT

Interleukin-27 (p28/EBI3) is an immunomodulatory cytokine expressed by activated antigen presenting cells. Although first discovered to be involved in Th1 cell differentiation, further studies demonstrated the immunosuppressive functions of IL-27 including inhibition of Th2 and Th17 differentiation, development of a tolerogenic phenotype in dendritic cells (DC), and promoting type 1 regulatory T cells (Tr1). The anti-inflammatory effects of IL-27 have been demonstrated *in vivo* in murine models of parasitic infections and autoimmune diseases. Despite the prevalence of studies detailing the induction of IL-27 expression and the role of IL-27 in Tr1 differentiation, little is known about factors that negatively regulate IL-27 expression and Tr1 differentiation. Prostaglandin E2 (PGE2), a lipid mediator abundant at inflammatory sites, was shown to act as a proinflammatory agent in models of inflammatory/autoimmune diseases primarily by promoting CD4 Th1/Th17 differentiation. Here we describe a novel proinflammatory mechanism for PGE2 through the inhibition of IL-27 production in conventional dendritic cells (cDC) and the inhibition of Tr1 differentiation. PGE2 inhibits IL-27 production in bone marrow-derived DC and macrophages, as well as in splenic cDC, through EP2/EP4 receptors, induction of cAMP, and downregulation of IRF1 expression and binding to the p28 IL-27 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 p28 expression *in vivo* in splenic DC following administration of dimethyl PGE2 in conjunction with LPS. In addition to the inhibition of IL-27 production in APCs, PGE2 also directly affects Tr1 differentiation by reducing IL-27-induced CD4⁺CD49b⁺LAG-3⁺Foxp3⁺ Tr1 cells and IL-10 production. The inhibitory effect is mediated by EP4 and induction of cAMP in differentiating CD4 T cells. IL-27-induced Tr1 differentiation and function depends primarily on the sustained expression of c-Maf in addition to AhR and Blimp-1. PGE2 significantly reduced expression of c-Maf without affecting AhR and only marginally reducing Egr-2/Blimp-1 expression. The effects of PGE2 on

Tr1 cells are independent of STAT1/STAT3 signaling and of IL-21 signaling. In addition, the effect of PGE2 on CD4⁺CD49b⁺LAG-3⁺ Tr1 differentiation was not associated with either induction of Foxp3 or IL-17 production, suggesting a lack of transdifferentiation into Foxp3⁺ Treg or effector Th17 cells. The effects of PGE2 on both IL-27 production and IL-27-induced Tr1 differentiation represent novel proinflammatory mechanisms of PGE2.

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LIST OF ABBREVIATIONS

8-CPT	8-pCPT-2'-O-Me-cAMP (EPAC activator)
AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
cDC	Conventional dendritic cell
ChIP	Chromatin immunoprecipitation
CIA	Collagen-induced arthritis
COX	Cyclooxygenase
dbcAMP	Dibutyl-cAMP
DC	Dendritic cell
dmPGE2	dimethyl PGE2
EAE	Experimental autoimmune encephalomyelitis
Ebi3	Epstein-Barr virus induced gene 3
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange protein activated by cAMP
FLTT	Fetal liver and thymus transplants
GVHD	Graft versus host disease
HLIL	Hodgkin's Lymphoma infiltrating lymphocytes
HSCT	Hematopoietic stem cell transplants
IBD	Inflammatory bowel disease

ICOS	Inducible T cell costimulator
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
LAG-3	Lymphocyte activation gene 3
MS	Multiple sclerosis
MT	Metallothioneins
qRT-PCR	Quantitative real-time polymerase chain reaction
SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
Tr1	Type 1 regulatory T cell
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E2
WT	Wild type

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CHAPTER ONE INTRODUCTION

Antigen Presenting Cells

Antigen presenting cells (APC) are the main bridge between innate and adaptive immunity. Depending on the inflammatory signal strength, duration, and surrounding milieu, APC produce an assortment of immune mediators. Professional APC (i.e. dendritic cells and macrophages) participate in the innate immune response through recognition of pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRR) (1-3). Other signals which can trigger an immune response by APC are extracellular DNA, ATP, and other damage associated molecular patterns (4, 5).

APC are also responsible for processing antigen in order to interact with antigen-specific T cells, leading to activation and differentiation of T cells. The most widely studied APC capable of activating T cells is the dendritic cell. Dendritic cells develop in the bone marrow from myeloid progenitors. Of the three types of dendritic cells, conventional, monocyte-derived and plasmacytoid, conventional dendritic cells (cDC) are most commonly studied for their production of immune modulators and ability to manipulate the immune response (6, 7). Activation of T cells by DC is dependent on engagement of the T cell receptor (TCR), co-stimulatory signals, and immune modulating cytokines (8-10). Antigen is processed by the cDC and complexed to MHC molecules, either class I for CD8 T cell activation, or MHC class II for CD4 T cell activation (7). Generally, costimulatory molecules CD80/CD86 and CD40 on DC bind to CD28 and CD40L, respectively (11).

CD4 T Cell Differentiation

The interaction between APC and naïve CD4 T cells, as well as the cytokines expressed by the APC, determines the fate of the T cell. Depending on the strength of the signal and the cytokine milieu during T cell stimulation, CD4 T cells are programmed to differentiate into regulatory T cells or a series of effector T cells. The cytokines present during APC-mediated stimulation play a key role in T helper subtype differentiation (12, 13).

Differentiation of a naïve CD4 T cell is initiated upon receipt of multiple signals from an APC, including engagement of the TCR by antigen complexed to MHC class II, ligation of the CD28 receptor by costimulatory molecules CD80/CD86, and cytokine signals (Fig. 1). In the context of Th1 differentiation, IL-12 and IFN γ signaling induces expression of the transcription factors Tbet, which drives production of IFN γ , IL-2 and IL-12R β 2, pushing the cells towards a Th1 phenotype (12, 14). Alternatively, the presence of IL-4 during T cell differentiation induces GATA-3 transcription. GATA-3, the master transcription factor for Th2, inhibits differentiation of Th1 cells, and promotes production of IL-4, IL-5 and IL-13, leading to development of a Th2 phenotype (13, 15). Finally, differentiation in the presence of TGF- β , IL-6, IL-1 β and/or IL-23 promotes the Th17 transcription factor, ROR γ t (15). ROR γ t is responsible for the induction of IL-17, IL-22, and IL-23R expression, consequently pushing CD4 T cells towards the Th17 phenotype (16). In addition to determining T cell subtype, cytokines can also affect the function of mature immune cells.

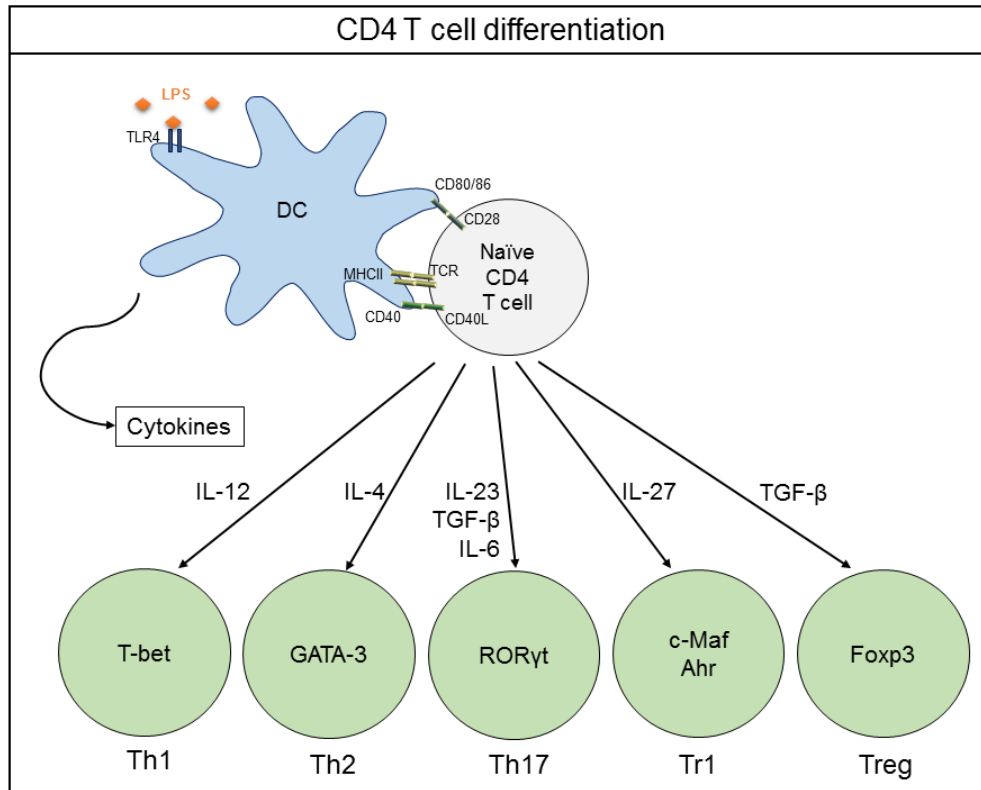


Figure 1. CD4 T cell differentiation. Naïve CD4 T cells are stimulated by APC to differentiate into a series of helper subsets, mainly Th1, Th2, Th17, Tr1 and Treg. Although a complex process, a simplified version is shown to highlight the key cytokines involved in each Th subset, i.e. IL-12 for Th1, IL-4 for Th2, etc. Cytokines initiate expression of key transcription factors which determine the fate of each T cell. Although a single master transcription factor has been identified for Th1, Th2, Th17 and Treg, respectively, several possible transcription factors have been implicated in the differentiation of Tr1 cells.

Many cytokines, like TGF-β and IL-10, have anti-inflammatory properties that promote the development of immunosuppressive cells such as Foxp3⁺ Treg (17) and

tolerogenic DC (18). In addition, IL-27 has been shown to affect early differentiation of CD4 T cells into Tr1 cells and inhibit the function of effector T cells (2, 19).

Interleukin-27

IL-27 Production

Interleukin-27 is expressed by activated dendritic cells, macrophages, microglia, mast cells, and endothelial cells (20-23). APC produce IL-27 in response to various TLR ligands and cytokines, including LPS, peptidoglycan, poly I:C, and both type I and type II interferons (20, 21, 24-26). While LPS is strong inducer of IL-27, the combination of LPS and IFN γ results in the highest amount of IL-27 production (24-26).

IL-27 Structure

Interleukin-27 is a heterodimeric cytokine belonging to the IL-12 family which, in addition to IL-27, is comprised of IL-12, IL-23, and IL-35 (27) (Fig. 2). Each cytokine within the family is composed of two subunits; IL-12 (p35/p40), IL-23 (p19/p40), IL-27 (p28/EBI3), and IL-35 (p35/EBI3) (20, 21, 27). Computational modeling of the p28 gene identified similar sequences and structural motifs between p28 and two other IL-12 family subunits; i.e. p19 and p35 (20, 28). The two IL-27 subunits are encoded by separate genes: *p28* and *Ebi3* (20, 29).

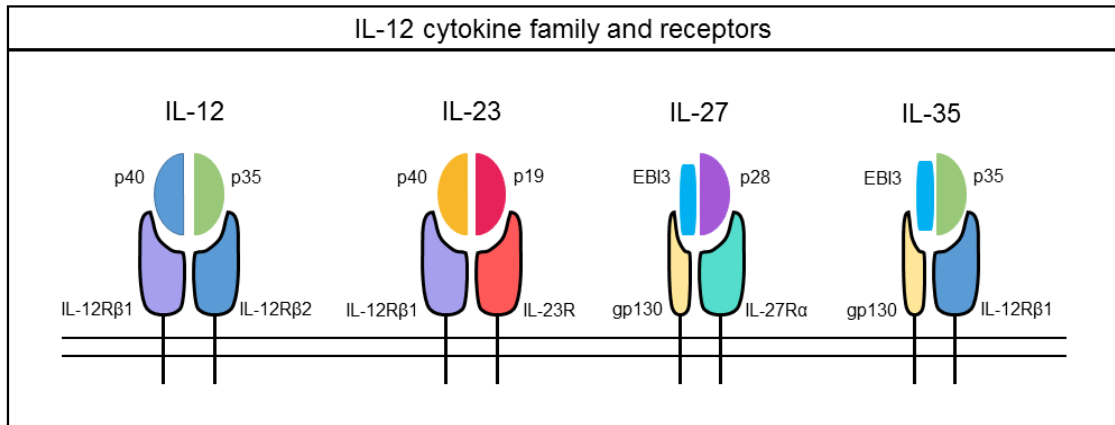


Figure 2. IL-12 family members and their receptors. The IL-12 family is comprised of IL-12, IL-23, IL-27 and IL-35. Each cytokine is composed of two subunits, many of which are shared between two members of the family (i.e. p40 is part of IL-12 and IL-23). Similarly, some receptor subunits are shared between the four receptors.

IL-27 Expression

The two IL-27 subunits are expressed differently. EBI3 is constitutively expressed by many cells including macrophages, DC, B cells, and epithelial cells (29, 30). EBI3 can also be upregulated in response to stimulation with ligands for TLR4, TLR2 and TLR9 through activation of transcription factors NF- κ B p50/p65 and PU.1 (30). Due to the constitutive expression of EBI3, p28 is the rate limiting factor of IL-27 and the subject of many IL-27 regulation studies.

Expression of p28 in response to LPS-stimulation is partially dependent on MyD88 and entirely dependent on TRIF (25). The p28 promoter contains two key binding sites whose roles in p28 expression have been examined extensively, an NF- κ B binding site (-3051) and an IRF binding site (-56) (24). Originally, c-Rel was identified to bind to the NF- κ B site, but further studies revealed the importance of the IRF binding site

in p28 promoter activation (24). IRF1, IRF3 and the ISGF3 complex (composed of STAT1/STAT2/IRF9) all bind to the IRF binding site (24-26). While deficiency in c-Rel leads to a slight reduction in *p28* expression in response to LPS-stimulation, cells lacking either IRF1, IRF3 or ISGF3 all produce significantly less p28 than cells without IRF deficiencies (25, 26). These studies confirmed the importance of the IRF binding site in p28 expression.

Differential expression of p28 in bone marrow-derived DC (BMDC) deficient in either IFNAR, IRF1 or IRF9 revealed a two-step process in p28 promoter activation. In response to LPS, IRF1 is recruited to the nucleus where it initiates production of both p28 and IFN- β . IFN- β then activates both IRF1 and ISGF3, leading to optimal expression of IL-27 (26). Initiation of p28 expression is dependent on IRF1 and IRF3, while recruitment of ISGF3 to the p28 promoter is responsible for sustained promoter activation and IL-27 production (Fig. 3) (26).

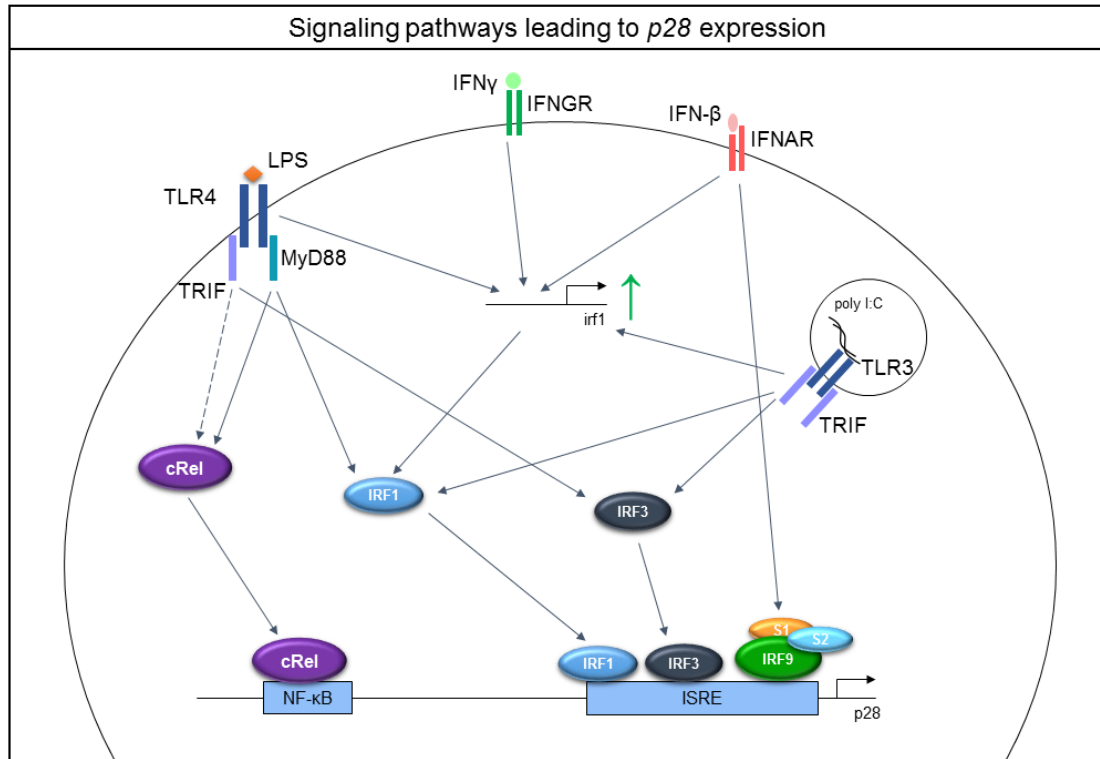


Figure 3. Signaling pathways involved in p28 expression. The signaling pathways involved in poly I:C-, LPS- and IFN-induced production of the p28 subunit of IL-27 are shown. LPS activates TLR4 signaling leading to activation of TRIF and MyD88. TRIF activates cRel and IRF3, while MyD88 leads to activation of cRel and IRF1, all of which bind to and activate the p28 promoter. IFN γ signaling activates IRF1, while IFN- β activated both IRF1 and the ISGF3 complex (STAT1/STAT2/IRF9). Finally, activation of TLR3 signaling leads to IRF1 and IRF3 activating the p28 promoter.

Negative Regulation of IL-27

In contrast to numerous studies on the positive regulation of IL-27 production, little is known about mechanisms that counteract IL-27 production (Fig. 4). Three molecules (eATP, C5a and histamine) have been reported to inhibit IL-27 expression (31-

33). Extracellular ATP has been shown to inhibit IL-27 production through the P2Y₁₁ receptor (31). C5a was identified as a specific inhibitor of TLR4-induced IL-27 production (TLR3-induced IL-27 production was not reduced) and shown to act through C5aR and the PI3K/Akt pathway (32). Finally, histamine reduced IL-27 production by LPS-stimulated BMDC (33). The possible role of inflammatory lipid mediators such as prostaglandins in IL-27 production and function is not known and represents the focus of the research reported in this thesis.

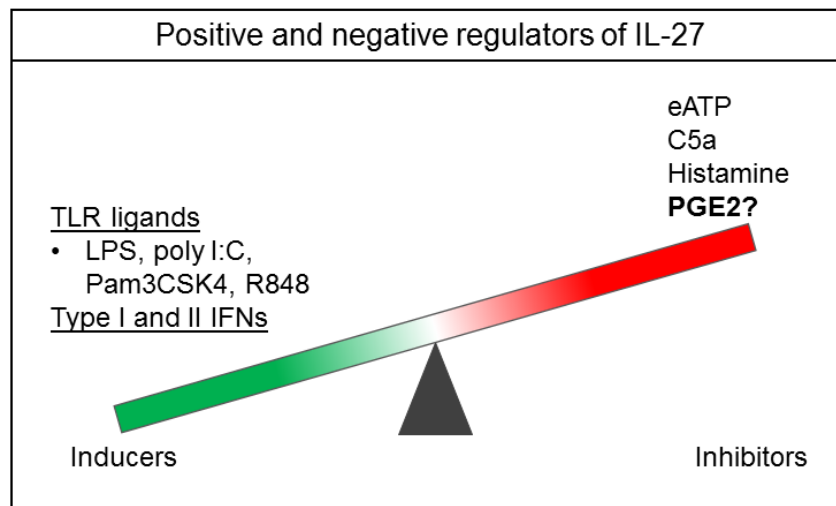


Figure 4. Positive and negative regulators of IL-27 production. Many molecules have been described to induce IL-27 production by APC, including multiple TLR ligands, and IFN α , IFN- β and IFN γ . In contrast, little is known about what might inhibit production of IL-27, leading to an informational imbalance in the literature. So far studies have implicated extracellular ATP, complement component C5a and histamine in the downregulation of IL-27. This study aims to determine whether PGE2 belongs to the inhibitor group and to investigate the relevant molecular mechanisms.

