In Vitro Differentiation of Dendritic Cells in the Presence of Prostaglandin E₂ Alters the IL-12/IL-23 Balance and Promotes Differentiation of Th17 Cells

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In Vitro Differentiation of Dendritic Cells in the Presence of Prostaglandin E$_2$ Alters the IL-12/IL-23 Balance and Promotes Differentiation of Th17 Cells$^1$

Tanzilya Khayrullina,*† Jui-Hung Yen,* Huie Jing,† and Doina Ganea$^2*$

Prostaglandin E$_2$ (PGE$_2$), an endogenous lipid mediator released in inflammatory conditions, affects both dendritic cell (DC) differentiation and maturation. Whereas the effect of PGE$_2$ on fully differentiated DC was studied extensively, little is known about its effects on DC differentiation. In this study, we show that bone marrow-derived DC generated in the presence of PGE$_2$ (DCp) acquire a proinflammatory profile; produce higher levels of proinflammatory cytokines/chemokines; express higher levels of MHC class II, costimulatory molecules, and TLRs; and exhibit increased activation of the NF-$\kappa$B-signaling pathway. In addition, DCp exhibit a different IL-12/IL-23 profile than DC generated in the absence of PGE$_2$. The low IL-12 and high IL-23 production in LPS-stimulated DCp is associated with the down-regulation of p35 and the up-regulation of p19 expression, respectively. In agreement with the DCp proinflammatory phenotype and especially with the altered IL-12/IL-23 balance which strongly favors IL-23, DCp also affect T cell differentiation. In contrast to DC which favor Th1 differentiation, DCp promote Th17 and inhibit Th1/Th2 differentiation, in vitro and in vivo. Previous in vivo studies indicated that PGE$_2$ had a proinflammatory effect, especially in models of autoimmune diseases. Our results suggest that the proinflammatory effects of PGE$_2$ could be mediated, at least partially, through effects on differentiating DC and subsequent alterations in CD4$^+$ T cell differentiation, resulting in the preferential development of pathogenic autoimmune Th17 cells. *The Journal of Immunology, 2008, 181: 721–735.

Dendritic cells (DC),$^3$ poised at the interface between innate and adaptive immunity, function as highly efficient APCs with a crucial role in both immunity and tolerance. In the last decade, a number of laboratories focused on in vitro procedures for generating either immunoreactive or tolerogenic DC for therapeutic purposes (reviewed in Refs. 1–3). Among various factors, the lipid mediator PGE$_2$ has been used in conjunction with the mixture of proinflammatory cytokines TNF, IL-1$\beta$, and IL-6 for maturation of human myeloid DC.

Whether PGE$_2$ interactions with DC lead to pro- or anti-inflammatory results depends to a large degree on the nature of the DC maturation signals. Several studies reported that PGE$_2$ presence during DC maturation with TLR ligands such as LPS or proteoglycan results in a significant inhibition of the expression and release of the proinflammatory cytokines and chemokines TNF, IL-6, IL-12p70, CCL3, CCL4, and CXCL10, and in the up-regulation of the potent anti-inflammatory cytokine IL-10 (4–10). This indicates an anti-inflammatory role for PGE$_2$. In contrast, DC matured with the cytokine mixture plus PGE$_2$ express increased levels of MHC class II (MHCII) and costimulatory molecules (CD80, CD86, and CD40), secrete high levels of IL-12p40 and IL-6, up-regulate CCR7, and are potent T cell stimulators, suggesting a proinflammatory effect (11–14). It is widely accepted that PGE$_2$ plays a critical role in myeloid DC migration, by promoting podosome dissolution through changes in $\beta_1$ integrins (15), facilitating CCL19/21-induced signaling through CCR7 (16, 17), and inducing matrix metalloproteinase 9 (18). The fact that PGE$_2$ promotes mature DC migration argues in favor of its proinflammatory effect. In autoimmune diseases, PGE$_2$ has been implicated mostly as a proinflammatory agent. For example, in animal models of rheumatoid arthritis, the role of PGE$_2$ was confirmed in PGES-1- and EP4-deficient mice, which have reduced disease incidence and severity (19, 20).

Both Th1 cells and the recently described Th17 cells play essential roles in acute and chronic inflammation. Murine Th17 effectors differentiate from naive T cells in the presence of TGF-$\beta$, IL-1, and IL-6 and require IL-23 primarily for survival/proliferation (reviewed in Refs. 21–24). In contrast, differentiation of human Th17 effectors depends primarily on IL-1$\beta$, IL-6, and IL-23, with TGF-$\beta$ acting as a negative regulator (25, 26). In addition, IFN-$\gamma$ and IL-4 were shown to inhibit Th17 differentiation (27, 28). There is substantial evidence that IL-23-dependent pathogenic Th17 cells are involved in chronic inflammatory processes and in the induction of autoimmune diseases. Increased expression of IL-17 was reported in rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (reviewed in Refs. 29 and 30). The role of IL-23 and IL-17 in autoimmune/inflammatory diseases is substantiated by the reduced susceptibility or resistance of IL-23- and IL-17-deficient mice to spontaneous and hapten-induced colitis, experimental autoimmune encephalomyelitis, and collagen-induced arthritis (31–36).

