

# Cyclooxygenase-2-Issued Prostaglandin E<sub>2</sub> Enhances the Production of Endogenous IL-10, Which Down-Regulates Dendritic Cell Functions

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PGE<sub>2</sub> is a well-known immunomodulator produced in the immune response by APCs, such as dendritic cells (DCs), the most potent APC of the immune system. We investigated the PGE<sub>2</sub> biosynthetic capacity of bone marrow-derived DC (BM-DC) and the effects of PG on the APC. We observed that BM-DC produce PGE<sub>2</sub> and other proinflammatory mediators, such as leukotriene B<sub>4</sub> and NO, after LPS exposure. Constitutively present in BM-DC, cyclooxygenase (COX)-1 did not contribute significantly to the total pool of PGE<sub>2</sub> compared with the LPS-induced COX-2-produced PGE<sub>2</sub>. Treatment of BM-DC with exogenous PGE<sub>2</sub> induced the production of large amounts of IL-10 and less IL-12p70. In addition, selective inhibition of COX-2, but not COX-1, was followed by significant decrements in PGE<sub>2</sub> and IL-10, a concomitant restoration of IL-12 production, and an enhancement of DC stimulatory potential. In contrast, we found no demonstrable role for leukotriene B<sub>4</sub> or NO. In view of the potential of PGE<sub>2</sub> to stimulate IL-10, we examined the possibility that the suppressive effect of PGE<sub>2</sub> is mediated via IL-10. We found that exogenous IL-10 inhibits IL-12p70 production in the presence of NS-398, a COX-2 selective inhibitor, while the inhibitory effects of PGE<sub>2</sub> were totally reversed by anti-IL-10. We conclude that COX-2-mediated PGE<sub>2</sub> up-regulates IL-10, which down-regulates IL-12 production and the APC function of BM-DC. *The Journal of Immunology*, 2002, 168: 2255–2263.

Dendritic cells (DC),<sup>3</sup> the most powerful APC of the immune system (1, 2), are critical for the induction of primary immune responses and immunological tolerance (3) as well as for the regulation of Th1 and Th2 immune responses (4). The Th1/Th2 balance is modulated by several APC-derived factors, among them, cytokines and PGs. PGE<sub>2</sub> is one of the most Th2-promoting factors and has been described as a potent modulator of both inflammation and immune responses (5, 6).

PGE<sub>2</sub> is thought to suppress cell-mediated immune response while enhancing humoral immune responses (7–9). In macrophages the down-regulation of cell-mediated immune responses by PGE<sub>2</sub> is suggested by a dramatic reduction in LPS-mediated TNF- $\alpha$  and IL-12 production (10, 11). PGE<sub>2</sub> modulates a variety of physiological processes, including APC function (12) and the production of inflammatory cytokines (13). Previous studies reported that PGE<sub>2</sub> is a strong inhibitor of IL-12 production (11, 14), which implies a feedback mechanism at the level of the APC (11). Both PGE<sub>2</sub> and IL-12 are produced by monocytes, macrophages, and other APCs in response to a variety of compounds, including

bacterial products (15), and have opposite effects on Th1 and Th2 responses.

PGE<sub>2</sub> is a potent inducer of IL-10 (11, 14, 16), which is produced by a variety of cells including monocytes (17), and exerts suppressive effects on Th1 responses (18–20). Similar to PGE<sub>2</sub>, IL-10 inhibits both the ability of DC to produce IL-12 (21, 22) and their stimulatory capacity (12, 21, 23), inducing the development of a tolerogenic type of DC (23, 24). Because PGE<sub>2</sub> triggers the synthesis of IL-10 that is known to inhibit important aspects of cell-mediated immunity (25), we investigated the hypothesis that PGE<sub>2</sub> modulates the DC-mediated immune response via the induction of IL-10.

PGE<sub>2</sub> is synthesized from arachidonic acid (AA), which can be rapidly liberated from membrane phospholipids by the action of phospholipases, in particular phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (26). The critical step in the formation of proinflammatory PGs is the oxygenation of free AA by cyclooxygenase (COX) enzymes (also named PGH synthase or PG endoperoxide synthase), (27). Two isoforms of COX exist. The type I enzyme (COX-1) is constitutively present in many cell types, whereas type II (COX-2) is usually absent under basal conditions but can be induced by several stimuli, such as cytokines and other factors (28). LPS is a strong inducer of COX-2, which is responsible for the elevated production of PGs during inflammatory processes (29, 30). LPS can also increase PLA<sub>2</sub> phosphorylation and AA release (31). To study the respective role of each isoform of COX in PGE<sub>2</sub> production and APC functions, we used drugs, such as indomethacin, a COX-1 preference inhibitor (32, 33); SC-560, a COX-1-selective inhibitor (34); and NS-398, a COX-2-selective inhibitor (35, 36).

Because IL-12 production by APC is central to the orchestration of both innate and acquired cell-mediated immune responses to many pathogens (37), in contrast to PGE<sub>2</sub> and IL-10, we examined the modulation of IL-12 production by bone marrow-derived DC (BM-DC). We used LPS-mediated stimulation and COX-selective inhibitors to investigate mouse BM-DC phenotype, function, and cytokine production.