Biological Activity of IL-27

IL-27 is recognized by the IL-27R, composed of IL-27R α (also known as WSX-1 or TCCR) and gp130 (20, 21). Engagement of both receptor subunits is required for signal transduction (34). IL-27R is expressed by many different cell types including APC, T cells, B cells, NK cells, mast cells, neutrophils, endothelial cells and epithelial cells (20). Due to the expression of IL-27R on many immune cells, IL-27 has both direct and indirect immunomodulatory effects on many different cell types.

Anti-inflammatory functions have been observed in numerous *IL-27R α ^{-/-}* studies. IL-27R-deficient mice have been shown to succumb to parasitic infection at greater rates than WT counterparts, largely due to uncontrolled inflammatory immune responses. *IL-27R α ^{-/-}* mice infected with *Leishmania major* developed larger lesions, increased numbers of Th17 cells and decreased numbers of IL-10⁺ CD4 T cells than WT mice (35). Additionally, *IL-27R α ^{-/-}* mice cleared *Plasmodium berghei* parasites more efficiently than WT controls, but exhibited decreased survival rates and high levels of IFN γ and IL-17 in the liver fourteen days post infection (36). Similarly, *IL-27R α ^{-/-}* mice infected with *Toxoplasma gondii* cysts were able to control parasitic infection, but developed uncontrolled T cell responses consisting of overproduction of IFN γ and increased proliferation of T cells *in vivo* (37). Furthermore, IL-27R deficiency in mice hindered regulation of pro-inflammatory cytokines IL-6, IL-17, IFN γ and TNF α in response to *Trypanosoma cruzi*, leading to increased inflammation and mortality (38). These studies detail the importance of IL-27 in regulating the immune response. Although the effects seen in these studies cannot be attributed to the effects of IL-27 on APC or T cells

individually due to the use of global knockout mice, there have been many studies showing the direct effects of IL-27 on individual immune cells.

Effects on APC

By shaping the response of APC, IL-27 can have indirect effects on many different immune cells. Treatment of DC with IL-27 promotes a more tolerogenic phenotype, characterized by IL-10 upregulation and reduced expression of the proinflammatory cytokines IL-6, IL-12 and IL-23 (39). Furthermore, treatment with IL-27 decreases expression of co-stimulatory molecules CD80, CD86, CD40 and MHCII (39). Consequently, co-culture of naïve CD4 T cells with IL-27-conditioned DC resulted in fewer Th1 and Th17 cells, and higher numbers of Tr1 cells, indicating that IL-27 promotes tolerogenic functions in DC (39). Moreover, treatment with IL-27 lead to expression of CD39, which increased the suppressive activity on T cells and inhibited activation of the inflammasome (39).

The anti-inflammatory functions of IL-27 on DC were confirmed in an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE). EAE mice that were reconstituted with IL-27R-deficient DC developed more severe clinical scores, and exhibited higher numbers of encephalitogenic Th1 and Th17 cells, and fewer IL-10-producing cells T cells in the CNS, as compared to mice reconstituted with WT DC (39). These studies demonstrate the immunosuppressive function of IL-27 on DC and indirectly on T cell differentiation (Fig. 5 upper panel).

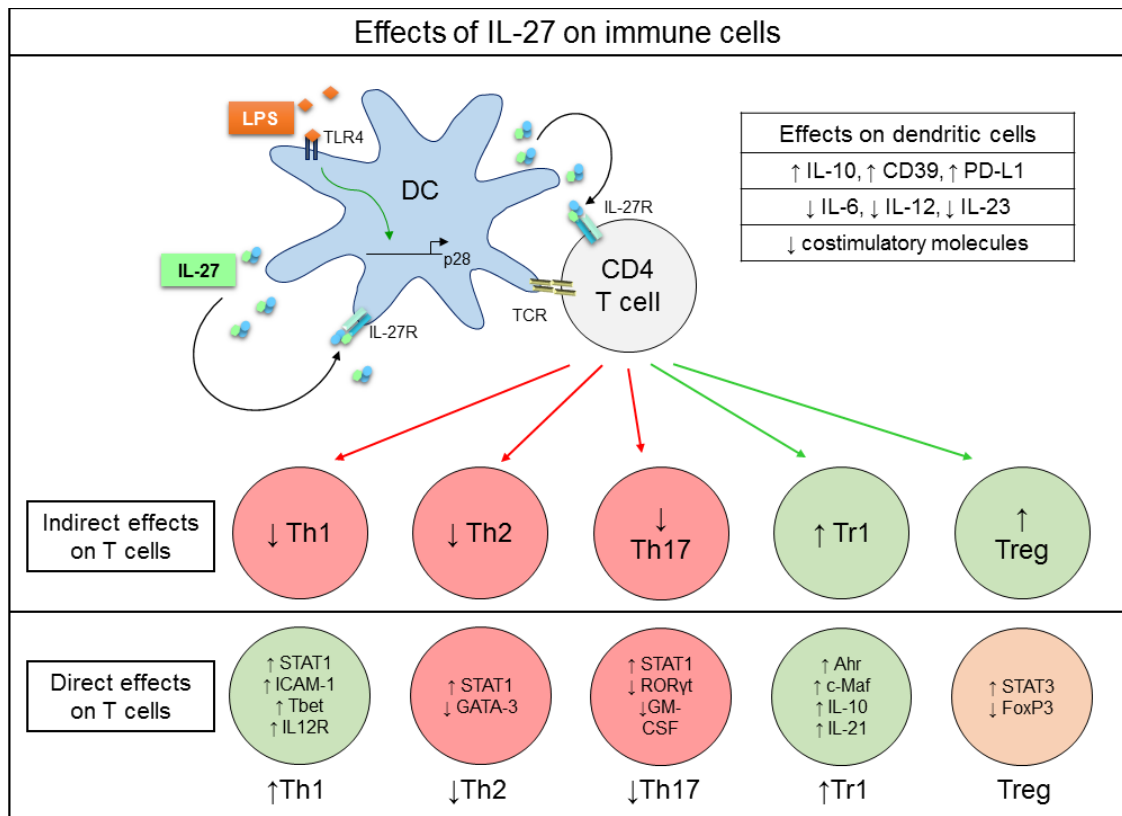


Figure 5. Effects of IL-27 on dendritic cells and CD4 T cells. (Upper panel) Treatment of DC with IL-27 results in an immunosuppressive phenotype, including an increase in IL-10, CD39 and PD-L1 expression, and a decrease in proinflammatory cytokines and costimulatory molecules. By acting on DC, IL-27 can indirectly inhibit differentiation of Th1, Th2 and Th17, while increasing regulatory T cells. (Lower panel) Conversely, IL-27 secreted by APCs can act directly on CD4 T cells to initiate early Th1 cell differentiation, and inhibit differentiation of both Th2 and Th17 cells. Despite conflicting reports of the effects of IL-27 on Foxp3-expressing Tregs, many studies have shown that IL-27 promotes Tr1 cell differentiation and function by increasing expression of transcription factors Ahr and c-Maf, and increasing production of IL-10 and IL-21.

Effects on CD4 T Cells

In CD4 T cells, recognition by IL-27R leads to signaling through the JAK/STAT pathway, utilizing different STATs depending on the activation state of the cell (40). In naïve CD4 T cells, IL-27 signaling activates STAT1 and STAT3 leading to an increase in T-bet and a decrease in GATA-3 respectively promoting Th1 at the expense of Th2 differentiation (40, 41), whereas in fully activated T cells, IL-27 acts as an anti-inflammatory cytokine through a reduction in pro-inflammatory cytokine secretion (40). Although IL-27 was first discovered to be involved in initiation of Th1 cell differentiation (20), further studies demonstrated the immunosuppressive functions of IL-27 including the inhibition of Th2 and Th17 cell development, and the induction of type 1 regulatory T cells (Tr1) and exhausted CD4⁺ T cells (Fig. 5 lower panel) (42-46).

IL-27 was initially thought to be pro-inflammatory due to its role in early Th1 differentiation. However, although IL-27 was shown to promote Tbet and IL-12Rβ2 (47) (48), it was not able to induce IFNγ production in the absence of IL-12 (41, 47). Despite the involvement of IL-27 in early Th1 differentiation, later studies have reported on the anti-inflammatory effects of IL-27. For example, in fully differentiated Th1 cells, IL-27 inhibited production of IL-2 (49). In addition, IL-27 did not support proliferation of memory T cells (20). Instead, IL-27 was shown to promote Th1 cell exhaustion, characterized by loss of effector function, decreased expression of pro-inflammatory cytokine secretion, increased IL-10 production and increased Tim-3 expression (46). Inhibition of IL-2, an essential cytokine for Th1 activation and function, and the promotion of an exhausted phenotype support the anti-inflammatory function of IL-27.

In Th2 polarizing conditions, IL-27 inhibited expression of GATA-3, IL-4, IL-5 and IL-13, thus inhibiting Th2 differentiation (41, 50). Treatment of fully differentiated Th2 cells with IL-27 resulted in reduced expression of GATA-3 and Th2 cytokines, indicating IL-27 inhibits both Th2 differentiation and function (50). Th2 inhibition by IL-27 has been confirmed *in vivo* in parasitic infections where parasite expulsion depends on Th2 responses (23, 50). In addition, IL-27R deficient mice were more susceptible to a Th2-mediated model of experimental allergic asthma (51). Taken together, these experiments show that IL-27 inhibits both differentiation of Th2 cells and the function of fully differentiated Th2 cells.

In Th17 polarizing conditions, IL-27 treatment inhibited expression of transcription factor ROR γ t and production of IL-17 by CD4 T cells (52). Mice deficient in IL-27R exhibited exacerbated Th17-mediated EAE (43). Mice injected with IL-27 after collagen-induced arthritis (CIA) was induced displayed less severe disease symptoms and fewer Th17 cells in the spleen (42). In a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, administration of IL-27 improved colon health, reduced colonic expression of IL-17, TNF- α , IL-1 β and IL-6 and reduced Th17 cell proliferation (44).

The ability of IL-27 to inhibit the function of fully differentiated Th17 cells depends on the presence or absence of IL-23. In an adoptive transfer EAE model, mice receiving MOG-specific CD4⁺ T cells restimulated *ex vivo* in the presence of IL-12 and IL-27 developed less severe EAE than those receiving T cells restimulated in the absence of IL-27 (53, 54). However, committed Th17 cells isolated from the CNS of EAE mice reactivated in the presence of IL-23 maintained their phenotype even upon exposure to

IL-27 (55). Later studies revealed that IL-27, in conjunction with IL-12, promotes Blimp-1 and IL-10 in Th17 cells, while IL-23 inhibits these effects (56). In conclusion, IL-27 inhibits both Th17 differentiation and the function of fully differentiated Th17 cells in the absence of IL-23.

There are conflicting reports in the literature about the role of IL-27 on Foxp3⁺ Tregs. A study on Tregs in the intestine showed that expression of GATA-3 was essential for their differentiation, and that treatment with IL-27 inhibited GATA-3 expression and subsequently inhibited Treg differentiation (57). Similarly, IL-27 was shown to inhibit TGF- β -induced Treg differentiation through STAT3 signaling (58, 59). However, other studies have shown that Foxp3⁺ Tregs restimulated with IL-27 displayed increased immunosuppressive function (60), and that despite not affecting Foxp3 expression, restimulation of Treg with IL-27 increased expression of LAG-3, leading to increased regulatory function (61). Further studies are needed to elucidate the role of IL-27 in Foxp3⁺ Treg differentiation and function.

Finally, many studies have identified a role of IL-27 in the differentiation of type 1 regulatory T cells (Tr1 cells) *in vivo* and *in vitro* (62-64). IL-27 signaling leads to activation of the transcription factors c-Maf and AhR which transactivate the IL-10 promoter and induce Tr1 cell differentiation (53, 65). IL-27 has been shown to induce both murine and human Tr1 cell differentiation, detailing a large portion of the anti-inflammatory function of IL-27 (66).

Effects on CD8 T Cells

Although IL-27 is largely anti-inflammatory in the context of CD4 T cells, it has been shown to enhance CD8 T cell activation. Stimulation of naïve CD8 T cells in the presence of IL-27 also leads to increased production of both IFN γ and granzyme B (67). IL-27 increases anti-tumor cytotoxic CD8 T cell responses (68, 69). Additionally, treatment with IL-27 promoted anti-tumor CD8 T cells to produce IL-10, and contributed to the development of memory CD8 T cells (70).

Effects on B Cells

The effects of IL-27 on B cells vary depending on the activation state of the cell. In naïve B cells, treatment with IL-27 leads to increased expression of Tbet, ICAM-I, CD86, FasR and isotype switching, which characterize activated B cells (71, 72). IL-27 also initiated differentiation of naïve B cells into germinal center B cells, both through direct activity on B cells and through stabilization of T follicular helper cells (73, 74). IL-27 increased proliferation of naïve B cells yet had little effect on the proliferation of memory B cells (71).

Effects on NK Cells

IL-27 has immunomodulatory effects on NK cells with increased expression of IL-10 and IFN γ in response to IL-27. Although IL-27 decreased NK cell proliferation (75), IL-27 also increased NK cell suppressive function and cytotoxic activity (75, 76). Moreover, IL-27 induced production of granzyme B in murine NK cells and perforin in human NK cells (75, 77). Furthermore, treatment with IL-27 promoted NK cell antibody-

dependent cellular cytotoxicity (76, 77). Through these functions, IL-27 acts as an activator of NK cell effector function.

Implications in Human Diseases

In multiple sclerosis (MS), the protective effect of IFN- β has been shown to be dependent on IL-27 (78). Pretreatment of human monocyte-derived DC with neutralizing IL-27 antibody reversed the inhibitory effect of IFN- β on IL-23 production (78). In addition, supernatants from human monocyte-derived DC treated with IFN- β , zymosan and neutralizing anti-IL-27 antibody lost the ability to reduce IL-17 production by allogeneic T cells, indicating that the inhibitory effect of IFN- β on Th17 differentiation is also mediated by IL-27 (78). Furthermore, monocyte derived DC from MS patients who respond to IFN- β treatment produced significantly higher IL-27 levels in response to IFN- β stimulation than DC derived from non-responding MS patients (78).

Decreased IL-27 levels have been reported in numerous autoimmune diseases. Significantly less IL-27 is found in the sera of patients with relapsing-remitting MS compared to healthy controls (79). Skin samples taken from moderate to severe psoriatic lesions expressed significantly less IL-27 and IL-27R compared to healthy controls (80). A systemic lupus erythematosus (SLE) study found that SLE patients had significantly lower levels of IL-27 in their sera compared to healthy controls, regardless of whether patients were in flare or remission (81, 82).

Furthermore, Genome Wide Association Studies have identified polymorphisms in *p28* associated with susceptibility to inflammatory bowel disease (IBD) (83, 84) and rheumatoid arthritis (85). Polymorphisms in *p28* have also been identified as potential

risk factors for development of type 1 diabetes (86). Additionally, depending on the haplotype, IL-27 polymorphisms were associated with either increased or decreased risk of SLE (87), chronic obstructive pulmonary disease (88) and ulcerative colitis(83, 84, 89). With the exception of a SNP identified by Imielinsky et al. that was associated with decreased production of IL-27 and increased susceptibility to IBD (84), further studies are needed to address whether polymorphisms affect IL-27 production and/or function.

Discovery of Tr1 Cells

The first indication of the existence of Tr1 cells came from studies of T cell reconstitution in patients with severe combined immunodeficiency (SCID) who received either HLA-mismatched fetal liver and thymus transplants (FLTT) or HLA-mismatched hematopoietic stem cell transplants (HSCT) (90, 91). Despite receiving HLA-mismatched transplants, the patients did not develop graft versus host disease (GVHD), even though CD8 T cells specific for recipient HLA type were detected (90, 91). Protection from GVHD was attributed to the presence of IL-10 producing host reactive CD4 T cells which inhibited proliferation and IL-2 production of host-reactive T cells (91, 92).

IL-10 producing T cells were further characterized and identified as a new type of Foxp3⁻ regulatory T cell called Tr1 in a model of colitis (93). The authors found that repeated stimulation of OVA-specific T cells in the presence of IL-10 resulted in IL-10^{high}IL-5^{high}IL-2^{low}IL-4^{low} T cells with low proliferation potential in response to stimulation with OVA. Although these cells produced IFN γ and TGF- β similar to Th0 and Th1 cells, they differed from Th1 effectors in terms of high levels of IL-10 and IL-5 (93). These Tr1 cells inhibited the proliferation of effector T cells *in vitro* through

secretion of IL-10 and TGF- β . Furthermore, transfer of Tr1 cells in a T cell transfer-induced model of colitis ameliorated disease symptoms, demonstrating the regulatory function of Tr1 cells *in vivo* (93). Following the identification of Tr1 cells as a separate CD4 T cell subset, other studies elucidated the differentiation process, phenotypic characteristics, and regulatory functions of Tr1 cells, including the importance of IL-27 in Tr1 differentiation.

Phenotypic Characteristics of Tr1 Cells

Tr1 Markers

Prior to the identification of Tr1 markers, many researchers defined Tr1 cells by a lack of Foxp3 expression and by the ratio of IFN γ /IL-10 production (94, 95). This made identification of Tr1 cells difficult and subject to variability. However, a recent study discovered that Tr1 cells could be identified by coexpression of CD49b and LAG-3 (96). CD49b⁺LAG-3⁺ CD4 T cells express significantly more IL-10 than single positive T cells (CD49b⁻LAG-3⁺ or CD49b⁺LAG-3⁻). Similarly, CD49b⁺LAG-3⁺ cells had greater suppressive function than single positive counterparts (96). Furthermore, the suppressive function of CD49b⁺LAG-3⁺ cells was confirmed *in vivo* in a transfer model of colitis. CD49b⁺LAG-3⁺ cells significantly reduced disease symptoms, while single positive cells had no effect on disease score or weight (96). Since the publication of these findings, many researchers have adopted CD49b and LAG-3 coexpression as a key identifier of Tr1 cells (97-101).

Transcription Factors

Unlike Treg cells, which can be defined exclusively by Foxp3 expression, Tr1 cells do not express a master transcription factor unique to Tr1 cells. However, Tr1 cells have been shown to express c-Maf, Ahr, Blimp-1 and Egr-2 (53, 65, 102).

Tr1 cells differentiated *in vitro* in the presence of IL-27 expressed both *AhR* and *Maf*, genes encoding the transcription factors AhR and c-Maf (53, 65). Additionally, IL-27 treated cells deficient in either c-Maf or AhR produced significantly less IL-10 than WT cells (53) and overexpression of c-Maf lead to an increase in expression of IL-10 by Tr1 cells (53). These results determined that c-Maf in particular is important for IL-27-induced Tr1 cell differentiation and IL-10 production in response to IL-27 (53, 65).

Expression of IL-10 by Tr1 cells has been shown to also involve Blimp-1 signaling (102). Blimp-1 deficient cells failed to increase expression of *Il10* in response to IL-27 (102). Further analysis identified Egr-2 as a transcription factor for Blimp-1, and determined that IL-27 signals through STAT3 to activate Egr-2, which then leads to production of Blimp-1 and Blimp-1 mediated IL-10 production in Tr1 cells (102, 103).

Although Tr1 cells do not express Foxp3, GATA-3 or ROR γ t, they do express low levels of Tbet (54). While absence of Tbet does not affect IL-10 production, indicating Tbet is not a key transcription factor for Tr1 differentiation (54), it is possible that Tbet may function as an inhibitor of ROR γ t activation during Tr1 differentiation (62, 104).

Despite the involvement of transcription factors c-Maf, AhR and Blimp-1 in the differentiation of Tr1 cells, mere presence of either of those transcription factors does not characterize a CD4 T cell as a Tr1 cell. c-Maf is also expressed by Th2 cells (105), while

AhR can be found in Th17 (106) and Blimp-1 in Th1 cells (103), hence the importance of using Tr1 markers in addition to transcription factor analysis in order to properly identify Tr1 cells.