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$^3$Abbreviations used in this paper: DC, dendritic cell; MHCII, MHC class II; DCp, dendritic cell differentiated in the presence of PGE$_2$; Tg, transgenic; BM-DC, bone marrow-derived DC; PCCF, pigeon cytochrome c fragment; PLP, proteolipid protein; DLN, draining lymph node; IRAK, IL-1R-associated kinase; IKK, I$\kappa$B kinase.

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The role of PGE2 in T cell differentiation is controversial. Whereas most studies agree on a reduction in IFN-γ production, presumably mediated through the inhibition of IL-12p70 release from PGE2-treated DC, there is disagreement on the PGE2-induced up-regulation of Th2 cytokines (11, 12, 37–40). The role of PGE2 in Th17 differentiation and/or activation is still uncharted territory. We reported recently that PGE2 induced p19 and reduced p35 expression in fully differentiated DC, resulting in the up-regulation of IL-23 at the expense of IL-12p70, and leading to increased production of IL-17 from activated CD4+ T cells (41, 42).

In the vast majority of studies, fully differentiated DC were exposed to PGE2 in addition to other maturation signals, i.e., treatment with PGE2 and either TLR ligands or cytokine mixtures. However, in vivo, both fully differentiated and developing DC become exposed to PGE2 in inflammatory conditions. In the present study, we show that DC exposure to PGE2 during differentiation results in DC priming for increased proinflammatory cytokine/chemokine production and up-regulation of MHCII and co-stimulatory molecule expression. We also show that DC differentiated in the presence of PGE2 (DCp) induce IL-17-producing T cells at the expense of IFN-γ-secreting Th1 effectors, both in vitro and in vivo.

Materials and Methods

Mice

B10.A (males, 8- to 10-wk old) were purchased from The Jackson Laboratory, and TCR-Cyt5.CC7-IRag1−/− transgenic (Tg; I-Ek) mice were bred and maintained in the Temple University School of Medicine animal facilities (Philadelphia, PA) under pathogen-free conditions.

Reagents

LPS (Escherichia coli 026:B6), PGE2, avidin-peroxidase, anti-mouse IgG were purchased from Sigma-Aldrich. Ibuprofen was purchased from Cayman Chemical. Recombinant murine GM-CSF was purchased from PeproTech. CD4 and CD11c MicroBeads were purchased from Miltenyi Biotec. Tetramethylbenzidine ELISA substrate was purchased from Pierce. Monoclonal and biotinylated anti-mouse IL-17, recombinant murine IL-17, recombinant murine IFN-γ, neutralizing anti-TGF-β, anti-p40 Ab, and the ELISA kit for murine IL-27p28 and PGE2, were purchased from R&D Systems. The IL-23 (p19/p40) ELISA kit was purchased from eBioscience. Neutralizing anti-P19 Ab were purchased from eBioscience. Capture and biotinylated anti-mouse IL-4, IL-6, IL-1β, IFN-γ, TNF, IL-10, IL-12p40, IL-23p19, IL-17, and IL-27 ELISA kits were purchased from R&D Systems.
IL-12p70 Abs, neutralizing anti-IFN-γ Abs, PE-conjugated anti-CD11c, FITC-conjugated anti-CD80, CD86, CD40, MHCII, CD11b, CD11c, CD4, allophycocyanin-conjugated IFN-γ, and FITC/PE/allophycocyanin-conjugated IgG, as indirect reagents, were purchased from BD Biosciences. Butaprost, misoprostol, sulprostone were purchased from Cayman Chemical.

Generation and purification of bone marrow DC (BM-DC)

DC were generated in vitro from bone marrow. Briefly, femur and tibiae were removed from 6- to 8-wk-old male B10.A mice. Both ends of the bone were cut open and bone marrow cells were flushed out and washed with ice-cold RPMI 1640 medium (Invitrogen Life Technologies Research Laboratory). Bone marrow cells (2 × 10^6 cells) were cultured in 100-mm petri dishes containing 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Atlanta Biological), 2 mM L-glutamine, 50 μM 2-ME, and 20 ng/ml murine rGM-CSF (DC) or 1 μM rGM-CSF plus PGE (DCp). After 3 days, another 10 ml of complete medium containing GM-CSF or GM-CSF plus PGE was added to each dish. On day 8, the nonadherent cells were harvested and used as immature DC. The percentage of CD11c+ DC in the nonadherent population averaged 70–80% by FACS analysis. CD11c+ DC were purified by immunomagnetic sorting using anti-CD11c-coated magnetic beads and the auto-MACS system according to the manufacturer’s instructions (Miltenyi Biotec). The purity of the sorted cells was determined by FACS analysis (>96% for CD11c+ cells).

Isolation of CD4+ T cells

Purified naive CD4+ T cells were isolated from the spleen of pigeon cytochrome c fragment (PCCF)-specific TCR-Tg mice by positive immunomagnetic selection using anti-CD4 mAb magnetic beads (Miltenyi Biotec). The purified T cells were 98% CD4+ as determined by FACS analysis.