Cytokine Profile

The earliest Tr1 studies showed that Tr1 cells express IL-10 in addition to IFN γ , IL-5 and TGF- β (93), while more recent studies have focused on expression of IL-10 and IFN γ (94, 95). Tr1 cells produce little or no IL-2 or IL-4 (93). Subsequent experiments identified the importance of IL-21 for driving differentiation of Tr1 cells (53, 65) and of IL-10 for maintaining Tr1 cell longevity *in vivo* (107).

IL-27 upregulated both c-Maf and AhR, leading to production of IL-10 and IL-21, the latter of which induces expression of more *Maf*, subsequently leading to increased production of IL-10, thus driving differentiation of Tr1 cells (Fig. 6) (53, 65). IL-21 deficiency resulted in significantly reduced production of IFN γ , IL-10 and c-Maf (65).

In addition to the role of IL-21 in promoting the Tr1 phenotype, IL-10 has also been shown to act on maintaining Tr1 cells *in vivo* (107). Tr1 cells differentiated *in vivo* expressed similar levels of *Maf*, *Ahr*, and *Prdm1* regardless of whether they were derived from WT or IL-10R α -deficient mice (107). However, five weeks after injecting Tr1 cells into *Rag1*^{-/-} mice, IL-10R α -deficient Tr1 cells produced significantly less IL-10 than WT Tr1 cells (107). These experiments showed the importance of IL-10 signaling in Tr1 cells, both for function and longevity (107).

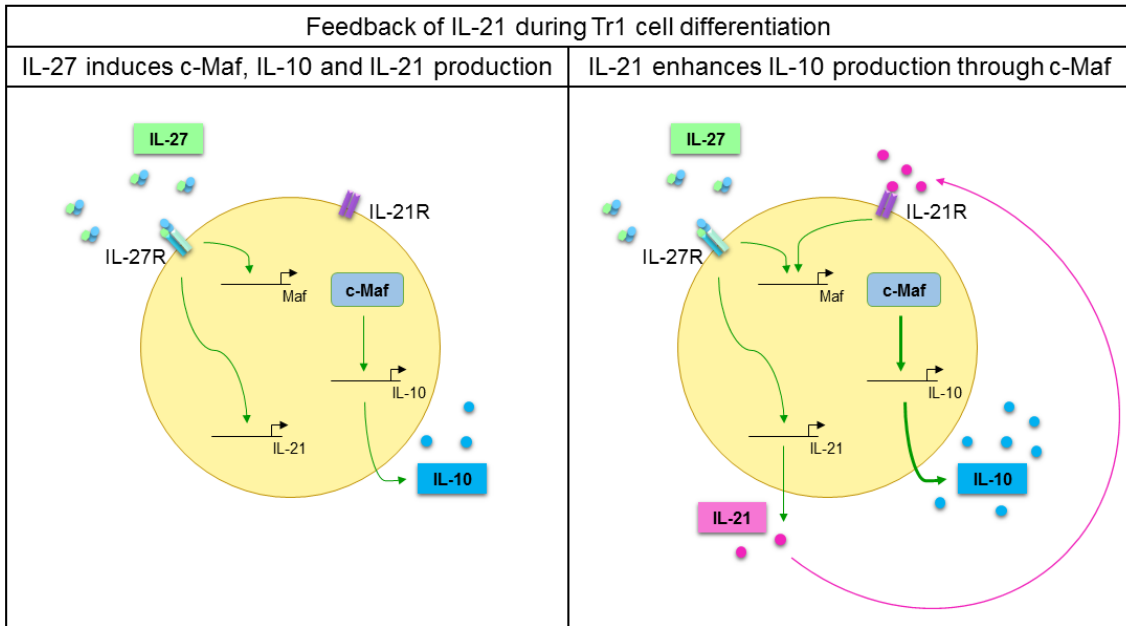


Figure 6. IL-27 induces production of IL-21 which feeds back on the Tr1 cell to increase production of IL-10 through c-Maf. During initial stages of IL-27-induced Tr1 cell differentiation (Left panel), IL-27 signaling initiates transcription of *Maf*, *Il21* and *Il10*. Feedback of IL-21 (Right panel) then increases c-Maf production and activity, resulting in increased production of IL-10.

Tr1 Differentiation In Vitro

Differentiation of Tr1 cells *in vitro* has been observed in multiple experimental scenarios, including co-cultures of naïve CD4 T cells with IL-10-producing APCs (108), and stimulation of naïve CD4 T cells with anti-TCR antibodies anti-CD3/anti-CD28 in the presence of various anti-inflammatory molecules (53, 65, 109).

Stimulation of naïve CD4 T cells in the presence of immunosuppressive drugs dexamethasone and bioactive vitamin D3 leads to differentiation of Tr1 cells which produce IL-10 and suppress effector cells (109). Alternatively, overexpression of IL-10 in

human CD4 T cells resulted in a Tr1-like phenotype, characterized by IL-10 production, absence of Foxp3, expression of ICOS, and suppression of effector T cells through IL-10 and TGF- β (110). Engagement of the complement regulator CD46 on CD4 T cells in the presence of IL-2 and anti-CD3 was shown to result in IL-10 producing, suppressive Tr1 cells (111, 112).

However, the most impressive finding has been the role of IL-27 in Tr1 cell differentiation. IL-27-induced differentiation of Tr1 cells was identified through a series of co-culture and purified T cell experiments. Priming DC with TGF- β^+ Tregs promoted expression of IL-27, IL-10 and TGF- β , which lead to DC inducing differentiation of Tr1 cells in co-culture experiments (113). Stimulation of whole splenocyte cultures with anti-CD3/anti-CD28 in the presence of IL-27 resulted in CD4⁺ T cells that expressed IL-10, IFN- γ and Tbet (54). Moreover, stimulation of purified naïve CD4 T cells with anti-CD3/anti-CD28 in the presence of IL-27 lead to differentiation of IL-10⁺ Tr1 cells (65, 113).

IL-27 signaling induced c-Maf and AhR, leading to production of IL-10 and IL-21 and differentiation of Tr1 cells (53, 65). Similarly, STAT1 and STAT3 were shown to be an integral part of the signaling pathway leading to IL-27-induced IL-10 production CD4 T cells (114), while neither STAT4, STAT5, nor STAT6 were involved in Tr1 differentiation (66, 94). Moreover, IL-27 signals through STAT3 to activate Egr-2, leading to Blimp-1 production and Blimp-1 mediated IL-10 production (102, 103). Although STAT3 has been shown to be important in the induction of c-Maf by IL-6 (115), a direct link between STAT1 or STAT3 and c-Maf activation in IL-27-induced Tr1 cells has yet to be defined.

Tr1 Differentiation In Vivo

In addition to the induction of Tr1 cells during immune responses to infections and during autoimmune diseases, treatment of mice with anti-CD3 has been shown to promote Tr1 cell differentiation *in vivo*. In mice, administration of anti-CD3 through the nasal passages resulted in increased numbers of IL-10-producing Tr1 cells in the cervical lymph nodes, which is dependent on IL-27 and TGF- β production by surrounding DC (116). Alternatively, intraperitoneal injections with anti-CD3 every three days for a total of four rounds increased the percentages of IL-10 producing CD4 T cells in both Peyer's patches and lamina propria (94). Finally, injecting anti-CD3 twice over the course of two days led to increases in CD49b+Lag-3+ CD4 T cells (117, 118). Tr1 cells induced using the latter protocol inhibited disease progression in a T cell transfer model of colitis (117, 118).

In addition, Tr1 cells also develop in response to infection in order to control the immune response. Tr1 cells were isolated from the lungs of mice infected with *Bordetella pertussis* (119). Infection with parasites *Plasmodium chabaudi* and *Leishmania donovani* also led to differentiation of Tr1 cells (120), and Tr1 cells have also been observed in some cases of chronic viral infection, including hepatitis C (121) and Epstein-Barr virus (122). As indicated in infectious disease models using *IL-27R α ^{-/-}* mice, induction of Tr1 cells is important to prevent immune responses from going unchecked and increasing morbidity (35-38).

In addition to the direct differentiation of Tr1 cells from naïve CD4 T cells, it has been suggested that Tr1 can develop from Th17 cells through transdifferentiation *in vivo* (118). Using a fate mapping model which tracked cells that expressed IL-17A at one time

but have since stopped, Gagliani et al. discovered that Th17 cells that no longer express IL-17A resemble Tr1 cells in both IL-10 expression and suppressive activity (118). Transdifferentiation of Th17 cells into Tr1 cells was observed during both EAE and *N. brasiliensis* infection (118). During initial differentiation of Tr1 cells, it has been suggested that IL-27 signals through STAT1 to activate Tbet, which inhibits ROR γ t activation (62, 104). Similarly, during transdifferentiation of Tr1 cells, TGF- β signals through SMAD3 to inhibit ROR γ t activation (118). Moreover, expression of IL-10 by Th17 treated by TGF- β was dependent on AhR (118).

Function

Tr1 cells have inhibitory effects on many cell types. Tr1 cells suppress effector T cell function through production of IL-10 and TGF- β (93) and CD39 (39). Additionally, Tr1 cells have also been shown to induce lysis of APC through granzyme B and perforin production (123, 124). Furthermore, Tr1 cells have been shown to inhibit NLRP3 inflammasome activation in macrophages through IL-10 (99).

The function of IL-27-induced Tr1 cells has been confirmed *in vivo* using a number of models of autoimmunity and transplantation. Transfer of IL-27-derived IL-10-producing Tr1 cells during EAE reduced incidence (54). Similarly, transfer of Tr1 cells inhibited disease development in a T cell transfer model of colitis (93, 96). Moreover, Tr1 cells have been shown to maintain graft tolerance (125-128). Treatment with IL-10 and rapamycin resulted in Tr1 differentiation in mice receiving pancreatic islet cell transplants, resulting in establishment of tolerance (127). Furthermore, Foxp3⁺ Treg

established tolerance in the proximity of the graft, while Tr1 cells maintained long-term tolerance originated in the spleen (128).

Human Tr1 cells

Phenotype

Human Tr1 cells display a phenotype similar to murine Tr1 cells. Human Tr1 cells express IL-10, IFN γ , TGF- β , and IL-5 (93). Like murine Tr1 cells, human Tr1 cells produce little to no IL-2 and IL-4 (93). Moreover, IL-27 treatment activates c-Maf and AhR mediated IL-10 production (129). Finally, human Tr1 cells can also be identified by coexpression of CD49b and LAG-3 (96).

Function

In addition to maintaining tolerance in SCID patients, Tr1 cells have also been shown to be involved in establishing tolerance in patients who received HSCT to treat beta-thalassemia major, a genetic disorder resulting in deficient production of the beta globin chain of hemoglobin, consequential anemia and enlargement of the hematopoietic system leading to possible hepatomegaly and splenomegaly (100, 130, 131).

The therapeutic potential of Tr1 cells for human transplant tolerance are being investigated in pancreatic islet cell (132), kidney (133), and liver transplantation (121, 134). Moreover, Tr1 cells are involved in establishing tolerance in allergic individuals (135-137), as demonstrated in immunotherapy studies performed to induce regulatory T cells which recognize birch pollen (138), bee venom antigen (136), nickel (139), and gliadin (140). These studies show the therapeutic potential of using Tr1 cells to combat

overactive immune responses, whether it be to autoantigens, alloantigens or allergens. However, the anti-inflammatory properties of Tr1 cells have also been implicated in tumor cell survival.

The possibility of Tr1 cells inhibiting anti-tumor immunity has been observed in many different cancers, including head and neck cancer (141), Hodgkin's lymphoma (142, 143), hepatocellular carcinoma (101), and colorectal cancer (144). Tr1 cells derived from tumor infiltrating lymphocytes collected from head and neck squamous cell carcinoma patients had greater suppressive activity on effector CD4 T cells than those derived from PBMC of healthy controls (141). A study on 24 Hodgkin's Lymphoma patients revealed that Hodgkin's Lymphoma infiltrating lymphocytes (HLIL) are composed of approximately 33% Tr1 cells (defined in this study as CD4⁺IL-10⁺ cells), versus 15% in Hodgkin's lymphoma patients' PBMC and 9% healthy control PBMC. Suppression assays revealed that HLIL, containing both Tr1 and Treg, were able to suppress proliferation of autologous PMBC through IL-10 and CTLA-4 (142). Additionally, a study of 108 colorectal cancer patients found that not only were Tr1 cells more prevalent in tumor tissue than surrounding healthy tissue, but the number of Tr1 cells present in tumor tissue correlated with increased disease, i.e. metastasis and stage of cancer (145). Finally, Tr1 cells have been identified in hepatocellular carcinoma tumor-infiltrating lymphocyte populations and much like Tr1 function in other cancers, the suppressive activity of Tr1 cells in liver cancer was mediated through IL-10 (101).

Considering both the advantages to having high Tr1 populations in autoimmune diseases or allergy, and the disadvantages of the presence of Tr1 cells during cancer treatments, the importance of determining the mechanisms by which Tr1 cells

differentiate, including Tr1 differentiation *in vitro*, and identifying any possible negative regulators of Tr1 cells became evident. There have been many studies detailing the differentiation process of Tr1 cells. However, not many have detailed inhibitors of Tr1 cell differentiation or function.

Inhibitors of Tr1 cells

To date, only a few inhibitors of Tr1 differentiation and function have been described. The costimulatory molecule OX40 (CD134), which is expressed by activated T cells and promotes survival of effector cells (146), was shown to inhibit differentiation of Tr1 cells induced by dexamethasone/vitamin D3 (109). In addition, ligation of OX40 after Tr1 differentiation decreased IL-10 expression in Tr1 cells, suggesting that OX40 inhibits both the differentiation and function of Tr1 cells (109). In another study, differentiation of Tr1 cells in response to IL-27 treatment was greater in the absence of metallothioneins (MT). MT-deficient Tr1 cells also expressed higher levels of both IL-10 and IL-21, possibly due to increased activity of STAT1 and STAT3 in response to IL-27, indicating that MT negatively regulate both Tr1 cell differentiation and function (94). Although a couple of negative regulators of Tr1 differentiation have been identified, little is known about the pathways and transcription factors that may be responsible for inhibition of Tr1 differentiation and function. We became intrigued in the possibility that PGE2, a lipid mediator previously described to act as a proinflammatory agent affecting CD4 T cell differentiation, might act as a direct or indirect negative regulator of Tr1 differentiation or function.

Prostaglandin E2

Prostaglandin E2 (PGE2) is a bioactive lipid derived from arachidonic acid in an enzymatic cascade utilizing COX1/2 and PGE synthase (147). PGE2 is composed of a 20 carbon fatty acid chain with a five carbon ring (148-150). Prostaglandins derived from arachidonic acid (i.e. PGE2, PGD2, PGF2) differ slightly in composition of the cyclopentane ring and side chains (148, 150). Prostaglandin structure determines their activity, with PGE2 having bioactive effects throughout the body. In addition, PGE2 is the most abundant of all prostaglandins and is released at high concentrations during inflammation (147, 151, 152).

PGE2 production

PGE2 is expressed in response to pro-inflammatory stimuli by virtually all cell types including APC, endothelial cells, and fibroblasts (147, 153). Activation of the arachidonic acid (AA) cascade triggers a series of multiple enzymes, ultimately resulting in production of leukotrienes, lipoxins, thromboxanes, prostacyclins and prostaglandins (147, 154). Phospholipase releases AA from the phospholipid bilayer, followed by oxidation into PGG2 and reduction of PGG2 into PGH2 by COX1/2 enzymes. PGH2 is converted into PGE2 by microsomal prostaglandin E synthase (mPGES-1) (Fig. 7) (147, 148).

The key enzymes responsible for the production of PGE2 are the constitutively expressed COX-1, the inducible COX-2, and mPGES-1 (152). Selective effects of PGE2 can be studied by targeting specific steps of the AA cascade, i.e. COX-2 or mPGES-1

deficiency, or by targeting PGE2 receptors via antagonists or genetic deletion (Fig. 7) (152, 155).

Although PGE2 can be produced by all cells in the body, the major producers are the immune cells during inflammatory conditions (147). Local concentrations of PGE2 *in vivo* have been reported to be as high as 10^{-6} - 10^{-7} M at the inflammatory site (156-158).

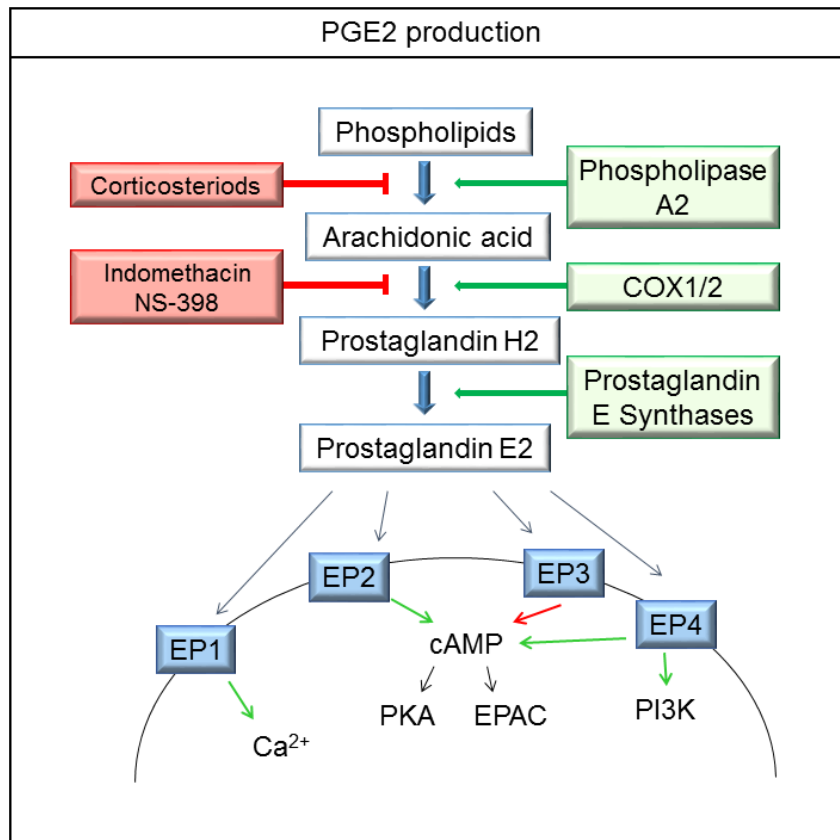


Figure 7. Production of PGE2. PGE2 is produced following a series of enzymatic reactions during the arachidonic acid cascade. Synthesis requires phospholipase A2, constitutive COX-1 or inducible COX-2, and prostaglandin E synthase (mPGES-1). Targeting specific segments of the pathway inhibits production of PGE2 (not shown are mPGES-1 inhibitors which are currently not commercially available). PGE2 is recognized by a series of EP receptors that signal through various intracellular pathways.

PGE2 receptors

PGE2 acts on various cell types through the plasma membrane-bound G-protein coupled receptor subtypes EP1-4. The inhibitory constant values (K_i expressed in nM) of PGE2 for EP1, EP2, EP3 and EP4 are 9.1, 4.9, 0.33, and 0.79 respectively for human receptors (159), and 20, 12, 1, and 2 respectively for mouse receptors (160, 161), indicating PGE2 has the highest affinity for EP3, followed by EP4, EP2 then EP1 in both mouse and human systems (147, 159-161). Downstream signaling varies depending on the receptors involved (162). EP1 signals through PLC and leads primarily to calcium influx (147, 163). Both EP2 and EP4 signal through G_s generating cAMP, with PKA and the exchange protein activated by cAMP (EPAC) as primary targets (147, 164). In addition, EP4 can signal through PI3K and other downstream signaling molecules such as ERK1/2 and Akt (147, 164). Signaling through EP3 is primarily inhibitory through G_i mediated inhibition of adenylate cyclase (165). However, unlike other EP receptors, EP3 can also activate adenylate cyclase through G_s (165). Activation of different signaling pathways by each of the EP receptor enables PGE2 to have differential effects on various cell types depending on the receptor subtype(s).

APC and T cells primarily express EP2 and EP4 receptors (147). Targeting each specific EP receptor, either with agonists, antagonists, or by EP receptor specific deletion, elucidated the role of EP2 and EP4 in immune cells. While PGE2 exerts functions on APC through EP2 and EP4, many of the pro-inflammatory effects of PGE2 on T cells are mediated by EP4 (166-169).

Biological activity of PGE2

PGE2 has been implicated in a number of biological processes including wound repair (148), angiogenesis (170), mucosal homeostasis (148, 171, 172), female reproduction (173) and cancer (148). While PGE2 is expressed by many cells throughout the body, most studies focused on the effects of PGE2 during the immune response.

The proinflammatory role of PGE2 has been confirmed *in vivo* in various inflammatory/autoimmune models where EP4 was identified as the relevant receptor. In a model of contact hypersensitivity, administration of EP4 antagonists reduced disease severity in association with reduced accumulation of antigen-specific Th1 and Th17 cells in the draining lymph nodes (166). In IBD models, disease exacerbation associated with colonic IL-23 upregulation and increases in Th17 was reported in TNBS-colitis following misoprostol administration (169), and endogenous PGE2→EP4 signaling associated with Th1 differentiation was shown to be involved in a transfer colitis model using conditional EP4-deficient T cells (174). In rheumatoid arthritis models, misoprostol was reported to exacerbate CIA in association with increases in IL-23 and Th17, and PGES-1^{-/-} and EP4^{-/-} mice exhibited significant attenuation of CIA clinical symptoms (175-177). Similar effects were reported in EAE models where disease suppression in EP4^{-/-} mice was associated with decreases in Th1 and Th17 cells, and in WT mice treated with EP4 antagonists at the time of immunization (167). In models of enteric and pulmonary bacterial infections, PGE2 inhibition was shown to be detrimental during early phase resulting in increased bacterial loads but beneficial during late/chronic phase enhancing host survival (178-180). These results are in agreement with a proinflammatory role of PGE2 supporting the immune response to control bacterial

growth during early phase, but also increasing the deleterious effects of sustained/chronic inflammation.

PGE2 effects on specific immune cells

The molecular mechanisms by which PGE2 affects immune responses have been dissected *in vitro* using various immune cell populations. A number of studies reported inhibitory or stimulatory effects of PGE2 on activation of macrophages, NK cells, B cells, and CD8 T lymphocytes (147, 181-188). The sometimes controversial results are probably due to the variety of receptors and the functional plasticity of many of these cell types.

A much larger number of studies have focused on PGE2 effects on the differentiation and function of DC and of the various CD4 T cell subsets.

Dendritic cells

The effects of PGE2 on cDC depends on the maturation stage. Exposure to PGE2 during differentiation of bone marrow-derived cDC accelerated maturation and promoted a pro-inflammatory phenotype, with increased production (upon TLR signaling) of the pro-inflammatory cytokines IL-23, IL-1 β and IL-6 (189-191). In contrast, the effects of PGE2 on mature cDC were more diverse, with sustained upregulation of IL-23, but inhibition of IL-12, suggesting differential downstream effects on Th1/Th17 differentiation (189-191) (192-194). In addition, PGE2 protected mature cDC from apoptosis induced by growth factor withdrawal, and promoted cDC migration to the draining lymph nodes primarily through dissolution of podosomes and upregulation of

MMP-9 (195). These events suggest a proinflammatory role for PGE2 through enhanced cDC viability and increased capacity to activate CD4 T cells by promoting the migration from the inflammatory site to secondary lymphoid organs.

Effector CD4 T cells

In addition to the indirect effects mediated through cDC, PGE2 has been shown to also directly affect T cell differentiation and cytokine production. Although initial *in vitro* experiments indicated that PGE2 reduced Th1 differentiation in DC/CD4 T cell co-cultures through inhibition of IL-12 production (192), *in vivo* studies reported a stimulatory effect on Th1 differentiation which was confirmed *in vitro* in purified CD4T cell cultures (166). PGE2 was shown to upregulate the expression of both IL-12R β 2 and IFN γ R1 during differentiation of naïve CD4 T cells in Th1 polarizing conditions which included exogenous IL-12, resulting in increased numbers of Th1 effectors (174). Furthermore, administration of an EP4 agonist reduced production of IFN γ by cells collected from lymph nodes of EAE mice, suggesting that endogenous PGE2 promoted *in vivo* Th1 differentiation (166).

In addition to promoting Th17 differentiation through upregulation of IL-23 production by cDC (168, 169, 175, 191), PGE2 also affects directly Th17 cell differentiation and function. During Th17 differentiation, treatment of purified CD4 T cells cultures with PGE2 led to increased expression of IL-23R and IL-1R, as well as ROR γ t (196, 197). Receptor knockout and antagonist studies determined these effects to be mediated primarily through EP4 (166, 167).

The pro-inflammatory role of PGE2 in association with increased Th1/Th17 differentiation and function has been confirmed in multiple *in vivo* models, i.e. CIA (175), IBD (169), kidney fibrosis (198), EAE, rheumatoid arthritis and contact hypersensitivity (199-202). Administration of EP agonists increased severity of disease in CIA (175), IBD (169) and kidney fibrosis (198), while receptor antagonists alleviated disease symptoms of EAE (167), and CIA (203). Receptor knockout studies and administration of selective EP4 receptor antagonists during the immunization phase in EAE demonstrated the role of EP2 and EP4 in promoting Th1 and Th17 cell differentiation. However, contrary to expectations, administration of an EP4 agonist at disease onset reduced symptoms instead of exacerbating disease. This was apparently due to the EP4-mediated protective effect on the blood-brain barrier, and suggested that endogenous PGE2 might have both a detrimental and protective effect in EAE/MS through actions on CD4 T cell differentiation versus maintenance of endothelial cell barrier function (167).

Regulatory CD4 T cells

In contrast to the effects on the differentiation of CD4 T effector subsets, the PGE2 effects on the generation and/or function of regulatory T cells are much less studied, with reports of both increases and decreases in Foxp3 expression and Treg function (204, 205). In addition, the existing reports suggest different effects in tumor compared to non-tumor microenvironments. Tumor-derived COX-2/PGE2 has been reported to promote Foxp3⁺Treg and Foxp3⁻Tr1 cells in cancer (206-210). In contrast,

PGE2 was reported to inhibit the differentiation of non-tumor murine Foxp3⁺Treg and of CD46-induced human Tr1 cells (205, 211).

To our knowledge, there are no reports at the present time on the role of PGE2 in IL-27 induced Tr1 differentiation. The first part of this thesis addresses the effect of PGE2 on IL-27 production and secretion from activated cDC. The second part addresses the direct effect of PGE2 on Tr1 differentiation and function. Taken together, the suppression of IL-27 production by cDC, and the direct inhibitory effect on Tr1 cell differentiation, highlight a novel proinflammatory mechanism for PGE2 which involves regulatory T cells, in addition to the previously described stimulatory effects on the differentiation of effector Th1/Th17 cells.

Project Aims

The IL-12 cytokine family, i.e. IL-12, IL-23, IL-27 and IL-35, have prominent and sometimes opposite effects on the immune response, partially mediated through effects of CD4 T cell differentiation. In contrast to IL-12 and IL-23 which play essential roles in the differentiation of pro-inflammatory Th1 and Th17 effector subsets, IL-27 and IL-35 are mostly anti-inflammatory. IL-27, an APC product, has been identified as a major inducer of the type 1 regulatory Tr1 cells. Previous studies indicated that PGE2 affects both IL-12 and IL-23 expression and production in cDC and macrophages, and supports in vivo differentiation of Th1 and Th17 cells in models of inflammatory and autoimmune diseases. Presently, there is little, if any, information on the effect of PGE2 on IL-27 production and on Tr1 differentiation and function. The aims of this thesis project consisted in studying the effects of PGE2 on IL-27 production in APC, with a

focus on conventional dendritic cells (cDC), and of the differentiation and function of Tr1 cells.

Aim 1. Effects of PGE2 on IL-27 production in APCs.

- a. Inhibition of IL-27 secretion by cDC and macrophages. Effects of various concentrations of PGE2 on IL-27 production of bone marrow-derived DC and macrophages, as well as splenic cDC stimulated through various TLR types.
- b. IL-27 is a heterodimer composed of the inducible p28 subunit and the constitutively expressed EBI3 subunit. Does PGE2 affect transcription of *p28* and/or *Ebi3*?
- c. Compare the effect on IL-27p28 with effects on MHCII, costimulatory molecules, IL-12 subunits, IL-23p19 and IL-10 in cDC.
- d. Signaling pathways involved in the PGE2 effects on IL-27 production. Analysis of PGE2 receptors, cAMP induction, EPAC, PKA, PI3K, and MAPK activation.
- e. Previous studies identified the involvement of NF- κ B (c-Rel), IRF1, and IRF3 in the expression of the *p28* gene. Investigate the effects of PGE2 on transcriptional factors involved in *p28* expression through microarrays, qRT-PCR and ChIP assays. Focus primarily on ISRE site binding factors: IRF1 and IRF3. Are the effects on *Irf1* mediated through STAT1 and/or STAT2?
- f. Are the PGE2 inhibitory effects on IL-27 mediated through induction of IFN- β ?
- g. In vivo effects of PGE2 on *p28* expression in splenic cDC.

Aim 2. Effects of PGE2 on Tr1 differentiation and IL-10 production

- a. Does PGE2 affect the differentiation of CD4⁺CD49b⁺LAG3⁺ Tr1 cells in splenocytes stimulated with LPS and anti-CD3 antibodies? Is the inhibitory effect solely dependent on the reduction in endogenous IL-27?
- b. Does PGE2 affect Tr1 differentiation in purified naïve CD4 T cells differentiated in Tr1 polarizing conditions (anti-CD3, anti-CD28, exogenous IL-27)? Does it affect Tr1 function (percentage of IL-10 producing Tr1 cells), proliferation, or does it promote transdifferentiation of Tr1 into Th17 cells (IL-17 producing cells)?
- c. Role of EP2/EP4 receptors and cAMP in the PGE2 inhibition of Tr1 differentiation.
- d. Effects of PGE2 on the major transcriptional factors involved in Tr1 differentiation and IL-10 production: c-Maf, AhR, Egr-2 and Blimp1.
- e. The sustained Tr1 phenotype and IL-10 production depends on the autocrine and paracrine effect of IL-27-induced IL-21. Is PGE2 inhibition of IL-27-induced Tr1 differentiation mediated through its effects on IL-21?
- f. During Tr1 differentiation, IL-27/IL-21 signal through STAT1 and STAT3. Is the inhibitory effect of PGE2 dependent on reduction in STAT1 or STAT3 activation?
- g. Tr1 cells can be induced *in vivo* in a model of anti-CD3 induced transient intestinal inflammation. By using this model, does PGE2 administration affect the *in vivo* Tr1 induction in Peyer's patches, spleen and small intestine intraepithelial lymphocytes?

These studies were designed to determine whether PGE2 has a suppressive effect on IL-27 production by cDC and on Tr1 differentiation and IL-10 production. We concluded that indeed PGE2 negatively affected both IL-27 production and Tr1 differentiation *in vitro* and *in vivo*, and that these effects were mediated through specific receptors and cAMP induction. In cDC, PGE2 reduced IRF1 expression and subsequent binding to the p28 promoter, and in CD4 T cells differentiated in Tr1 polarizing conditions, PGE2 inhibited c-Maf expression, a transcription factor required for both Tr1 differentiation and IL-10 expression in Tr1 cells.

These studies describe novel pro-inflammatory mechanisms for PGE2 involving IL-27 production in APC and Tr1 differentiation, and identify possible new targets for the treatment of inflammatory diseases.

CHAPTER TWO METHODS

Mice

C57BL/6 (6-10 weeks old), B6.129S2-*Irf1*^{tm1Mak} (*Irf1*^{-/-}) and B6(Cg)-*Il10*^{tm1.1Karp} (*Il10*^{gfp}) 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 (GM-CSF), macrophage colony-stimulating factor (M-CSF) 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. Recombinant mouse IL-27p28 and neutralizing anti-mouse IL-27 antibodies were from R&D Systems. Recombinant mouse IL-23 was purchased from eBiosciences. Recombinant IL-4, IL-6, IL-12, IL-10 and TGF- β 1 were from Peprotech. Recombinant antibodies anti-CD3 and anti-CD28, were

purchased from BioLegend. Capture and biotinylated anti-mouse IL-27p28 antibodies, anti-mouse IL-21 antibodies and recombinant mouse IL-27p28, IL-17 and IL-21 were from R&D Systems. Capture and biotinylated anti-mouse IFN- β antibodies were from BioLegend. 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 blotting 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. Anti-mouse CD49b PE antibody and isotype control were from BioLegend. APC-conjugated anti-mouse LAG3, anti-mouse LAG3 PE-Cy7, anti-mouse CD4 PerCP-Cy5.5, Foxp3 PerCP-Cy5.5, Egr-2 APC, c-Maf eFluor660, FITC pSTAT3 and isotype control antibodies were purchased from eBioscience. FITC conjugated anti-mouse CD4 and AlexaFluor 647-conjugated anti-mouse Blimp-1 was from BD Biosciences.

DC and Macrophage Culture

Primary DCs were derived from bone marrow cells as previously described (195). Briefly, bone marrow was flushed from the femur and tibia of mice, passed through a 70 μ m cell strainer and centrifuged for 5 min at 1,100 rpm. Cells were suspended 10⁶/ml in complete RPMI (RPMI supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% antibiotics) in the presence of 20ng/ml GM-CSF and plated 10⁷ per

100mm tissue culture plate. On day 3, cultures were supplemented with 10ml complete RPMI containing GM-CSF per plate. Alternatively, macrophages were derived from bone marrow cells cultured in the presence of 10 ng/ml M-CSF. Bone marrow was prepared as described and cells were cultured in IMDM containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% antibiotics and M-CSF. Cultures were supplemented with fresh IMDM and M-CSF on day 3, and adherent cells were collected on day 7.

To collect DC from spleens, spleens were dissociated in HBSS with calcium and magnesium in the presence of 0.5 mg/ml liberase TL (Roche Diagnostics) and 1mg/ml DNase at 37C for 30 min. Tissue was then passed through a 70 μ M strainer and subjected to red blood cell lysis per manufacturer's instructions (eBioscience). Single cell suspensions were then magnetically sorted using CD11c MicroBeads per manufacturer's instructions (Miltenyi Biotec). Briefly, cells were incubated with anti-CD11c microbeads, washed, then passed through an LS column (Miltenyi) to purify CD11c⁺ cells. Purity was determined by FACS analysis (>93% CD11c⁺).

T Cell Culture

Naïve CD4⁺CD62L⁺ T cells were isolated from spleens of 6-8 week old mice using a T cell isolation MicroBead kit per manufacturer's instructions (Miltenyi Biotec). Briefly, spleens were mechanically dissociated and passed through a 70 μ M strainer. Cells then underwent negative selection to obtain CD4⁺ cells by removing any cells expressing CD8a, CD45R, CD11b, CD25, CD49b, TCR γ/δ and Ter-119. CD4⁺ cells then underwent positive selection to purify CD4⁺CD62L⁺ cells. Purified, naïve T cells were cultured *in vitro* in fresh RPMI supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine,

1% antibiotics and 50 μ M β ME. Cells were stimulated with plate-bound anti-CD3 (3 μ g/ml) and soluble anti-CD28 (1 μ g/ml) in the presence of recombinant IL-27 (50 ng/ml) for three days to derive Tr1 cells. Recombinant IL-2 (10 ng/ml) was added to all cultures on day 2.

qRT-PCR

Gene expression analysis of *Il-10*, *Il12a*(p35), *Il12b*(p40), *Il23a*(p19), *Ifnb*, *p28*, *Irf1*, *Maf*, *Ahr*, 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'-TGCTGGATTGC AGAGCAGTAA-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'-TAGCCCTGAACCTCAGAGAGCA-3'; *p28* (*in vivo* studies) (26) forward 5'-ATCTCGATTGCCAGGAGTGA-3' and antisense 5'-GTGGTAGCGAGGAAGCAGAGT-3'; *Irf1* sense 5'-CCCACAGAAGAGCATAGCAC-3' and antisense 5'-AGCAGTTCTTTGGGAATAGG-3'; *Ahr* sense 5'-CCACCCCTGCTGACAGAAAT-3' and antisense 5'-AGCCATTCAGCGCCTGTAAC-3', *Maf* sense 5'-AGCAGTTGGTGACCATGTTCG-3' and antisense 5'-

TGGAGATCTCCTGCTTGAGG-3', *β-actin* sense 5'-
AGCTTCTTTGCAGCTCCTTCGTTGC-3' and antisense 5'-
ACCAGCGCAGCGATATCG TCA-3'; *Gapdh* sense 5'-GGAGCGAGACCCCACTAA-
3' and antisense 5'-ACATACT CAGCACCGGCCTC-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 using TRI reagent (Sigma) per manufacturer's instructions and
reverse transcribed using the RT² First Strand kit (Qiagen). Expression levels of
transcription factors were determined using the RT² Profiler PCR Array Mouse
Transcription Factors (Qiagen).

Chromatin Immunoprecipitation Assay (ChIP)

BMDCs (1x10⁷) were stimulated for 3-4hr then fixed with 1% formaldehyde for
15 min. Cells were treated with 125 mM glycine for 5 min to stop fixation, washed twice
with PBS containing Protease Inhibitor Cocktail (Sigma) and phenylmethylsulfonyl
fluoride, lysed, sonicated using a sonic Dismembrator (Fisher Scientific) and processed
as previously described (212). 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'-AGCCATCTCCTGG
GTAGG-3'. Results were analyzed using the $\Delta\Delta$ CT method.

***In Vivo* DC Experiment**

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

***In Vivo* T Cell Isolation**

Il10^{gfp} mice were injected intraperitoneally with anti-CD3 (20 µg/mouse) or vehicle (PBS) in addition to either dmPGE2 (200µg/kg) or vehicle (0.4% DMSO in PBS) twice, 48 hours apart. Four hours later, mice were euthanized and Tr1 cell populations within the Peyer's patches, spleens and small intestines were analyzed. Briefly, Peyer's patches were removed from the small intestine, and tissue was dissociated in HBSS with calcium and magnesium in the presence of 0.5 mg/ml liberase TL (Roche Diagnostics) and 1mg/ml DNase at 37C for 30 min to obtain single cell suspensions. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from the small intestine according to Current Protocols in Immunology Unit 3.19 (213). Briefly, visible fat, connective tissue and mucous were mechanically removed from the small intestine. Intestines were then flushed with HBSS (calcium and magnesium free) containing 1x HEPES and 0.2% FBS. Intestines were then cut into small segments, washed multiple times and subjected dissociation in the presence of 0.5mg/ml liberase TL and 1mg/ml

DNase. Cells were subjected to Percoll gradients at different wash steps in order to isolate IEL and LPL. Samples were analyzed by FACS for Tr1 cell markers.

FACS Analysis

Cells were treated as indicated, collected, washed with FACS buffer (2mM EDTA, 0.5% BSA in PBS), then incubated at 4°C for 30 min with anti-mouse CD11c, CD80, CD86, CD40, MHCII or corresponding isotype controls. Cells were washed three times and collected using BD FACSCalibur. For analysis of Tr1 markers, cells were treated or isolated as indicated, washed twice in FACS buffer, incubated at 4°C for 30 min with anti-mouse CD4, CD49b, LAG-3, or corresponding isotype controls then washed three times. Cells were then either collected immediately using BD FACSCanto or underwent further staining for transcription factors. For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm per manufacturer's instructions, incubated at 4°C for 30 min with anti-mouse Foxp3, c-Maf, Blimp-1, Egr2 or isotype control. Alternatively, cells were fixed at room temperature for 60 min with eBioscience IC Fixation buffer then permeabilized on ice with 100% methanol for 30 min (eBioscience Protocol C). Cells were washed using eBioscience Flow Cytometry Staining Buffer then stained at 4°C for 30 min with anti-mouse pSTAT3 antibody. Cells were washed three times then collected using BD FACSCanto.

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. BMDC or BMDM were plated in 12-well culture plates ($1-2 \times 10^6/\text{ml}$) and stimulated for 24hr. Alternatively, splenocytes or T cells were plated in 24-well culture plates ($1-2 \times 10^6/\text{ml}$) and stimulated for 72hr. Detection limit was 5 pg/ml for IL-27, 15 pg/ml for IFN- β , 62.5 for IL-21, and 15.6 pg/ml for IL-17.