**FIGURE 2.** p19/p35/p40 expression and IL-23/IL-12p70 secretion in DCp and DC. A, Purified CD11c+ DC and DCp were treated with LPS (1 μg/ml) for various periods of times (3, 6, and 12 h). Total RNA was isolated and the expression of p19/p35/p40 was determined by real-time RT-PCR. One representative experiment of three is shown. B and C, DC and DCp were stimulated with LPS (1 μg/ml) and supernatants were harvested at different time points (3, 6, 12, and 24 h for IL-23) and (24 and 48 h for IL-12p40 and IL-12p70). The levels of cytokines were measured by ELISA. Data represent the mean ± SD of three experiments performed in triplicate.
Cytokine production

To analyze cytokine production by T cells, DC or Dcp (1 x 10^5 cells/well) were placed in 24-well plates, stimulated with LPS for 24 h, and pulsed with PCCF (5 μM) or PLP (20 μg/ml) for 2 h at 37°C (5% CO₂). After extensive washing, the DC/DcP were cocultured with PCCF-specific Tg CD4⁺ T cells (1 x 10^5 cells) for 4 days. Purified CD4⁺ T cells were rested in complete medium supplemented with 10 ng/ml IL-2 for 2 days and subjected to a secondary stimulation with Ag-pulsed DC/DcP, or restimulated with PMA (10 ng/ml) plus ionomycin (500 mM) for 4 h for intracellular cytokine staining.

Table II. Effect of PGE₂ on chemokine expression

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Chemokines

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*C Total RNA was isolated from DC cultured in the absence (DC) or presence of PGE₁ (1 μM). Expression of proinflammatory chemokines and chemokine receptors was evaluated using the RT² Profiler PCR array. Fold change indicates gene expression levels in DC generated in the presence of PGE₁ (DCP) divided by gene expression levels of regular DC. Two experiments are shown.

Flow cytometry analysis

For immunophenotyping, DC (1 x 10^6) were washed in PBS and incubated for 20 min at 4°C with FITC-conjugated anti-CD80, CD86, CD40, MHCI mAb and PE-conjugated anti-CD11c mAb (BD Biosciences). The specificity of the primary Abs was established with appropriate isotype-matched controls. After extensive washing, stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Results were collected for 10,000 cells and analyzed using CellQuest software from BD Biosciences.

Mouse proinflammatory chemokine and chemokine receptors microarray, and TLR-signaling pathway microarray

The expression of proinflammatory chemokines and chemokine receptors and mediators of the TLR-signaling pathway was evaluated by using the RT² Profiler PCR array based on SYBR green-containing PCR technique.
RNA was prepared from purified CD11c<sup>+</sup> DC and DCp. cDNA was prepared as described above. One microgram of cDNA was diluted with double-distilled H<sub>2</sub>O to a total of 100 μl. The experimental mixture was prepared by mixing the following components: 1225 μl of 2X SYBR-green containing PCR master mix, 98 μl of diluted cDNA, and 1127 μl of double-distilled H<sub>2</sub>O. A total of 25 μl of the mixture was loaded in each well of a 96-well plate precoated with primers for different genes. The PCR array was performed using the Stratagene Mx3005P<sup>®</sup> detector. PCR cycling conditions were 15 s at 95°C, 1 min at 60°C for 40 cycles followed by a melting point determination of dissociation curves. Cycle threshold values were determined by automated threshold analysis based on standard curves. Results were normalized to the housekeeping gene β-actin.

**Determination of NF-κBp65 nuclear translocation**

Nuclear protein extracts were isolated as described below, and the levels of NF-κBp65 were determined by using an ELISA kit (Imgenex).

**Preparation of nuclear extracts**

DC/DCp were plated in 6-well plates at a density of 1 x 10<sup>7</sup> cells/well in serum-free medium X-VIVO 20 (Cambrex BioScience), rested overnight, stimulated with LPS (1 μg/ml), PGE<sub>2</sub> (1 μM), or in combination for indicated time periods and washed twice with ice-cold PBS. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml apronin, 10 μg/ml leupeptin, 10 μg/ml pepstain, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM NaN<sub>3</sub>). After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration, and nuclei were isolated by centrifugation at 12,000 x g for 40 s. Pellet containing nuclei were washed once with 0.2 ml of ice-cold buffer A and lysed by incubation for 30–60 min on ice in 0.1 ml of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml apronin, 10 μg/ml leupeptin, 10 μg/ml pepstain, and 1 mM NaN<sub>3</sub>). Supernatants containing nuclear proteins were collected by centrifugation for 10 min at 14,000 rpm at 4°C. The protein concentration was determined by the Lowry’s method, and aliquots were stored at -80°C for further use. We used a 3 mg/ml protein concentration for the NF-κBp65 ELISA.

**FACS analysis for phosphorylated NF-κBp65**

DC/DCp were cultured in 24-well plates (1 x 10<sup>6</sup> cells/ml), rested overnight in serum-free medium, stimulated with LPS (1 μg/ml), PGE<sub>2</sub> (1 μM), or in combination for the indicated time periods. Following incubation, the cells were fixed and permeabilized with Cytofix/Cytoperm according to the manufacturer’s instructions. The cells were incubated with rabbit polyclonal anti-phosphorylated NF-κBp65 or total NF-κBp65 Abs at room temperature for 60 min, followed by the appropriate Alexa-conjugated goat anti-rabbit IgG for 30 min (Invitrogen). After extensive washing, the cells were analyzed by FACS.