Western Blot Analysis

BMDCs ($2-3 \times 10^6$) were serum starved for 4hr prior to treatment with LPS and PGE2. Samples were lysed, denatured (95°C , 5 min) 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.

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.

CHAPTER THREE 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 $\mu\text{g/ml}$). 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 $\mu\text{g/ml}$ unless otherwise noted. The PGE2 concentrations were chosen in agreement with the concentrations reported *in vivo* in inflammatory conditions (10^{-6} - 10^{-7}M) (156-158). PGE2 reduced IL-27 production in a dose-dependent manner in both LPS- and LPS+IFN γ stimulated BMDCs (Fig. 8A). A similar inhibitory pattern was observed in BMDCs stimulated with peptidoglycan or poly I:C (Fig. 8B-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. 8D) and in BMDM stimulated with LPS, LPS+IFN γ , and poly I:C (Fig. 8E-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. 8G). These results support a role for endogenous PGE2 in the reduction of IL-27 production.

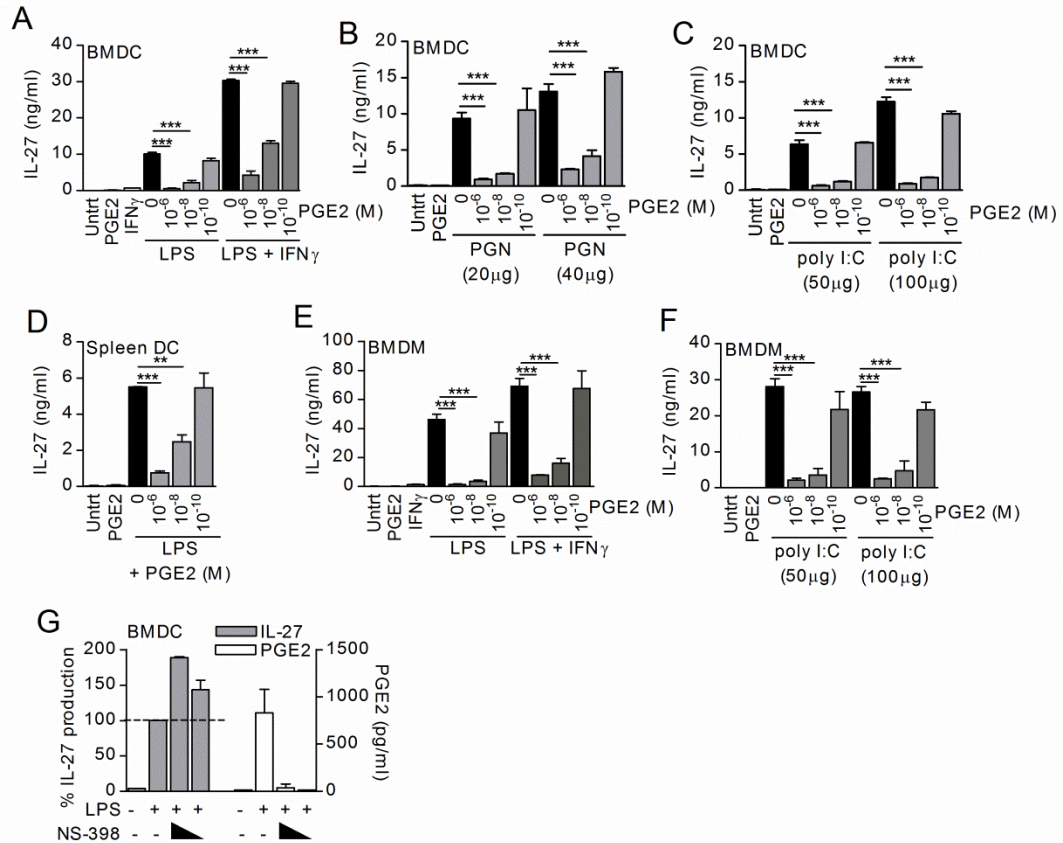
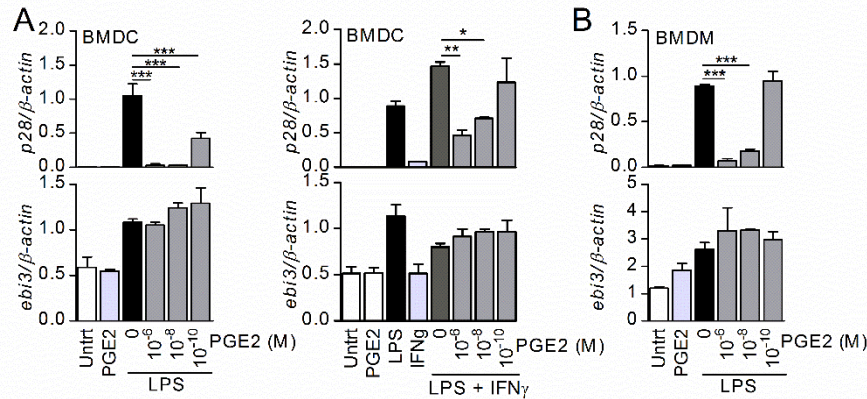


Figure 8. PGE2 inhibits IL-27 production in CD11c⁺ DC and macrophages. BMDC were stimulated with (A) LPS (TLR4; 100 ng/ml) or LPS+IFN γ (500 U/ml), (B) peptidoglycan (PGN, TLR2; 20 or 40 μ g/ml), or (C) poly I:C (TLR3; 50 or 100 μ g/ml) in the presence or absence of PGE2 for 24hr. One representative 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.

PGE2 reduced mRNA expression levels of *p28*, but not *ebi3*, in both LPS- and LPS+IFN γ -treated DCs (Fig. 9A) and in LPS-treated BMDM (Fig. 9B), indicating that the reduction in IL-27 is transcriptional through inhibition of *p28* expression.



**Figure 9. PGE2 inhibits IL-27

p28 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 *** $P < 0.001$.

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. 10A) 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. 10B).

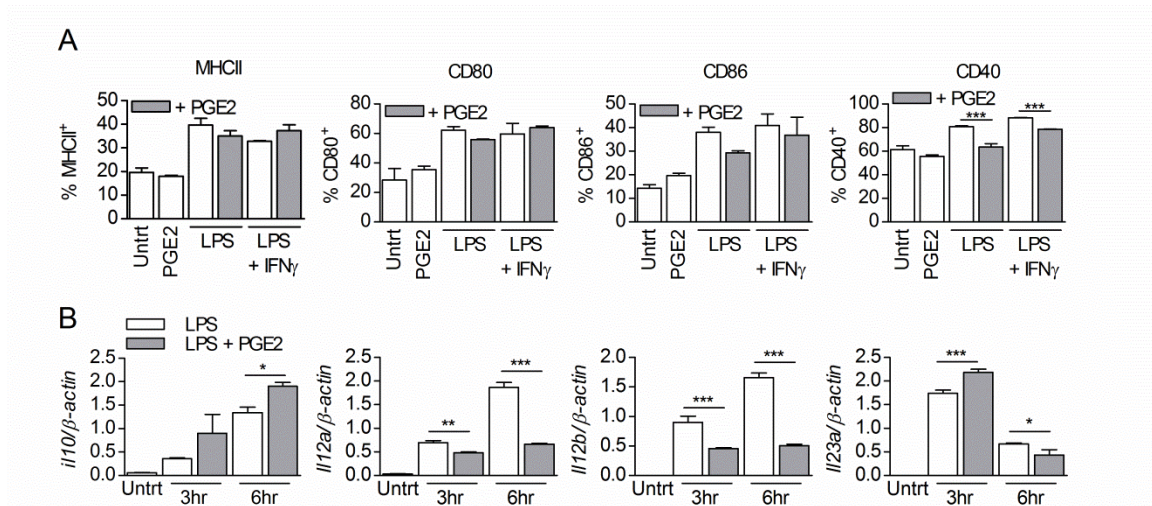


Figure 10. Effects of PGE2 on MHCII, costimulatory molecules and cytokine expression in activated DCs. (A) BMDCs were stimulated with LPS (1 μ g/ml), LPS+IFN γ (500U/ml) +/- PGE2 (10⁻⁶M) for 24hr. Surface expression of co-stimulatory molecules CD40, CD80, CD86 and MHCII was measured by FACS. Each sample was tested in triplicate and results represent means \pm SD. (B) BMDCs were stimulated with LPS (1 μ g/ml) in the presence or absence of PGE2 for 3hr or 6hr and analyzed for *il-10*, *Il12a*(p35), *Il12b*(p40) and *il23a*(p19) expression. One of two independent experiments is shown. Results represent means \pm SD. * P <0.05, ** P <0.01 and *** P <0.001.

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 (EP3; K_i values of 110nM for EP2 and greater than 3.3 μ M for EP1, EP3 and EP4), misoprostol (EP3, EP4>EP1; K_i values of 120nM for EP1, 250nM for EP2, 67nM for both EP3 and EP4), PGE₁OH (EP4>EP3; K_i values of

330nM for EP3, 190nM for EP4 and greater than 3.3 μ M for both EP1 and EP2) and sulprostone (EP3>EP1; K_i values of 21nM for EP1, 0.6nM for EP3, and greater than 3.3 μ M for both EP2 and EP4) (160, 161). Butaprost, misoprostol and PGE₁OH, but not sulprostone, inhibited IL-27 production (Fig. 11A), 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 min 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. 11B), 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 (214). 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. 11C). Furthermore, addition of the adenylate cyclase activator forskolin also inhibited IL-27 production (Fig. 11D). These results indicate that PGE₂ signals through cAMP to inhibit IL-27 production in LPS-stimulated DCs.

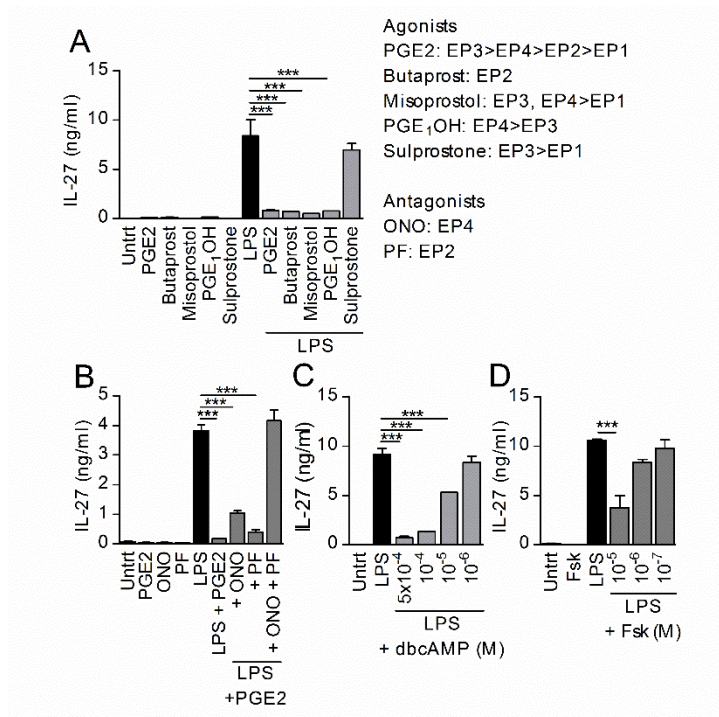


Figure 11. PGE2 signals through EP2/EP4 and cAMP to inhibit IL-27 production.

(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; *** $P < 0.001$.

Increased intracellular cAMP levels lead to activation of a number of downstream signaling pathways, primarily through activation of EPAC and PKA (215, 216). To assess the involvement of EPAC, we treated DCs with LPS in the presence of the

selective EPAC activator, 8-CPT. In contrast to PGE2, 8-CPT had no effect on LPS-induced production of IL-27 (Fig. 12A). 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. 12B-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 (217). 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. 12D). 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. 12E), 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 (26) 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. 13A) (24-26, 218-220).

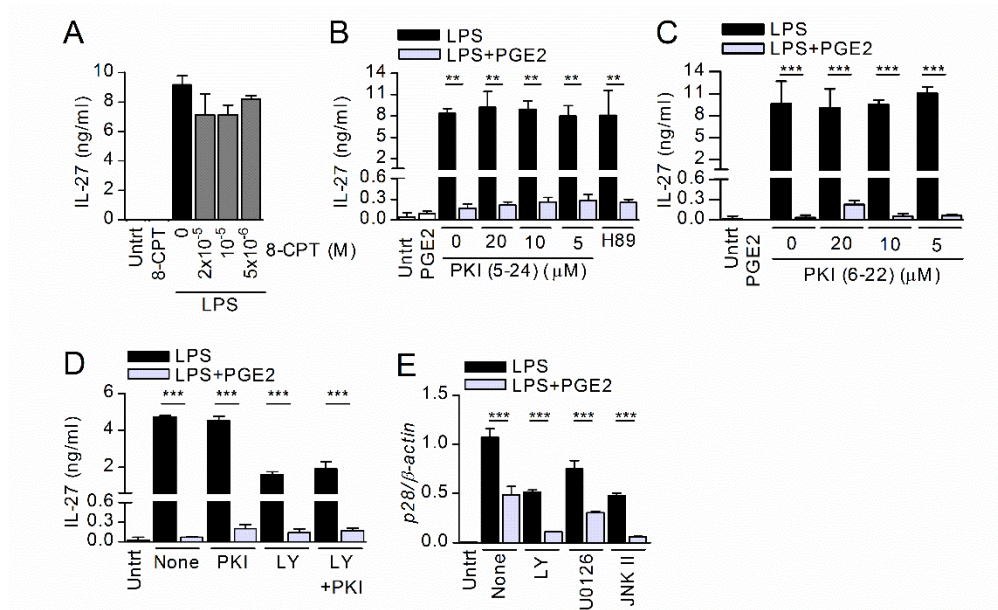


Figure 12. Inhibition of IL-27 by PGE2 is not mediated through EPAC, PKA, PI3K, or MAPK pathways. (A) BMDCs were stimulated with LPS in the absence or presence of the selective EPAC activator 8-CPT for 24hr. One of two independent experiments is shown. BMDCs were pretreated for 3hr with the PKA inhibitors 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 *** $P < 0.001$.

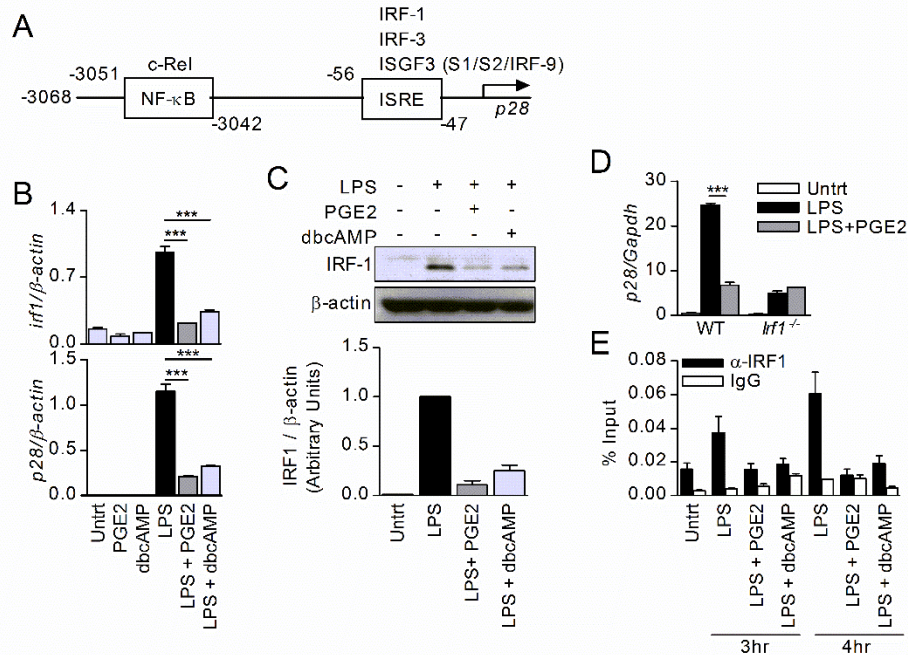


Figure 13. Effects of PGE2 on IRF1. (A) Schematic of the murine *p28* promoter with NF- κ B and ISRE sites. (B) BMDC were stimulated with LPS in the presence of either PGE2 (10^{-6} M) or dbcAMP (10^{-4} M) for 2hr. *Irf1* and *p28* expression was determined by qRT-PCR. One of two independent experiments 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; *** $P < 0.001$.

To identify transcription factors (TF) affected by PGE2 in LPS-stimulated DCs, we used an RT² profiler PCR assay and compared early expression (90 min) 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 (Table 1). *Irf1* downregulation by PGE2 was confirmed by qRT-PCR (Fig. 13B), Western blot (Fig. 13C) 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. 13B-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. 13D). 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. 13E). 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.

Table 1. Gene expression fold change in LPS+PGE2 vs LPS treated BMDC.

Gene symbol	Fold Change	Gene symbol	Fold Change	Gene symbol	Fold Change
Ar	1.70	Gtf2f1	-1.07	Nfyb	1.01
Arnt	1.06	Hand1	-1.09	Nr3c1	-1.41
Atf1	1.02	Hand2	-1.09	Pax6	1.41
Atf2	1.01	Hdac1	1.12	Pou2af1	-1.39
Atf3	-4.01	Hif1a	1.29	Ppara	-1.09
Atf4	1.02	Hnf1a	-6.31	Pparg	-1.39
Cebpa	-1.87	Hnf4a	1.5	Rb1	-1.01
Cebpb	-1.08	Hoxa5	-1.51	Rel	1.28
Cebpg	-1.20	Hsf1	1.07	Rela	1.09
Clasrp	-1.20	Id1	1.05	Smad1	1.04
Creb1	1.04	lrf1	-2.61	Smad4	1.04
Crebbp	1.10	Jun	-1.37	Smad5	1.05
Ctnnb1	-1.01	Junb	1.09	Smad9	1.03
Dr1	-1.03	Jund	-1.52	Sp1	-1.13
E2f1	-1.03	Kcnh8	-1.09	Sp3	-1.05
E2f6	1.05	Max	-1.04	Stat1	1.04
Egr1	-1.41	Mef2a	-1.08	Stat2	-1.02
Esr1	1.06	Mef2b	1.23	Stat3	1.17
Ets1	1.73	Mef2c	1.43	Stat4	1.49
Ets2	1.10	Myc	-1.12	Stat5a	-1.26
Fos	3.96	Myf5	-1.09	Stat5b	-1.10
Foxa2	-1.09	Myod1	-1.09	Stat6	-1.05
Foxg1	10.38	Nanos2	-1.09	Tbp	1.14
Gata1	1.05	Nfat5	1.16	Tcf7l2	1.22
Gata2	1.43	Nfatc2	-1.01	Tfap2a	3.09
Gata3	-1.17	Nfatc3	1.01	Tgif1	1.60
Gli1	-2.23	Nfatc4	1.37	Trp53	1.09
Gtf2b	-1.11	Nfkb1	1.62	Yy1	1.01

Shown are results from BMDC treated for 90 min with 1 μ g LPS +/- 10⁻⁶M PGE2; expressed as fold change values obtained using RT² Profiler PCR Array for Mouse Transcription Factors (Qiagen).

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 (26). STAT1 and STAT2 are essential components of ISGF3 and participate in IRF1 expression (221-223). Therefore, we investigated whether the inhibitory effect of PGE2 on IL-27 was 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. 14A). 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. 14B). 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. 7C). PGE2 retained its inhibitory capacity in the *Stat2*^{-/-} DCs (Fig. 14C), and this was associated with a reduction in *Irf1* expression (Fig. 14D), suggesting that, similar to STAT1, the effects of PGE2 on IL-27 and IRF1 are STAT2-independent.

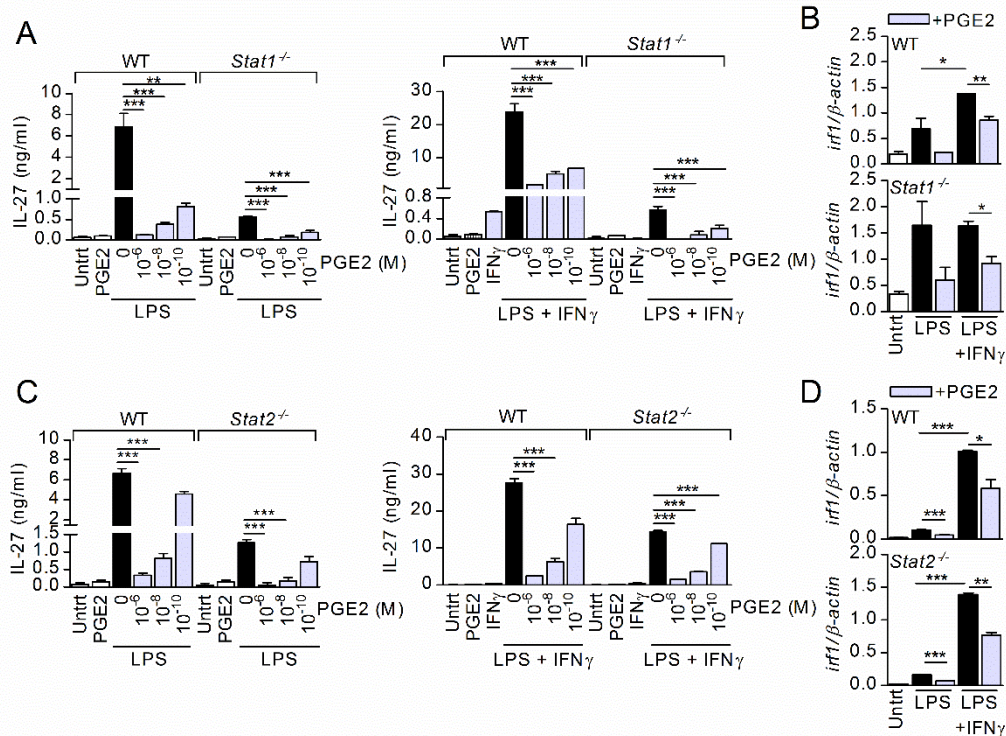


Figure 14. The inhibitory effect of PGE2 on IL-27 and *Irf1* expression is not mediated through STAT1/STAT2. BMDC from WT 129S6 mice and *Stat1*^{-/-} mice (A) or WT C57BL/6 mice and *Stat2*^{-/-} mice (C) were stimulated with LPS or LPS+IFN γ in the presence or absence of PGE2 for 24hr. Supernatants 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 *** $P < 0.001$.

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 (26). PGE2 suppresses IFN- β production in macrophages (224).

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. 15A). 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^{-6} M, and 50% for 10^{-8} M) (Fig. 15B). 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. 15C).

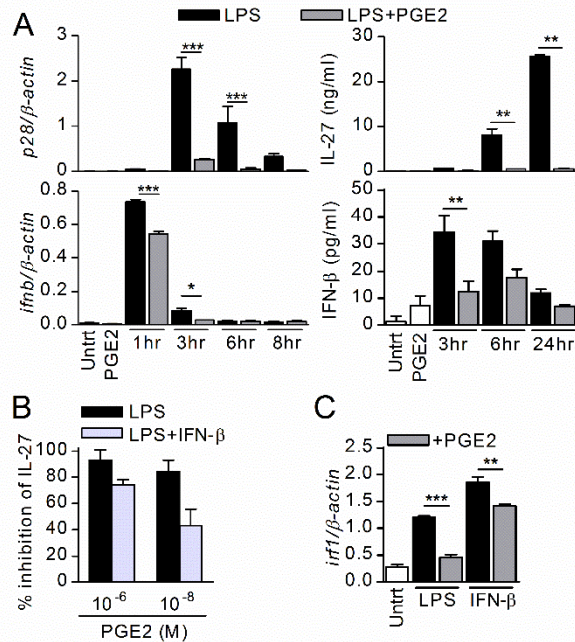


Figure 15. Inhibition of IL-27 by PGE2 is not mediated by the reduction in endogenous IFN- β . (A) BMDCs were stimulated with LPS with or without PGE2 for 1, 3, 6 and 8hr to analyze *p28* and *ifnb* expression levels by qRT-PCR or for 3, 6 and 24hr to analyze IL-27 and IFN- β protein levels by ELISA. 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^{-6} M, 10^{-8} M) 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. *Irfl* 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 *** $P < 0.001$.

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. 16A). Splenic CD11c⁺ cells were collected 4hr 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. 16B).

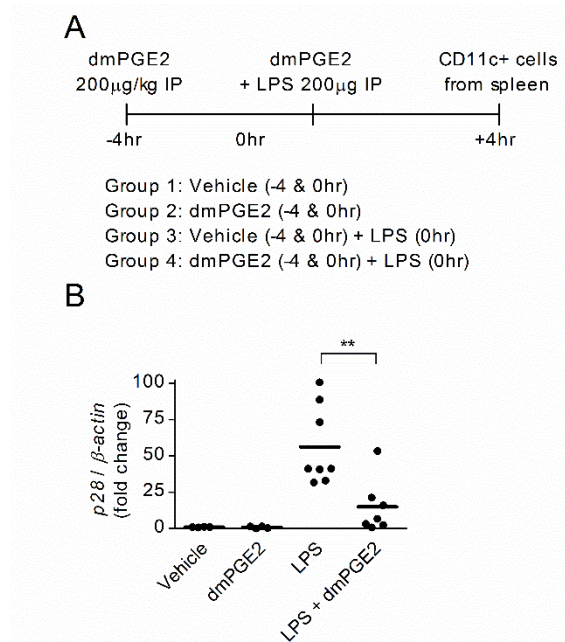


Figure 16. *In vivo* effects of PGE2 on splenic DC *p28* expression. (A) Schematic of experimental timeline and groups. (B) C57BL/6 mice (n=4-8) were injected intraperitoneally with vehicle (0.4% DMSO in PBS) or dmPGE2 (0.2mg/kg) twice at 4hr interval. LPS (10mg/kg) was given intraperitoneally to groups 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.

PGE2 Inhibits Tr1 Differentiation of TCR-stimulated CD4 T Cells

IL-27 functions as an essential factor in the differentiation of CD4⁺IL-10⁺Foxp3⁻ Tr1 cells (53, 54, 63, 113). Recently, co-expression of CD49b and LAG-3 was identified as a characteristic of Tr1 cells (96). We confirmed that IL-27 induces differentiation of naïve CD4⁺ T cells into CD49b⁺LAG-3⁺Foxp3⁻ Tr1 cells, in contrast to TGFβ1 which

induces CD49b⁻LAG-3⁻Foxp3⁺Treg (Fig. 17). In cells treated with IL-27, PGE2 reduced the percentage of Tr1 cells, without inducing Foxp3 expression (Fig. 17).

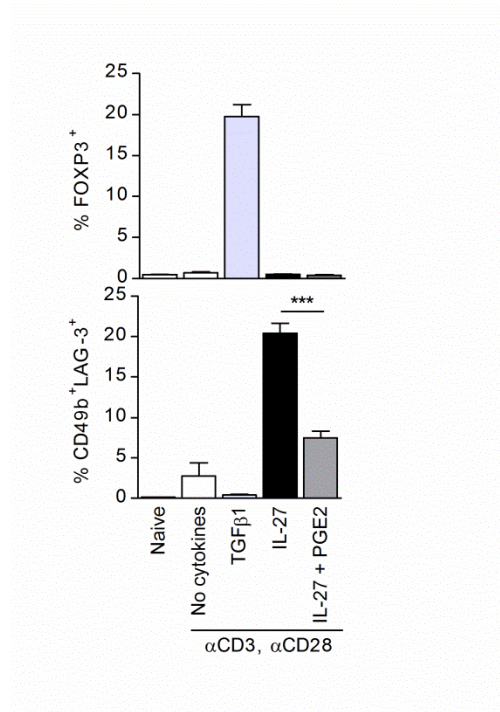


Figure 17. Differentiation of Tr1 and Treg cells. Naive CD4⁺CD62L⁺ cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of 5 ng/ml TGF-β1 or 50 ng/ml IL-27 for three days. Cells were collected on day 3 and analyzed by flow for intracellular Foxp3 or Tr1 markers CD49b & LAG-3. Each sample was tested in duplicate and results represent means ± SD. Significance was determined using one-way ANOVA; ****P*<0.001.

Next we confirmed the role of endogenous IL-27 in Tr1 differentiation by measuring the percentage of CD4⁺CD49⁺LAG-3⁺ cells in spleen cell cultures treated with LPS and anti-CD3 in the presence and absence of neutralizing anti-IL-27 antibodies. As expected, neutralization of endogenous IL-27 resulted in a decrease in

CD4⁺CD49b⁺LAG-3⁺ cells (Fig. 18A). In addition to reducing IL-27 production by BMDC (225), PGE2 had a similar effect in unfractionated spleen cells treated with LPS/anti-CD3 and PGE2 (Fig. 18B). To determine whether PGE2 affects Tr1 differentiation solely through the reduction in endogenous IL-27, we tested its effect in the presence and absence of exogenously added IL-27 (concentrations 50 fold higher than the amount of endogenous IL-27 determined by ELISA). As expected, PGE2 reduced the percentage of CD4⁺CD49b⁺LAG-3⁺ cells. However, we observed similar levels of inhibition in the presence and absence of exogenous IL-27 (Fig. 18C-D), suggesting that PGE2 also inhibits Tr1 differentiation independent of its effect on IL-27 production.

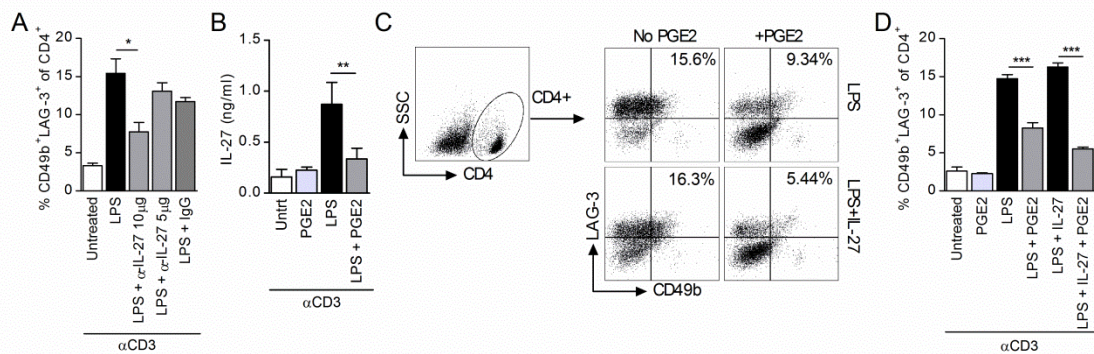


Figure 18. PGE2 inhibits Tr1 cell differentiation in splenocyte cultures. (A) *il10^{gfp}* splenocytes were stimulated with 3 μg/ml anti-CD3 and 1 μg LPS in the presence of neutralizing IL-27 antibodies (5, 10 μg/ml) or IgG control (20 μg/ml). Cells were collected on day 3 and CD4⁺CD49b⁺LAG-3⁺ Tr1 cells were identified by FACS. Data are cumulative from two independent experiments. (B-D) Splenocytes were stimulated with anti-CD3 and LPS in the presence of IL-27 (50 ng/ml) and PGE2 (10⁻⁶M). (B) Supernatant was collected on day 3 and IL-27 levels analyzed by ELISA. (C) Representative sample shows gating strategy to determine percentage of CD49b⁺LAG-3⁺ within CD4⁺ T cells and (D) graph presents representative data from three independent experiments. Each sample was tested in duplicate and results represent means ± SD. Significance was evaluated by one-way ANOVA; ***P* < 0.01.

To characterize the possible direct effect of PGE2 on T cells, we induced Tr1 differentiation in purified naïve CD4 T cells in polarizing conditions (anti-CD3/anti-CD28/IL-27). PGE2 reduced the percentages of CD4⁺CD49b⁺LAG-3⁺ T cells in a dose-dependent manner (Fig. 19A-B), as well as the percentage of IL-10 expressing cells within the CD4⁺CD49⁺LAG-3⁺ population (Fig. 19C). The inhibitory effect could be due to a general reduction in T cell proliferation. Indeed, PGE2 reduced proliferation in Th0 cells (anti-CD3/anti-CD28 stimulated in the absence of exogenous cytokines). However, PGE2 did not substantially affect proliferation in Tr1 polarizing conditions, at concentrations that reduced the percentage of CD4⁺CD49b⁺LAG-3⁺ Tr1 cells (Fig. 19D). Therefore, the inhibitory effect of PGE2 on the percentage of Tr1 cells and on IL-10 production within the Tr1 population does not appear to result from a reduction in T cell proliferation.

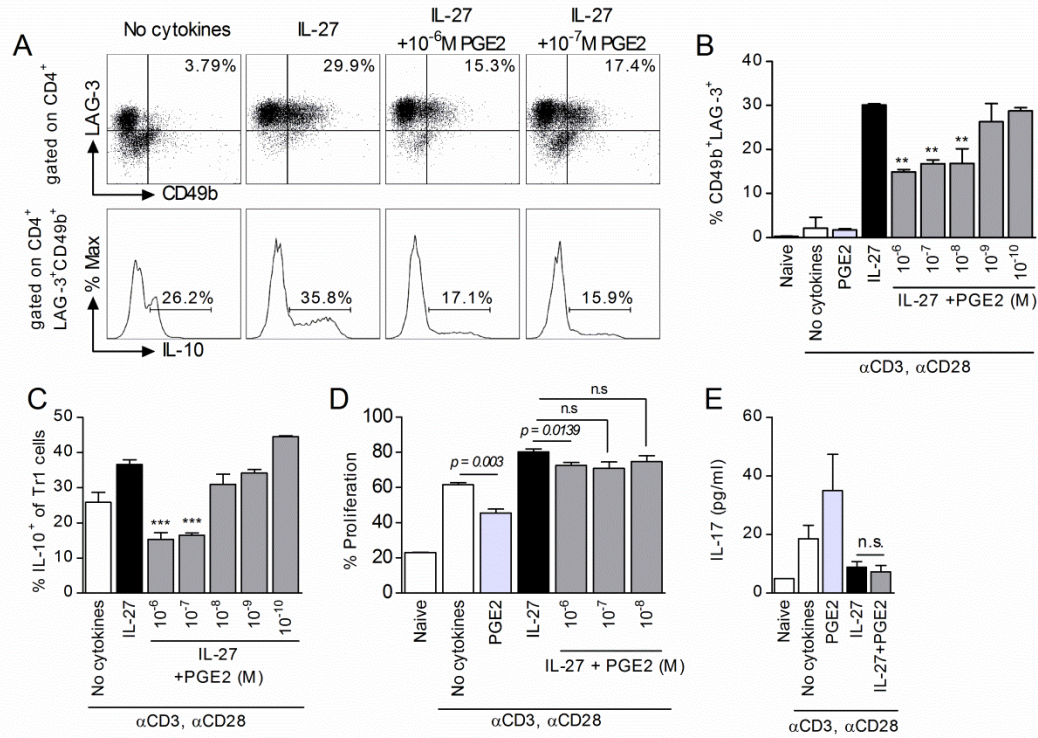


Fig 19. PGE2 inhibits IL-27 induced differentiation of Tr1 cells. (A-C) Naïve CD4⁺CD62L⁺ cells from *il10^{gfp}* mice were stimulated with plate-bound anti-CD3 (3 µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence of 50 ng/ml IL-27 and various concentrations of PGE2 for three days. Cells were collected on day 3 and analyzed by FACS for Tr1 markers, and IL-10 within CD49b⁺LAG-3⁺ populations. Representative samples show CD49b⁺LAG-3⁺ populations (upper panel) and histograms of IL-10 (lower panel). Data are representative of four independent experiments. Each sample was tested in duplicate and results represent means ± SD. Significance was determined using one-way ANOVA and * represents the P value for a sample versus IL-27 control. ***P* < 0.01, ****P* < 0.001. (D) For cell proliferation experiments, naïve CD4⁺CD62L⁺ cells were stained with CFSE per manufacturer's instructions prior to stimulation with anti-CD3, anti-CD28, IL-27 and PGE2. Incorporation of CFSE was analyzed by FACS on day 3. Data are representative of two independent experiments. Each sample was tested in triplicate and results represent means ± SD. Significance was determined using unpaired t-test. (E) Naïve CD4⁺CD62L⁺ cells were stimulated in the presence of 50 ng/ml IL-27 and 10⁻⁶M PGE2. Supernatant was collected on day 3 and subjected to ELISA to determine IL-17 levels. Data are representative of three independent experiments. Each sample was tested in duplicate and results represent means ± SD.

Recently Th17 cells were reported to transdifferentiate *in vivo* into IL-10 producing Tr1 cells during resolution of inflammation or in conditions favoring IL-27 at the expense of IL-23 signaling (56, 118). It is not known however whether a reciprocal transdifferentiation from Tr1 into Th17 cells can occur *in vivo* or *in vitro*. Since we and others reported that PGE2 contributes to Th17 differentiation/function by increasing IL-23 production in cDC and upregulating IL-23R expression in CD4 T cells (166, 168, 174, 191), we investigated whether PGE2 can induce IL-17 production in Tr1 polarizing conditions. In the absence of IL-27, PGE2 increased IL-17 production from activated CD4 T cells. In contrast, the levels of IL-17 secreted by CD4 T cells in Tr1 polarizing conditions were significantly lower, and they were not altered by PGE2 (Fig. 19E). This suggests that inhibition of Tr1 differentiation by PGE2 is not associated with transdifferentiation to the Th17 phenotype.

The Inhibitory Effect of PGE2 on Tr1 Differentiation is Mediated Through EP4 and cAMP

Immune cells, including activated CD4 T cells preferentially express the PGE2 receptors EP2 and EP4 (196, 226). EP4 was shown to be the major contributor to Th1 and Th17 differentiation and expansion *in vitro* and *in vivo* in several autoimmune/inflammatory models (166, 167, 196). To investigate which receptors are involved in the effects of PGE2 on Tr1 differentiation, we differentiated naïve CD4 T cells in the presence of IL-27 and various EP receptor agonists. Misoprostol (EP3, EP4>EP1) mimicked the effects of PGE2, while neither Butaprost (EP2) nor Sulprostone (EP3>EP1) altered Tr1 cell populations (Fig. 20A). These results suggest that PGE2

signals through EP4 to inhibit Tr1 cell differentiation. We confirmed the involvement of EP4 by using the EP4 antagonist ONO-AE3-208 which reversed the inhibitory effect of PGE2 on both CD4⁺CD49⁺LAG-3⁺ Tr1 differentiation and on the percentage of IL-10⁺ Tr1 cells. In contrast, the EP2 antagonist PF-04418948 did not have any effect (Fig. 20B and C).

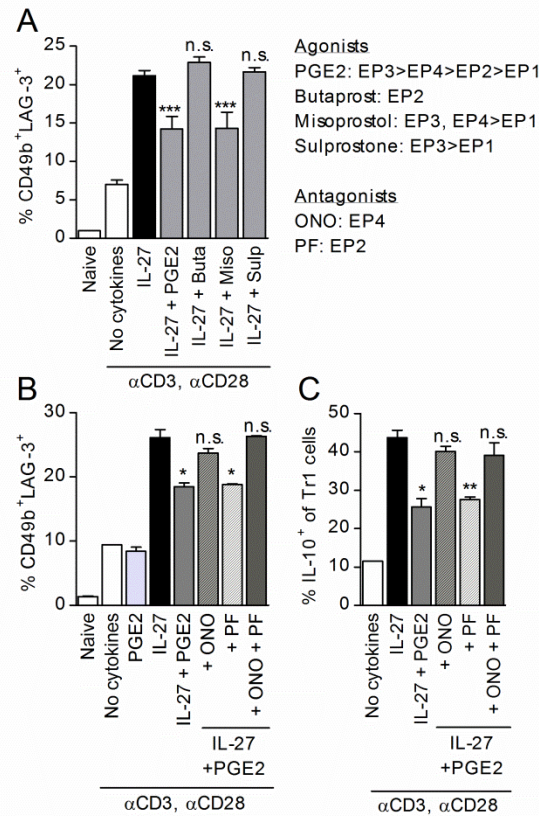


Figure 20. PGE2 signals through EP4 to inhibit Tr1 cell differentiation and expression of IL-10. (A) Naïve CD4⁺CD62L⁺ cells were stimulated in the presence of IL-27 and 10⁻⁶M PGE2 or 10⁻⁵M of EP receptor agonists: Butaprost (EP2), Misoprostol (EP3, EP4>EP1) or Sulprostone (EP3>EP1). Cells were collected on day 3 and analyzed by FACS for Tr1 cell markers. (B,C) Naïve CD4⁺CD62L⁺ cells from *il10^{gfp}* mice were pretreated for 30 min with 10⁻⁶M EP receptor antagonists, ONO-AE3-208 (ONO; EP4) or PF-04418948 (PF; EP2) prior to treatment with 50 ng/ml IL-27 and 10⁻⁷M PGE2. Cells were collected on day 3 and analyzed by FACS for Tr1 markers and IL-10 expression. Data are representative of three independent experiments. Each sample was tested in duplicate and results represent means ± SD. Significance was evaluated by one-way ANOVA and * symbolizes significance of experimental condition versus IL-27 control; **P*<0.05, ***P*<0.01, ****P*<0.001.