**In vivo experiments**

DC or DCp generated from B10.A mice were treated with LPS and pulsed with PCCF (5 μM) or PLP (20 μg/ml) overnight. After washing, 1 x 10<sup>7</sup> DC or DCp were i.p. injected into syngeneic PCCF-specific TCR-Tg mice (six mice per group). PLP-pulsed DC/DCp were used as control. One week later, CD4<sup>+</sup> T cells were harvested from spleen or from pooled inguinal and mesenteric lymph nodes and restimulated ex vivo with fresh DC or DCp in the presence or absence of PCCF (5 μM). The ratio of DC: T cells was 1:10. Supernatants were harvested 4 days later and assayed for IL-17 and IFN-γ by ELISA. In other experiments, spleen and lymph node cells were harvested from mice inoculated with Ag-pulsed DC/DCp, cultured in the presence or absence of PCCF (5 μM), and the supernatants were tested for cytokine release 4 days later by ELISA. In a parallel experiment, splenocytes were restimulated with PMA (10 ng/ml) plus ionomycin (500 nm) for 4 h and the numbers of CD4<sup>+</sup>IL-17<sup>+</sup> and IFN-γ<sup>-</sup> cells was detected following intracellular staining.

**Statistics**

The results were expressed as mean ± SD of at least three independent experiments. The Student t test was used to compare control and experimental groups. Statistical significance was based on p values <0.05.

**Results**

**DCp cytokine and chemokine profile following LPS stimulation**

DCp were differentiated from bone marrow cells in the presence of GM-CSF and PGE<sub>2</sub> (added on days 1 and 3), whereas DC were differentiated in the presence of GM-CSF without PGE<sub>2</sub>. Immature DC/DCp harvested as nonadherent cells on days 7–8 were washed extensively and immunomagnetically purified. CD11c<sup>+</sup> DC and DCp were stimulated with LPS, and cytokine levels were determined by ELISA. Compared with immature DC and DCp, the LPS-stimulated DC/DCp produced high levels of IL-1β, IL-6, and TNF-α (Fig. 1A). Compared with DC, DCp secreted consistently higher levels of cytokines, particularly TNF-α (Fig. 1A). However, in contrast to DC, LPS-stimulated DCp were deficient in IL-12p70.
DCp express higher levels of MHCII and costimulatory molecules

Immature and LPS-stimulated DC and DCp were analyzed in terms of MHCII and CD80, CD86, and CD40 expression by FACS. Compared with DC, immature DCp express higher levels of MHCII and costimulatory molecules.

In terms of chemokine profile, DCp expressed higher levels of proinflammatory chemokines, with maximum induction of CXCL4 and CXCL10 (~27- and 23-fold increase over the basal levels) for 3 and 8 h. Total RNA was isolated and the cytokine/chemokine profile, the higher level of MHCII and costimulatory molecule expression suggests that DCp are primed for enhanced proinflammatory activity following TLR signaling.

The effect of PGE$_2$ on p19/IL-23 expression is mediated through EP2/EP4 receptors and is mimicked by forskolin

To determine which PGE$_2$ receptors are involved in the up-regulation of p19/IL-23 by PGE$_2$, DC were differentiated in the presence of PGE$_2$, sulprostone (EP1/EP3 agonist), butaprost (EP2-specific agonist), or misoprostol (EP2/EP4/EP3 agonist). Following purification, CD11c+ DC were treated with or without LPS and the levels of p19 expression were determined by real-time RT-PCR. LPS treatment of DC differentiated in the presence of sulprostone resulted in p19 levels similar to control (DC generated in the absence of LPS). The p19 expression also differ in terms of kinetics, with p19 maximum expression in unstimulated and LPS-stimulated DC/DCp. P19 is highly expressed in DCp, whereas p35 is expressed primarily in DC (Fig. 2A). Both DC and DCp express similar levels of p40. The RNA results were confirmed at the protein level. Although DC and DCp secrete similar levels of IL-12p40, they differ in terms of IL-12p70 and IL-23 production, with DCp secreting IL-23 and very little, if any, IL-12p70, and DC producing high levels of IL-12p70 and less IL-23 than DCp (Fig. 2, B and C). The p19 and p35 expression also differ in terms of kinetics, with p19 maximum expression at 3 h, and p35 at 6 h (Fig. 2A). At the protein level, we observed maximum IL-23 and IL-12p70 release at 12 and 24 h, respectively.

In terms of chemokine profile, DCp expressed higher levels of proinflammatory chemokines, with maximum induction of CXCL4 and CXCL10 (~27- and 23-fold increase over the amounts produced by DC, respectively) (Table I).

DCp exhibit higher levels of TLR-4 expression. CD11c+ DCs were generated in the absence (DC) or presence (DCp) of PGE2 (1 μM). A. RNA was isolated from DC and DCp cultured for 3 h in medium and expression of TLR4 was determined by real-time RT-PCR. One experiment of two is shown. B. DC or DCp were cultured in the absence or presence of LPS (1 μg/ml) for 3 and 8 h. Total RNA was isolated and the expression of TLR4 was determined by real-time RT-PCR. One experiment of three is shown.

Table III. Effects of PGE$_2$ on NF-κB-signaling molecules

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* Total RNA was isolated from DC generated in the absence (DC) or presence of PGE$_2$ (1 μM) (DCp). Expression of NF-κB signaling molecules was evaluated by using a RT2 Profiler PCR array as described in Materials and Methods. Three experiments are shown.
cAMP in p19/IL-23 expression, we compared DC differentiated in the presence of PGE2 (DCp) with DC differentiated in the presence of forskolin (DCFK). DC, DCp, and DCFK were treated with or without LPS followed by measurements of p19 expression and IL-23 release. Both DCp and DCFK expressed higher levels of p19 and released higher levels of IL-23, as compared with DC (Fig. 3, B and C), suggesting that agents that increase cAMP are capable of priming differentiating DC to produce higher levels of IL-23.

Effects of PGE2 on TLR expression and NF-κB-related genes

Exposure to PGE2 during differentiation leads to DC priming, as manifested by enhanced production of a number of cytokines and chemokines (Fig. 1 and Table I). A higher PGE2-induced expression of TLR4 is an obvious possibility. We compared unstimulated DC and DCp in terms of TLR4 mRNA expression, and detected a slight, but statistically significant, increase in DCp TLR4 expression (Fig. 4A). Upon treatment with LPS, TLR4 expression (at 3 and 6 h) was significantly reduced in both DC and DCp, but remained consistently higher in DCp (Fig. 4B).

Next, DC, DCp, and fully differentiated DC treated with PGE2 for 24 h (DC plus PGE2) were analyzed in terms of expression of a panel of genes relevant to TLR signaling using the RT2 Profiler PCR array for molecules involved in TLR-signaling pathways. DCp expressed higher levels of various TLRs, particularly TLR2 and 9, of genes related to the NF-κB pathway such as IL-1R-associated kinase 1 (IRAK1) and IκB kinase (IKK), and of MKK3 (involved in the JNK/p38 pathway) (Table III). These results are in agreement with our previous observation that exposure to PGE2 during DC differentiation has a stimulatory effect on the expression of most cytokine/chemokine genes. The RT2 Profiler array data suggest that the effects of PGE2 are mediated mostly through effects on NF-κB signaling.

PGE2 affects LPS-induced NF-κB p65 phosphorylation and nuclear translocation

To confirm the effect of PGE2 on differentiating DC in terms of NF-κB activation, we quantified the amounts of phosphorylated p65 in DC/DCp treated with LPS. Even in the absence of LPS, DCp express higher levels of Pp65, indicative of a preactivated stage (Fig. 5A, left panels). An increase in Pp65 was observed in both LPS-treated DC and DCp at 15 and 30 min. The numbers of cells expressing Pp65 were higher in LPS-treated DCp than regular DC. In addition, the amounts of active p65 in nuclear extracts, as determined by ELISA, were higher for LPS-treated DCp than regular DC (Fig. 5B).
**DCp preferentially induce IL-17-producing T cells**

To determine the effect of DC exposed to PGE2 during differentiation (DCp) on T cell differentiation, we cocultured various numbers of DC/DCp stimulated with LPS and pulsed with PCCF with TCR-Tg PCCF-specific splenic CD4+ T cells. DC and DCp induced similar levels of T cell proliferation (results not shown). In contrast, we observed significant differences in the Th17/Th1/Th2 profiles, based on secretion of IL-17, IFN-γ, and IL-4. Whereas DC induced mostly IFN-γ, and to a lesser degree IL-4 production (Th1/Th2), and no significant IL-17 release, DCp had the opposite effect, inducing high levels of IL-17, and reduced levels of IFN-γ and IL-4 (Fig. 6A). The preferential induction of IL-17-producing T cells by DCp was confirmed by FACS analysis for intracellular expression of IFN-γ and IL-17. T cells cocultured with DC contained a substantial IFN-γ population, whereas T cells cocultured with DC had few high expressing IFN-γ cells (Fig. 6B, upper panels). In contrast, there were few IL-17-expressing T cells in cultures containing DC, whereas T cells cocultured with DCp contained a substantial population of IL-17+ T cells (Fig. 6B, lower panels).

Differentiation and proliferation/survival of murine Th17 effectors requires IL-6, TGF-β, and IL-23, and is negatively affected by IFN-γ. To investigate the possible role of these cytokines in the DCp induction of IL-17-producing T cells, we cocultured LPS-stimulated, PCCF-pulsed DCp with PCCF-specific Tg T cells in the presence of various neutralizing Abs or rIFN-γ. Anti-IL-6, anti-TGF-β, anti-p40 (IL-12 and IL-23), and rIFN-γ partially inhibited the induction of IL-17 release, whereas the combined treatment (all three Abs plus IFN-γ) reduced IL-17 production to control levels (nonspecific Ag–PLP) (Fig. 7, upper panel). This indicates that the effect of DCp is mediated through the release of IL-6, TGF-β, and IL-23, and the inhibition of IFN-γ production. As a control, we determined the effects of rIFN-γ and anti-IFN-γ Abs on IL-17 induction by regular DC. As expected, IFN-γ reduced IL-17, and the inclusion of the neutralizing anti-IFN-γ Ab enhanced IL-17 production (Fig. 7, lower panel).

**DCp capacity to affect the IL-17/IFN-γ balance depends on the timing of exposure to PGE2**

To evaluate the effect of PGE2 exposure at different times during DC differentiation, we generated DC in the presence of PGE2 added on days 0, 4, and 6. First, we determined p19 expression in purified unstimulated and LPS-stimulated DC/DCp. LPS-stimulated DC (generated in the absence of PGE2) expressed low p19 levels. In contrast, DC generated in the presence of PGE2 (DCp) expressed higher p19 levels, and the expression increased with prolonged PGE2 exposure (highest for DCp(D0)–DC exposed to PGE2 since day 0; lowest for DCp(D6)–DC exposed to PGE2 on day 6) (Fig. 8A). As expected, DCp(D0) induced the highest levels of IL-17 production when cocultured with PCCF-specific Tg T cells (Fig. 8B). In agreement with our previous results (Fig. 6), the increase in IL-17 was associated with a decrease in IFN-γ production (Fig. 8C), suggesting that DCp affect Th17/Th1 differentiation in an opposite manner.
neutralizing anti-p19 (20 μg/ml) and stimulated with PCCF-pulsed DC/DCp, with PCCF-specific CD4+ T cells (1 × 106/well) in the presence or absence of neutralizing anti-p19 (20 μg/ml), anti-p40 (20 μg/ml), anti-IFN-γ (10 μg/ml), rIFN-γ (10 ng/ml), or αAb mixtures for 72 h. PLP (20 μg/ml) was used as a negative control. Supernatants were tested for IL-17 production by ELISA. Data represent the mean ± SD of three experiments performed in triplicate.