EP4 signaling includes both generation of cAMP and activation of PI3K (214). In agreement with previous reports showing that activation of PI3K is essential for T cell proliferation and survival (227-229), the PI3K inhibitor LY294002 proved to be detrimental for the generation of Tr1 cells in the presence as well as absence of PGE2 (Fig. 21A). Due to the effects on proliferation and differentiation, the use of LY294002 cannot exclude the role of PI3K in the inhibition of Tr1 differentiation by PGE2. To investigate the potential involvement of cAMP in the inhibitory effect of PGE2, we used the cell-permeable cAMP analog dibutyryl-cAMP (dbcAMP). At high concentrations (10^{-4} M) dbcAMP affected T cell viability. However, 5×10^{-5} M dbcAMP, which did not affect T cell viability, reduced Tr1 cell differentiation similar to PGE2 (Fig. 21B). We next investigated the role of exchange protein activated by cAMP (EPAC) by utilizing the specific EPAC activator 8-CpT and found that 8-CpT did not reduce Tr1 cell differentiation (Fig. 21C). To determine whether PKA activation is involved, we pretreated naïve CD4 T cells with two PKA inhibitor peptides, PKI 6-22 and PKI 5-24 prior to treatment with IL-27 and PGE2. Neither PKI 6-22 nor PKI 5-24 affected Tr1 differentiation in the absence of PGE2, or reversed the inhibitory effect of PGE2 (Fig. 21D). Although inhibition of the PKA pathway actually increased the percentage of IL-10⁺ cells within the Tr1 cell population in the absence of PGE2, it did not reverse the inhibitory effect of PGE2 on the percentage of CD49b⁺LAG-3⁺IL-10⁺ T cells (Fig. 21E). These results suggest that the inhibitory effect of PGE2 on Tr1 differentiation is dependent on cAMP through a signaling pathway independent of EPAC or PKA activation.

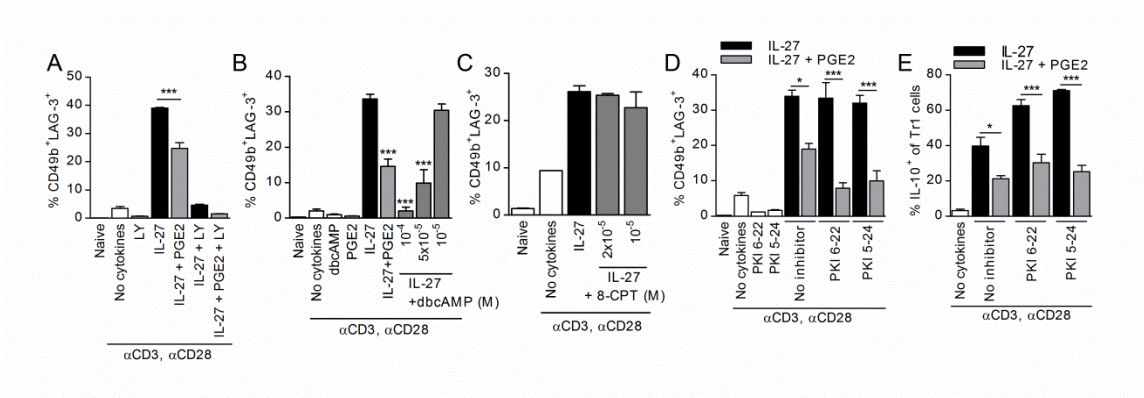


Figure 21. PGE2 signals through cAMP, but not EPAC or PKA, to inhibit IL-27

mediated Tr1 differentiation. (A) *il10^{gfp}* naïve CD4⁺CD62L⁺ cells were pretreated with PI3K inhibitor LY294002 (LY) for 30 min prior to stimulation in the presence of IL-27 and PGE2. (B) Naïve CD4⁺CD62L⁺ cells were stimulated as described in the presence of dbcAMP. (C) Cells were stimulated in the presence cell-permeable EPAC activator 8-CPT. Cells were collected on day 3 and analyzed by FACS for Tr1 markers. (D, E) Naïve CD4⁺CD62L⁺ cells were pretreated with PKA inhibitors PKI 6-22 and PKI 5-24 (2x10⁻⁵M) for 30 min prior to stimulation with IL-27 and PGE2 and analyzed on day 3 for Tr1 markers (D) and IL-10 within Tr1 cell populations (E). Data are representative of three independent experiments. Each sample was tested in duplicate and results represent means ± SD. Significance was evaluated by one-way ANOVA and * symbolizes significance of experimental condition versus IL-27 control (A); **P*<0.05, ***P*<0.01, ****P*<0.001.

PGE2 Inhibits Expression of c-Maf

Previous studies identified c-Maf and AhR as major transcription factors involved in both the differentiation of Tr1 cells and the production of IL-10 by Tr1 cells (53, 65).

To explore the effects of PGE2 on c-Maf and AhR in IL-27 differentiating Tr1 cells, we analyzed first *Maf* and *Ahr* expression by qRT-PCR. Expression of *Maf* increased gradually during Tr1 cell differentiation, while *Ahr* peaked at 24hr (Fig. 22A). PGE2 inhibited expression of *Maf* at both 48hr and 72hr, with no effect on *Ahr* expression (Fig. 22A). Both PGE2 and dbcAMP inhibited *Maf* expression (Fig. 22B), supporting the role of cAMP as a signaling intermediate. The inhibitory effect of PGE2 on *Maf* expression occurred also in unfractionated spleen cell cultures stimulated with LPS and anti-CD3 in the presence and absence of IL-27 (Fig. 22C). Next, we confirmed the inhibitory effect of PGE2 and dbcAMP on c-Maf protein levels in differentiating Tr1 cells by using intracellular flow cytometry. Similar to the qRT-PCR data, both PGE2 and dbcAMP significantly reduced intracellular c-Maf in both the total CD4 T cell population and in the CD49b⁺LAG-3⁺ gated Tr1 cells (Fig. 22D).

In addition to c-Maf and AhR, Egr-2 and downstream Blimp-1 have been implicated in IL-10 expression in Tr1 cells (102, 103). Analysis of intracellular Egr-2 in CD4 T cells differentiated in the presence of IL-27 showed a statistically significant reduction by PGE2, but not by dbcAMP, in the total CD4 T population, and a trend towards reduction without reaching statistical significance in the gated Tr1 cells (Fig. 22E). The effects on Blimp-1 tested by intracellular flow cytometry did not reach statistical significance and were minimal (Fig. 22F). These data indicate that the inhibitory effect of PGE2 on IL-27-induced Tr1 differentiation is associated primarily with substantial reductions in c-Maf expression, with dbcAMP mimicking the effects of PGE2.

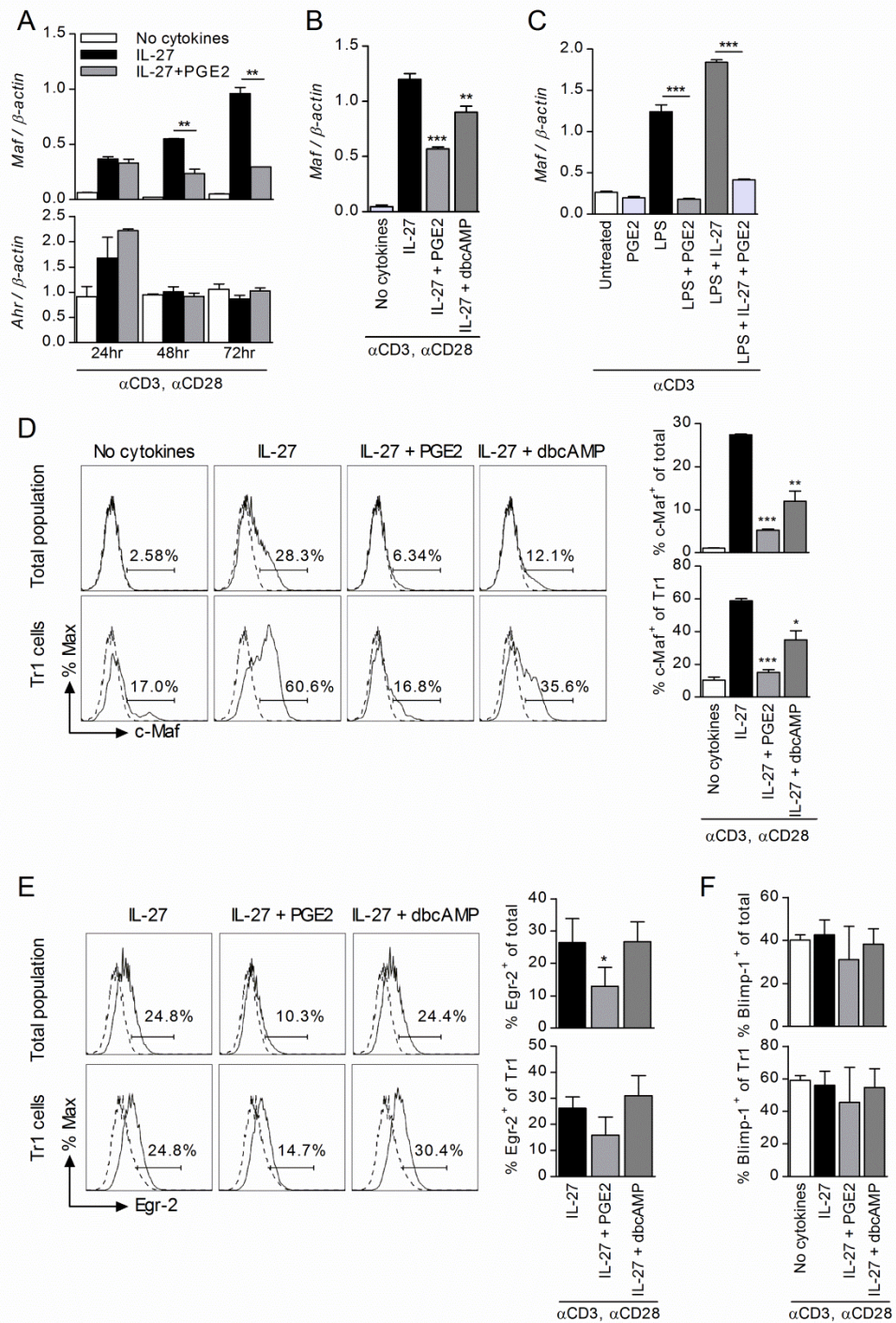


Figure 22. PGE2 inhibits c-Maf in CD4 T cells differentiated in the presence of IL-27. (A) Naïve CD4⁺CD62L⁺ cells were stimulated in the presence of IL-27 and PGE2. RNA was collected at 24, 48 and 72 hr and analyzed by qRT-PCR for *Maf* and *Ahr* expression. (B) Naïve CD4⁺CD62L⁺ cells were stimulated in the presence of IL-27 and PGE2 or dbcAMP. Cells were collected on day 3 and analyzed by qRT-PCR for *Maf* expression. (C) Splenocytes from *il10^{gfp}* mice were stimulated with 3 μ g/ml anti-CD3 and

1 μg LPS in the presence of IL-27 and PGE2. RNA was collected on day 3 and *Maf* expression was analyzed by qRT-PCR. (D) Naïve CD4⁺CD62L⁺ cells were stimulated in the presence of IL-27 and PGE2 or dbcAMP. Cells were collected on day 3 and analyzed by FACS for intracellular c-Maf within the whole cell population (upper panel) and within CD49b⁺LAG-3⁺ populations (lower panel). Graphical representation of data is on the right. (E) Naïve CD4⁺CD62L⁺ cells were stimulated as in D. Cells were collected on day 3 and analyzed by FACS for intracellular Egr-2 within the whole cell population (upper panel) and within CD49b⁺LAG-3⁺ populations (lower panel). (F) Naïve CD4⁺CD62L⁺ cells were stimulated as in D. Cells were collected on day 3 and analyzed by FACS for intracellular Blimp-1 within the whole cell population and within CD49b⁺LAG-3⁺ populations. Data are representative of two to three independent experiments. Each sample was tested in duplicate and results represent means \pm SD. Significance was determined by one-way ANOVA; ** $P < 0.01$, *** $P < 0.001$.

The Effect of PGE2 on IL-27-induced Tr1 Differentiation is Independent of IL-21

Tr1 cells differentiated in the presence of IL-27 secrete substantial amounts of IL-21 that precedes IL-10 secretion (65). Although IL-21 cannot induce Tr1 differentiation by itself, it plays a crucial role as an autocrine growth factor in the expansion and functional stability of IL-27-induced Tr1 cells (63). To determine whether PGE2 inhibits the differentiation of Tr1 cells through its effect on IL-21, we first investigated the effect of PGE2 on IL-21 production in IL-27-induced Tr1 cells. Indeed, PGE2 and dbcAMP reduced IL-21 production from Tr1 cells differentiated in the presence of IL-27 (Fig. 23A). However, the effect on Tr1 function was not mediated through the reduction in IL-21, since we observed similar decreases in CD49b⁺LAG-3⁺IL-10⁺ T cells in the presence or absence of exogenous IL-21 (Fig. 23B).

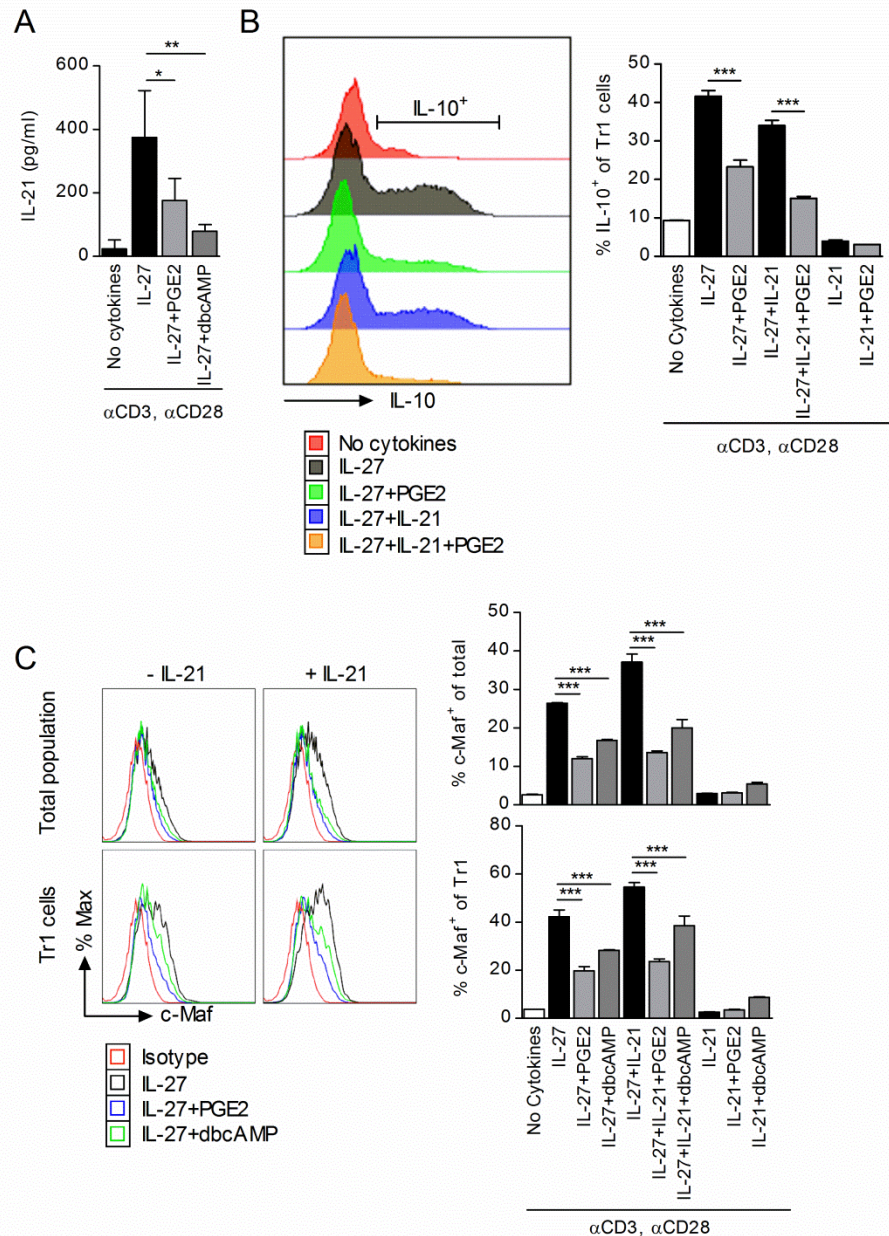


Figure 23. Inhibition of c-Maf by PGE2 is independent of IL-21. (A) Naive CD4⁺CD62L⁺ cells were stimulated in the presence of IL-27 and PGE2 or dbcAMP. Supernatant was collected on day 3 and analyzed by ELISA for IL-21. Data are cumulative from two independent experiments. (B) Naive CD4⁺CD62L⁺ cells from *il10^{gfp}* mice were stimulated in the presence of IL-27, 100 ng/ml IL-21 and PGE2. Cells were collected on day 3 and analyzed by FACS to determine IL-10 production within CD49b⁺LAG-3⁺ CD4 T cells. Data are representative of two independent experiments. (C) Naive CD4⁺CD62L⁺ cells from C57BL/6 mice were stimulated as above. Cells were collected on day 3 and analyzed by FACS to determine presence of intracellular c-Maf within CD4 T cells (top) and CD49b⁺LAG-3⁺ CD4 T cells (bottom). Histograms show representative samples, while graphs present data from one of three independent

experiments. Each sample was tested in duplicate and results represent means \pm SD. Significance was determined by one-way ANOVA; ** $P < 0.01$, *** $P < 0.001$.

The effect of IL-21 in the amplification and sustenance of the Tr1 phenotype was attributed to maintenance of c-Maf expression (63). Therefore, we investigated whether the inhibitory effect of PGE2 and dbcAMP on c-Maf expression observed during IL-27-induced Tr1 differentiation also occurred in the presence of exogenous IL-21. Similar to their effects on CD49b⁺LAG-3⁺IL-10⁺ Tr1 cell differentiation, PGE2 and dbcAMP reduced intracellular c-Maf even in the presence of exogenous IL-21 (Fig. 23C).

The Effects of PGE2 on Tr1 Differentiation and c-Maf Expression Do Not Depend on STAT1 or STAT3

In T cells, engagement of the IL-27 receptor leads to activation of STAT1 and STAT3 signaling, resulting in inhibition of Th17 and promotion of Tr1 differentiation (62, 66, 114). In contrast to Th17 differentiation which is inhibited by STAT1, Tr1 differentiation and IL-10 production are promoted by both STAT1 and STAT3 activation (62, 65, 114). Therefore, we investigated whether the inhibitory effect of PGE2 was mediated through effects on STAT1 or STAT3 activation. To determine the role of STAT1, we assessed the effect of PGE2 on Tr1 differentiation of WT and *Stat1*^{-/-} CD4 T cells. Although IL-27 treatment of *Stat1*^{-/-} T cells resulted in fewer CD49b⁺LAG-3⁺ Tr1 cells (~40% reduction), PGE2 inhibited Tr1 differentiation at the same level as in WT CD4 T cells (Fig. 24A). Similarly, overall expression of *Maf* was reduced in IL-27 treated *Stat1*^{-/-} T cells, but PGE2 maintained its inhibitory activity (Fig. 24B). These results confirmed the role of STAT1 in Tr1 differentiation but excluded STAT1 as a

mediator for the inhibitory effect of PGE2. To examine whether PGE2 affected STAT3 activation, we treated naïve CD4 T cells with IL-27 in the presence or absence of PGE2 or dbcAMP, and determined the levels of STAT3 phosphorylation (Tyr705) by flow cytometry. While IL-27 increased STAT3 phosphorylation, neither PGE2 nor dbcAMP affected IL-27-induced STAT3 phosphorylation (Fig. 24C), suggesting that the inhibitory effect of PGE2 was not mediated through a reduction in STAT3 activation.