**FIGURE 7.** DCp promote IL-17 responses through multiple cytokines. Purified CD11c+ DC/DCp (1 × 106/well) were stimulated with LPS (1 μg/ml) for 24 h, pulsed with PCCF (5 μM), and cocultured with PCCF-specific CD4+ T cells (1 × 106 cells/well) in the presence or absence of neutralizing anti-p19 (20 μg/ml), anti-p40 (20 μg/ml), anti-IL-6 (20 μg/ml), anti-TGF-β (20 μg/ml), anti-IFN-γ (10 μg/ml), rIFN-γ (10 ng/ml) or Ab mixtures for 72 h. PLP (20 μg/ml) was used as a negative control. Supernatants were tested for IL-17 production by ELISA. Data represent the mean ± SD of three experiments performed in triplicate.

**Effects of DCp on naive and activated T cells**

To assess whether DCp have similar effects on naive and activated T cells, we cocultured LPS-stimulated, PCCF-pulsed DC/DCp with unstimulated or anti-CD3/-CD28-stimulated T cells. Although the levels of both IL-17 and IFN-γ were higher in cultures containing activated T cells, the effect of DCp followed a similar pattern in both unstimulated and anti-CD3/-CD28-stimulated T cells, inducing IL-17 and reducing IFN-γ production (Fig. 9A).

In a second set of experiments, we tested the effects of DC/DCp during primary and secondary Ag-specific stimulation. We cocultured LPS-stimulated, PCCF-pulsed DC/DCp with PCCF-specific Tg CD4+ T cells (primary stimulation) and determined the amounts of secreted IL-17 and IFN-γ. As shown previously, DCp induced higher levels of IL-17 and inhibited IFN-γ release (Fig. 9B). T cells were then repurified from the DC/DCp cocultures and re-exposed to LPS-stimulated, PCCF-pulsed DC or DCp (secondary stimulation). T cells exposed only to DC (both primary and secondary stimulation) did not produce IL-17. As expected, T cells exposed to DCp either during primary or secondary stimulation secreted detectable levels of IL-17. The highest IL-17 amounts by far were secreted by T cells exposed to DCp during both primary and secondary stimulation (Fig. 9C, upper panel). However, in contrast to primary stimulation where DCp reduced IFN-γ secretion, restimulated T cells secrete similar levels of IFN-γ regardless of their exposure to DC or DCp (Fig. 9C, lower panel).

**Role of endogenous PGE2 in Th17 differentiation**

DC were reported to produce and secrete PGE2 upon LPS stimulation. To assess the role of endogenous PGE2, we included the Cox (1/2) inhibitor ibuprofen during the LPS treatment of DC/DCp, and during T cell differentiation in the presence of PCCF-pulsed DC/DCp. As described above, DCp induced high levels of IL-17, and reduced IFN-γ and IL-4 production, in comparison to control DC. However, the effect does not appear to be due to endogenous PGE2, because the presence of ibuprofen only slightly reduced the levels of secreted cytokines (Fig. 10C). The concentration of ibuprofen used in these experiments does not affect cytokine release by purified T cells stimulated with anti-CD3/-CD28 (Fig. 10D), although it substantially reduces the levels of secreted PGE2 in DC/DCp (Fig. 10B).

**In vivo effects of DCp on IL-17/IFN-γ production**

To determine whether DCp induce preferentially an IL-17 response in vivo, we injected PCCF- or PLP-pulsed DC/DCp i.p. into MHCII-compatible PCCF-TCR Tg mice. One week later, spleen and draining lymph node (DLN; inguinal and mesenteric lymph nodes) cells were restimulated ex vivo with PCCF, and proliferation and IL-17 and IFN-γ release were determined 4 days later. Also, spleen cells harvested from mice inoculated with PCCF-pulsed DC or DCp were restimulated ex vivo with ionomycin and PMA and analyzed by FACS for surface CD4 and intracellular IFN-γ and IL-17 expression. We observed a slight increase in the proliferation of spleen and DLN cells from mice inoculated with PCCF-pulsed DCp (Fig. 11A). Spleen cells from mice inoculated with PCCF-DC produced low IL-17 and high IFN-γ levels. In contrast, spleen cells from mice inoculated with PCCF-DC had the opposite cytokine profile, with high IL-17 levels and essentially no IFN-γ (Fig. 11B). Similar results were obtained with DLN cells (results not shown). In agreement with the ELISA results, we observed an increase in the percentage of IL-17+ and a decrease in the percentage of IFN-γ- cells in the CD4+ T cell population obtained from PCCF-pulsed DCp-inoculated animals (Fig. 11C).

In a second series of experiments, purified CD4+ T cells from mice inoculated with PLP-DC/DCp or PCCF-DC/DCp were restimulated ex vivo with PCCF-pulsed DC/DCp. In vivo exposure of T cells to PCCF-pulsed DC followed by restimulation with control DC results in high IFN-γ, intermediate IL-4, and no IL-17 production. If, however, T cells exposed in vivo to PCCF-pulsed DC were restimulated ex vivo with DCp, there was an increase in IL-17 and a decrease in IFN-γ and IL-4 release (Fig. 11D). In contrast to initial exposure to PCCF-pulsed DC, the in vivo exposure to PCCF-pulsed DCp followed by restimulation with DC or DCp results in higher IL-17 and lower IFN-γ release. IL-17 production was increased ∼10-fold when both the initial exposure and
the restimulation occurred in the presence of DCp, compared with regular DC (Fig. 11D).

Discussion
In addition to bone marrow-derived steady-state DC residing in lymphoid and nonlymphoid tissues, inflammatory DC, such as monocyte-derived DC, develop following infection or inflammation (reviewed in Ref. 43). Because PGE₂ is produced by bone marrow stromal cells and by various cell types during inflammatory processes, both steady-state and inflammatory DC may be exposed to PGE₂ during differentiation and/or maturation.

The effect of PGE₂ on DC function has been studied extensively. However, the majority of studies focused on PGE₂ effects during DC maturation, not differentiation. Presently, there are only two studies on PGE₂ effects during human monocyte-derived DC differentiation reporting opposite results in terms of Th1/Th2 differentiation (12, 37). We found that murine bone marrow-derived control DC secrete high levels of IL-12p70 and very little, if any, IL-10, whereas DC differentiated in the presence of PGE₂ (DCp) have the opposite cytokine profile. Although the induction of IL-10 and inhibition of IL-12p70 suggest an anti-inflammatory response, a more comprehensive analysis of the cytokine and chemokine profile showed that the presence of PGE₂ during differentiation primes DC for a strong, widespread proinflammatory response to LPS. DCp produce significantly higher levels of proinflammatory cytokines (IL-1β, TNF-α, IL-6, IL-23) and chemokines (CCL12, CCL19, CXCL4, CXCL10), and show increased expression of MHCII and costimulatory molecules. In addition, DCp pulsed with specific Ag induce a preferential Th17 response both in vitro and in vivo, supporting their proinflammatory role.

The expression of most proinflammatory cytokines/chemokines depends on NF-κB p65/p50 transactivation. LPS is a strong inducer of NF-κB activation through the TLR-4-signaling cascade, which involves the MyD88/IRAK/TNFR-associated factor 6 (TRAF6) pathway (reviewed in Refs. 44 and 45). Our results indicate that DCp exhibit higher levels of TLR-4 expression, higher levels of IRAK1 and IKK, and higher levels of MEKK3, the p38 activator. IRAK1, IKK, and p38 are essential participants in NF-κB activation, with IRAK1 and IKK as components of the cascade transducing the signal from multiple TLRs to NF-κB activation (46, 47), and p38 MAPK participating in the phosphorylation and activation of p65RelA (48). In agreement with the role of p65 in proinflammatory cytokine/chemokine gene expression, our results indicate enhanced p65 phosphorylation and nuclear translocation in DCp, compared with regular DC. The effects on differentiating DC appear to be long-lasting, because bone marrow cells were exposed to PGE₂ on days 1 and 3, harvested and purified on day...
several authors reported that PGE2 substantially decreased IFN-DC and TCR-Tg CD4 different, consisting of LPS-stimulated, Ag-pulsed DCp or control DC or pDC in the presence of PCCF for 4 days (secondary stimulation). Supernatants were collected and subjected to ELISA for IL-17 and IFN-γ. Data represent the mean ± SD of three experiments performed in triplicate.

In two recent publications, cAMP has been shown to be involved in the down-regulation of IL-12p70 production in DCp. Because the IL-12/IL-23 balance is important in T cell differentiation, we compared DCp and DC in terms of IL-12 (p35/p40) and IL-23 (p19/p40) expression and release. Exposure to PGE2 during DC differentiation results in an almost complete IL-12p70 shutoff and a significant up-regulation of IL-23, paralleled by the up-regulation of p19 and down-regulation of p35 expression. The effect of PGE2 on p19 expression involves EP2, and possibly EP4 receptors, and appears to be mediated through increases in intracellular cAMP, because the effect of PGE2 is mimicked by forskolin. In two recent publications, cAMP has been shown to be involved in the down-regulation of IL-12p35 and up-regulation of IL-23p19 in human DC (49, 50). Analysis of the p19 promoter indicates additional putative binding sequences for the transcription factors C/EBP and CREB. PGE2 has been previously described to phosphorylate CREB and to induce C/EBP nuclear translocation (51–54). Therefore, both CREB and C/EBP are potential targets for the positive transcription effect of PGE2 on p19 gene expression.

Following antigenic stimulation, CD4+ T cells differentiate into three major effector subsets, i.e., Th1, Th2, and Th17. The role of PGE2 in Th1/Th2 differentiation is still controversial. Whereas several authors reported that PGE2 substantially decreased IFN-γ production, implying a suppressive effect on Th1 differentiation (37–40), some reports indicate that PGE2 may actually support the development of Th1 cells (11, 55). Our experimental system is different, consisting of LPS-stimulated, Ag-pulsed DCp or control DC and TCR-Tg CD4+ T cells. Although DCp were differentiated in the presence of PGE2, there was no direct exposure to PGE2 of the fully differentiated DC/DCp or of T cells. In this system, we consistently saw decreases in both IFN-γ and IL-4 production for T cells exposed to DCp, suggesting impairment in both Th1 and Th2 differentiation.