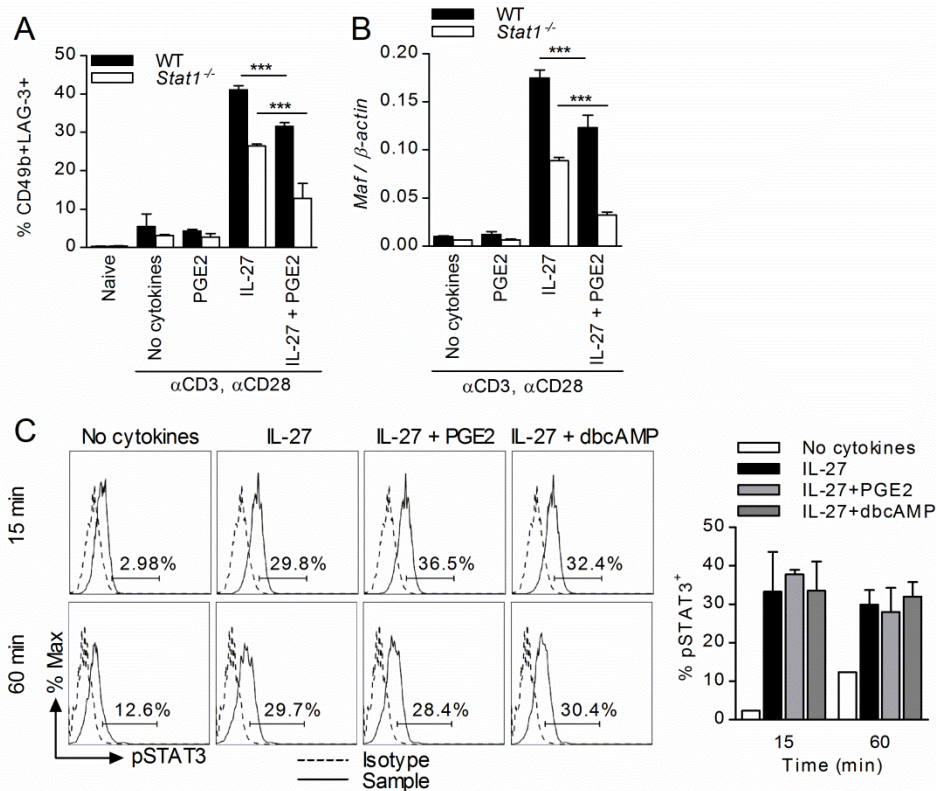


Figure 24. Inhibition of Tr1 differentiation by PGE2 is independent of STAT1/3.

Naïve CD4⁺CD62L⁺ cells from WT 129S6 (WT) and *Stat1*^{-/-} mice were stimulated in the presence of IL-27 and PGE2. (A) Samples were collected on day 3 and cells were analyzed for Tr1 markers. (B) Cells were subjected to RNA extraction and subsequent analysis by qRT-PCR for *Maf* expression. (C) Naïve CD4⁺CD62L⁺ cells from C57BL/6 mice were stimulated in the presence of IL-27, PGE2 and 10⁻⁴M dbcAMP. Cells were collected 15 and 60 min later and analyzed by FACS for intracellular phospho-STAT3 (Tyr705). Data are cumulative from three independent experiments. Each sample was tested in duplicate and results represent means ± SD. Significance was determined using one-way ANOVA; ****P* < 0.001.

The *In Vivo* Effect of PGE2 on Tr1 Cells

To determine whether treatment with PGE2 affects Tr1 generation/amplification *in vivo*, we used a model of anti-CD3-mediated transient intestinal inflammation (117). *IL10^{gfp}* mice were inoculated i.p. with anti-CD3 and the stable PGE2 analog dimethyl PGE2 (dmPGE2), and cells from Peyer's patches, spleen, and intraepithelial lymphocytes (IEL) from the small intestine were obtained 4h after the last inoculation (Fig. 25A). The percentage of CD4⁺CD49b⁺LAG-3⁺ Tr1 cells and of IL-10⁺ cells within total CD4 T cell populations were determined by flow cytometry. Both CD4⁺CD49b⁺LAG-3⁺ Tr1 cells and the percentage of IL-10⁺ CD4 T cells increased following anti-CD3 inoculation in all tissues examined. Administration of dmPGE2 led to a reduction in CD4⁺CD49b⁺LAG-3⁺ cells and in IL-10 expressing CD4 T cells, reaching statistical significance in spleen and Peyer's patches for CD4⁺CD49b⁺LAG-3⁺ cells and in IELs for IL-10 expressing CD4 T cells (Fig. 25B-C).

CHAPTER FOUR DISCUSSION

Multiple reports have detailed the regulatory function of IL-27 in both innate and adaptive immunity and its crucial role in the generation of Tr1 cells (2, 230, 231). Initially, IL-27 was described as promoting Th1 differentiation (20, 232). However, in contrast to expectations, mice deficient in IL-27R were able to mount adequate Th1 responses against intracellular pathogens (233), but succumbed due to uncontrolled severe tissue inflammation associated with exaggerated T cell responses and enhanced TNF and IFN γ production (37). These observations led to the conclusion that IL-27 acted primarily by limiting the response of effector T cells and by maintaining and/or reestablishing immune homeostasis (234, 235). Subsequent studies demonstrated the role of IL-27 in inducing Tr1 cells *in vitro* and *in vivo*, and revealed its therapeutic potential (54, 113, 114, 116, 236).

Although the mechanisms involved in the induction of IL-27 in cDCs and macrophages and IL-27 involvement in Tr1 differentiation have been studied extensively, little is known about factors and mechanisms that negatively impact IL-27 expression or IL-27-mediated Tr1 differentiation. We were the first to report that PGE2 inhibited IL-27 production by BMDCs (175), and a later publication detailed a similar finding in THP-1 cells (237). 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 addition, we report for the first time that PGE2 inhibits the IL-27-induced differentiation of regulatory Tr1 cells. The inhibitory effect of PGE2 on the differentiation and IL-10 production by CD4⁺CD49b⁺LAG-3⁺ Tr1 cells is mediated by EP4 and c-AMP leading to a significant reduction in c-Maf expression, and is independent of STAT1/STAT3 activation. The effect of PGE2 on CD4⁺CD49b⁺LAG-3⁺ Tr1 differentiation is not associated with either induction of Foxp3 or IL-17 production, suggesting a lack of transdifferentiation into Foxp3⁺ Treg or effector Th17 cells.

PGE2, a major prostanoid abundant at inflammation sites, has multiple effects on the immune response primarily through EP2 and EP4 receptors expressed on immune cells (226). *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 (166, 167, 169, 174-177). *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 (189, 195, 238). Although PGE2 was reported initially to inhibit Th1 differentiation, this was apparently due to the PGE2-induced suppression of IL-12 production in APCs (192, 239). In contrast to the reported inhibitory effect on Th1 differentiation in APC/CD4 T cell co-cultures, *in vivo* administration of PGE2 receptor antagonists reduced production of IFN γ by cells collected from the draining lymph nodes of EAE mice, suggesting that endogenous PGE2 promoted *in vivo* Th1 differentiation (166). This was confirmed *in vitro* in purified CD4 T cell cultures, where PGE2 was shown to upregulate the expression of both IL-12R β 2 and IFN γ R1 during differentiation of naïve CD4 T cells in Th1 polarizing conditions that included exogenous IL-12 (174).

Several laboratories, including our own, reported that PGE2 also stimulates IL-23 production in DCs and upregulates IL-23R in T cells, promoting the generation of pathogenic Th17 cells (166, 168, 191, 240-242). Despite its role in IL-12 and IL-23 production by APCs, little is known about the effect of PGE2 on another IL-12 family member, IL-27.

Previously, we reported that PGE2 inhibited IL-27 expression in BMDCs (169), and Zhu et al (237) 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 the inhibition occurs regardless of the TLR involved in IL-27 production. Both EP2 and EP4 receptors, previously identified in DCs and shown to mediate most PGE2 effects in immune cells, were involved in the inhibition of IL-27 (153, 194, 243). 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 (24, 218-220). 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 (26). 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 (26).

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 (approximately 50%) as compared to more than 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. Although IL-27 production was reduced in *Stat1*^{-/-} DCs, 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 (147, 244). 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 (245, 246). We were able to eliminate a number of possible intermediaries such as PKA, EPAC, PI3K, MAPKs, STAT1 and STAT2. Presently, the link between cAMP and IRF1 in the inhibition of IL-27 by PGE2 in cDC remains to be identified.

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 IFN-β 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. A possible mechanism by which PGE2 inhibits IRF1 could involve sirtuin 1 (SIRT1). SIRT1, a histone deacetylase, has been reported to deacetylate IRF1, leading to reduced IL-27 production (247). Although little is known

about the regulation of SIRT1 by PGE2, a recent report determined that cAMP binds directly and activates SIRT1, *in vitro* (247). Despite the interaction between cAMP and SIRT1 not being confirmed *in vivo*, the involvement of SIRT1 in the inhibition of IL-27 by PGE2 remains a possibility.

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 (177) 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 (248). Therefore, the development and use of a conditional temporal mPGES1 KO might be a better option.

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. In addition to the inhibition of IL-27 production by APC, we reported that PGE2 inhibits Tr1 differentiation in splenocyte cultures even in the presence of exogenous IL-27. This suggested the possibility that PGE2 acted directly on the differentiating CD4 T cells, inhibiting Tr1 differentiation.

Previous studies using EP2 and EP4-deficient mice and specific EP2/EP4 antagonists concluded that PGE2 induced Th1 differentiation and facilitated Th17 expansion in models of immune inflammation (200). More recently, PGE2 was shown to promote Th1 differentiation *in vivo* and *in vitro* through upregulation of IL-12R β 2 and IFN γ -R1 on CD4 T cells (167, 174). PGE2 was also reported to promote differentiation of Th17 cells through upregulation of IL-1R and IL-23R, and Th17 expansion by altering the IL-23/IL-12 balance in favor of IL-23 in cDC (191, 196).

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 (206-210). In contrast, PGE2 inhibits the differentiation of non-tumor murine Foxp3⁺Treg and of CD46-induced Tr1 cells (205, 211).

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 (39, 54, 113, 114). In DCs, IL-27 reduced costimulatory molecules, IL-12, IL-6 and IL-23, and inhibited DC capacity to generate Th1/Th17 cells (39). In addition, adoptive transfer of IL-27-treated DC reduced EAE and suppressed recall responses of Th1 and Th17 cells (39). 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 \rightarrow Blimp1 (53, 102).

Here we report that, in addition to reducing IL-27 production by APCs, PGE2 directly inhibits Tr1 differentiation and Tr1 IL-10 production. Inhibition of both Tr1

differentiation and IL-10 production in Tr1 cells brings a novel mechanistic perspective to the proinflammatory role of PGE2. CD4 Tr1 cells, characterized by co-expression of CD49b, LAG-3 and CD226, high levels of IL-10 production, and lack of constitutive Foxp3 and CD25 expression, play a major role in tolerance and immune homeostasis (108, 249). Tr1-mediated immune suppression occurs through multiple mechanisms including secretion of high levels of IL-10 and TGF β , generation of extracellular adenosine, direct killing of APCs through granzyme B/perforin release, and PD-1/PD-L and CTLA-4-mediated inhibition of the stimulatory capacity of APCs (249, 250). The generation of Tr1 cells has been studied extensively (249, 251). In addition to IL-27, which was identified as the crucial factor in inducing Tr1 differentiation (62, 231), both IL-21 and IL-10 were reported to act as autocrine factors in Tr1 amplification and maintenance of functional stability (63, 107).

In contrast to factors that positively affect Tr1 differentiation, much less is known about the suppression of Tr1 development and/or function. It is conceivable that factors present in acute inflammatory conditions, and possibly in chronic inflammation as well, might have a negative impact on Tr1 development and function. A recent study reported that extracellular ATP and hypoxia, both increased at inflammatory sites, inhibit Tr1 differentiation through AhR inactivation (252). Along those lines, most prostanoids and especially PGE2, found at high concentrations at inflammatory sites, could impact the generation and function of Tr1 cells either indirectly through effects on IL-27 production or directly by acting on the differentiating Tr1 cells. In addition to PGE2 inhibiting the expression and production of IL-27 from TLR-stimulated cDC by reducing IRF1 expression and binding to the *Il27p28* promoter, PGE2 can also directly affect IL-27-

induced Tr1 differentiation by reducing the expression of the essential transcription factor c-Maf in Tr1 differentiating CD4 T cells.

c-Maf is a critical factor in Tr1 differentiation and IL-10 expression, acting in conjunction with AhR for the transactivation of both *I110* and *I121* transcription (53, 65). The essential role of c-Maf and AhR was also demonstrated *in vivo* for the IL-27-mediated induction of Tr1 cells (116). In addition to c-Maf and AhR, IL-10 production in Tr1 cells is also dependent on the Egr-2→Blimp-1 pathway controlled primarily through STAT3 activation (102). We found that PGE2 significantly reduces c-Maf expression at both mRNA and protein level during Tr1 differentiation, with no effect on AhR and minimal effects on Egr-2→Blimp-1 expression. Kuchroo and colleagues (65) proposed that Tr1 differentiation occurs in two phases, *i.e.* the initiation phase induced by IL-27, and the IL-21/IL-10-dependent Tr1 amplification/stabilization. c-Maf was shown to play a major role in both the initiation and amplification phase. In the initiation phase, signaling through IL-27R induces expression of c-Maf, IL-21R and ICOS, and c-Maf transactivates the *I121* and *I110* promoters. In the amplification phase, IL-21 acts in an autologous manner activating and maintaining high levels of c-Maf expression. Our finding that PGE2 inhibits c-Maf expression during Tr1 differentiation is in agreement with the observed inhibitory effect on the production of IL-21 in the initiation phase. The inhibitory effect of PGE2 on Tr1 differentiation could be entirely mediated through the inhibition of IL-21 expression. However, we found that PGE2 inhibits the differentiation of CD49b⁺LAG-3⁺IL10⁺ Tr1 and reduces c-Maf levels in Tr1 cells even in the presence of exogenous IL-21. These observations suggest that the inhibitory effect of PGE2 is

mediated primarily through reduction in both IL-27- and IL-21-induced c-Maf expression, affecting both Tr1 induction and amplification/stabilization.

Although human and murine CD4 T cells express both EP4 and EP2 receptors, loss of function experiments and use of selective receptor antagonists identified EP4 as a major mediator of PGE2-induced Th1/Th17 differentiation and expansion in models of immune inflammation (200). We found that the inhibitory effect of PGE2 on Tr1 cells is also mediated through EP4. The effects of PGE2 on IL-21 production, Tr1 differentiation and c-Maf expression were mimicked by dbcAMP, strongly suggesting that the effect of PGE2 is mediated through cAMP. Similar conclusions were previously reached for the effects of PGE2 on Th1 and Th17 differentiation (200). However, in contrast to previous reports which identified PKA or EPAC as downstream mediators, we found that neither one was involved in the inhibitory effect of PGE2 on Tr1 differentiation. We eliminated STAT1 and STAT3, known intermediates in IL-27-mediated Tr1 differentiation (114, 253), as possible targets for the inhibitory effect of PGE2 and dbcAMP. The link between cAMP induction and inhibition of c-Maf expression remains unidentified at the present time. Future transcriptome studies are required to elucidate the signals involved in the PGE2→cAMP→c-Maf pathway.

Fate mapping experiments underlined the plasticity of both Th17 and Foxp3⁺ Treg, and a recent study reported on the transdifferentiation of Th17 into Tr1 cells during resolution of inflammation (118, 254, 255). PGE2 was previously reported to promote Th17 expansion and to induce Foxp3 expression and regulatory function in human T cells (166, 204). Therefore, we took into consideration the fact that PGE2 might switch Tr1 differentiation towards either Foxp3 expressing- or IL-17-producing T cells. This was not

the case. However, the questions whether already differentiated Tr1 cells could be induced to transdifferentiate into effector T cells in an inflammatory microenvironment, and whether PGE2 could contribute to this transdifferentiation, remain to be addressed.

Taken together, these results detail a novel PGE2 proinflammatory mechanism, through inhibition of IL-27 production by APCs, and direct and indirect inhibitory effects on Tr1 differentiation. We propose that in inflammatory conditions, PGE2 can shift the balance of CD4 T cell subsets promoting Th1/Th17 and inhibiting Tr1 differentiation and function (Fig. 26).

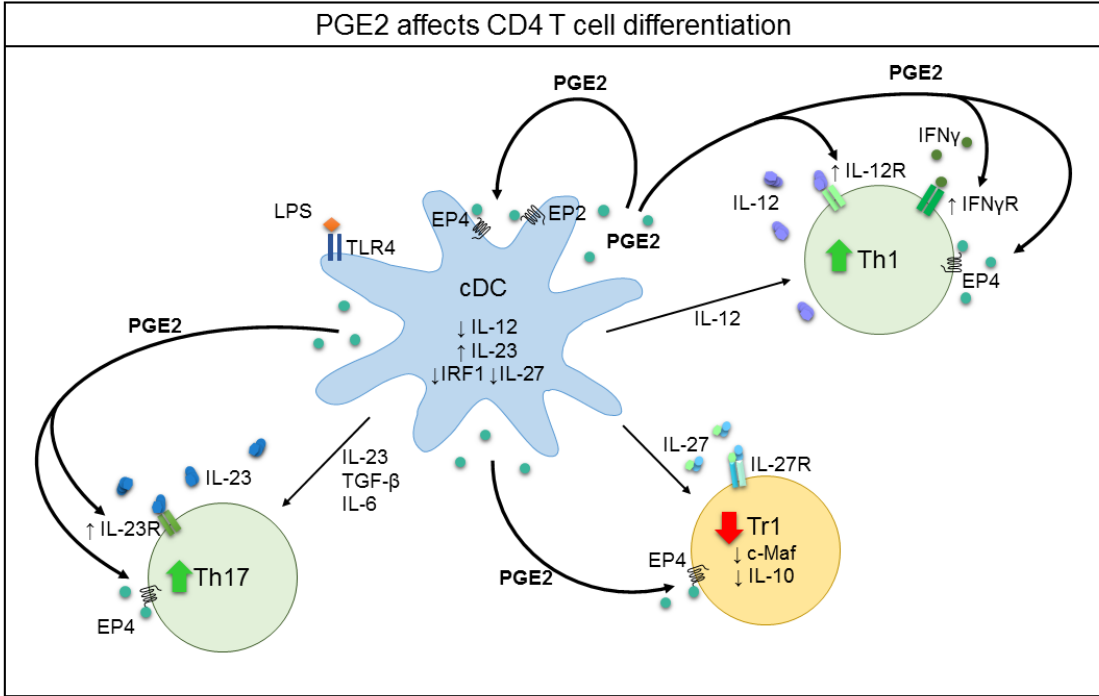


Figure 26. PGE2 affects CD4 T cell balance. By acting on both APC and CD4 T cells, PGE2 has multiple effects on CD4 T cell differentiation. Upon TLR stimulation, cDC produce multiple cytokines including IL-12, IL-23 and IL-27 leading to differentiation of Th1, Th17 and Tr1 cells, respectively. Although Th1 differentiation is initially reduced by PGE2 due to a reduction in IL-12 production by DC, upregulation of IL-12R and IFN γ R on T cells in response to PGE2 ultimately leads to increased Th1 differentiation in vivo and sustains Th1 effectors in vitro. PGE2 increases and sustains Th17 differentiation through upregulation of both IL-23 in DC, and IL-23R on T cells. Finally, PGE2 can inhibit Tr1 differentiation through a reduction in IL-27 production by DC, and by inhibiting c-Maf expression in Tr1 cells.

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APPENDIX

THE JOURNAL OF IMMUNOLOGY PUBLICATION INFORMATION

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