Th17 cells were discovered recently as producers of IL-17, IL-22, and IL-25, and to a lesser degree of TNF-α and IL-6. IL-17 is a potent inflammatory cytokine, which induces the expression of numerous proinflammatory cytokines and chemokines, and metalloproteinases, and plays a major role in neutrophil proliferation/survival and chemotaxis (reviewed in Refs. 23 and 30). There is recent evidence that Th17 effectors are the major participants in autoimmune/inflammatory disorders (reviewed in Refs. 24, 29, 56, 57). In mice, Th17 differentiation requires TGF-β and IL-6, is amplified by IL-1β, and Th17 proliferation and/or survival requires IL-23 (reviewed in Refs. 29 and 56).

The role of PGE2 in Th17 differentiation is not known. We were the first to report that PGE2-treated immature and mature DC stimulate IL-17 production by anti-CD3-activated CD4+ T cells, partially through the induction of IL-23, and that in vivo administration of the PGE analog misoprostol results in IL-23→IL-17-mediated exacerbation of murine collagen-induced arthritis and hapten-induced colitis (41, 42, 58). In the present study, we assessed the capacity of DC differentiated in the presence of PGE2 (DCp) to induce Th17 differentiation. We found that naive TCR-Tg CD4+ T cells cultured in the presence of Ag-pulsed DCp produced much higher levels of IL-17 compared with T cells cultured with control DC. This was associated with a decrease of ~40 and 60% in IFN-γ and IL-4 production, respectively. The increase in IL-17 production appears to be due to a combination of cytokines, including IL-6, TGF-β, and IL-23 and low levels of IFN-γ, because a combined treatment with the corresponding neutralizing
Abs and rIFNγ reduced IL-17 levels to control values. Because DCp produce high levels of IL-6, IL-1β, and IL-23, they are the most probable source for the cytokines involved in IL-17 production. The capacity of DCp to affect the IL-17/IFN-γ balance correlates with the levels of p19 expression, suggesting that although IL-23 is not the only responsible factor, it might be the determining one.

Although anti-CD3/CD28-activated T cells produce much higher levels of IL-17 and IFN-γ than naive T cells, in both cases the IL-17/IFN-γ balance is in favor of IL-17 when T cells are cultured with DCp, and in favor of IFN-γ in cultures with control DC. If T cells saw the Ag presented by DCp either during primary stimulation or during restimulation they produced higher levels of IL-17, compared with T cells that were exposed only to control DC. If both the initial stimulation and the restimulation were done with DCp, the IL-17 levels increased an additional 4-fold. This suggests that DCp provide the signals not only for the initial Th17 differentiation but also for the amplification of the IL-17 response. We consider the increased levels of IL-23 expression in DCp as the most probable factor responsible for this amplification step.

Because LPS is a known inducer of endogenous PGE2 in DC, the possibility exists that the effect of DCp might be mediated by endogenous PGE2. The first indication that this is probably not the case was the fact that DCp treated with LPS expressed much lower levels of Cox2 and secreted approximately three times less PGE2 than LPS-treated DC. If endogenous PGE2 was responsible for the induction of IL-17 and inhibition of IFN-γ, the reverse should be observed in the presence of a Cox inhibitor. However, the Cox1/Cox2 inhibitor ibuprofen did not reverse the IL-17/IFN-γ pattern, although, as expected, it did inhibit PGE2 release from both LPS-treated DC and DCp.

In previous studies, we established a correlation between the exacerbation of clinical symptoms following administration of PGE2 analogs in models of RA and inflammatory bowel disease and increased IL-23 and IL-17 expression in draining lymph nodes and affected tissue/organisms (42, 58). In this study, we present more direct evidence that DC exposed to PGE2 play a role in the induction of IL-17-producing T cells. Spleen and DLN cells harvested from mice inoculated with Ag-pulsed DCp secrete higher levels of IL-17 and lower levels of IFN-γ, and
contain higher percentages of IL-17+ CD4+ T cells. In addition, using a first stimulation in vivo with DC/DCp, and a second ex vivo restimulation with Ag-pulsed DC or DCp, we were able to determine that a single exposure to Ag-pulsed DCp, whether in vivo or ex vivo, results in increases in IL-17, and decreases in IFN-γ production. Based on these results, we propose that DC generated in the presence of PGE2 acquire the ability—upon maturation and migration to lymph nodes and Ag presentation to cognate naive T cells—to induce differentiation of TH17 effectors, and to promote their subsequent survival/proliferation. This is most probably mediated through a broad range of proinflammatory cytokines, including IL-1β, IL-6, and IL-23, and through the inhibition of TH1-derived IFN-γ production.

One of the important questions raised by this model is where and when would differentiating DC encounter PGE2. One possible site is the bone marrow itself, because macrophages, mesenchymal stem cells, and stromal preadipocytes produce and secrete PGE2 (59–61). In addition, DC also differentiate from transendothelial migrating blood monocytes (62). Because the migration occurs in response to an inflammatory process in the neighboring tissue, the differentiating DC could be exposed to high levels of PGE2 generated by immune and nonimmune cells at the inflammatory site.

Disclosures
The authors have no financial conflict of interest.

References