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Received for publication July 31, 2001. Accepted for publication January 2, 2002.

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<sup>1</sup> This work was supported by La Ligue Régionale Contre Le Cancer Comité Départemental des Charentes and Comité Départemental de Gironde.

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; AA, arachidonic acid; BM-DC, bone marrow-derived DC; COX, cyclooxygenase; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; cPLA<sub>2</sub>, cytosolic PLA<sub>2</sub>; L-NMMA, N<sup>G</sup>-monomethyl-L-arginine; 5-LO, 5-lipoxygenase; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; NDGA, nor-dihydroguarinic acid; SNAP, S-nitroso-N-acetyl-D,L-penicillamine.

We demonstrate that although BM-DC express both isoforms of COX, production of PGE<sub>2</sub> derives largely from COX-2 after LPS exposure. In agreement with other investigators (16), our study shows that COX-2-produced PGE<sub>2</sub> is a strong inducer of IL-10 and plays a pivotal role in the reciprocal regulation of IL-10 and IL-12 balance. By contrast, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and NO produced in response to LPS did not affect BM-DC cytokine production. In these studies we clearly demonstrate that the suppressive activity of PGE<sub>2</sub> on DC-mediated responses is induced by the endogenously produced IL-10.

## Materials and Methods

### Media and reagents for cell culture

Complete medium was RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (Dominique Dutscher, Brumath, France), 1% streptomycin (Life Technologies; 1000 µg/ml), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 50 µM 2-ME (Sigma-Aldrich), and 2 mM sodium pyruvate (Life Technologies). Growth factors used for the generation of DC were recombinant mouse GM-CSF (20 ng/ml), IL-4 (10 ng/ml; purchased from PeproTech, Rocky Hill, NJ), and TNF-α (10 ng/ml; Valbiotech, Paris, France).

### COXs and lipoxygenase inhibitors

Inhibitors used in our experiments were indomethacin, a COX-1 preference inhibitor; nor-dihydroguaric acid (NDGA), a 5-lipoxygenase (5-LO) inhibitor (Sigma-Aldrich); NS-398, a COX-2-specific inhibitor; and SC-560, a COX-1 selective inhibitor (Cayman Chemicals, Ann Arbor, MI). These drugs were dissolved in absolute ethanol (Merck, Darmstadt, Germany) and stored as stock solutions of 1 mM at -20°C. The required dilutions were prepared immediately before use, and equivalent quantities of ethanol were added to the cultures to serve as controls for these agents.

### DC generation

DC were generated *in vitro* from mouse bone marrow as we previously reported (12, 38, 39). At the end of the culture period DC were positively purified using anti-CD11c (N418) microbeads and a MACS system column (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified BM-DC (routinely >98% CD11c<sup>+</sup>) were used for phenotypic, molecular, and functional analyses.

### FACS analysis

Cells were subjected to analysis with a three-color FACSCalibur (BD Biosciences, Mountain View, CA) using the following mouse Abs: anti-CD11c-PE, anti-CD40-FITC, anti-CD80-PE, anti-CD86-PE, and anti-I-A<sup>d</sup>-PE (BD PharMingen Europe, Pont de Claix, France), and data were collected for 1 × 10<sup>4</sup> cells. The primary Ab was directed toward a panel of cell surface markers and compared with their appropriate isotype-matched controls: hamster IgG1A-PE, rat hamster IgM-FITC, hamster IgG-PE, rat IgG2a-PE, and mouse IgG2b-PE, respectively (BD PharMingen, San Diego, CA). Cells showing a typical dendritic morphology were always >95%, and the DC marker CD11c was always present on >98% of the cells. Less than 1% of cells expressed the CD14 marker. Expression of these markers was also analyzed by FACS on DCs matured for 48 h in the presence of COX inhibitors.

### Preparation of DC cytoplasmic extracts and Western blot analysis

BM-DC obtained at 98% purity from bone marrow cells *in vitro* were washed twice with PBS and lysed in ice-cold lysis buffer containing 10 mM HEPES (pH 7.6), 3 mM MgCl<sub>2</sub>, 40 mM KCl, 2 mM DTT, 0.5% Nonidet P-40, 8 µg/ml aprotinin, 8 µg/ml leupeptin, and 10 µg/ml PMSF. Nuclei were removed by centrifugation (1250 × g) at 4°C for 5 min. The bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) was used for analyzing the protein concentration. Cytoplasmic extracts (15 µg protein/lane) were resolved on 7% SDS-polyacrylamide gels, and Western blotting analysis was performed using an ECL kit (Amersham, Little Chalfont, U.K.). The blots were probed with specific Abs directed against COX-1 (1/1,000 dilution; Cayman Chemicals), COX-2 (1/500 dilution; BD Transduction Laboratories, Lexington, KY), cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>; 1/1,000; Santa Cruz Biotechnology, Santa Cruz, CA), or α-tubulin (2/10,000; Sigma-Aldrich). The blots were subsequently incubated with the secondary Ab (peroxidase-labeled anti-mouse Ab; 2/1,000 dilution; Amersham) and an-

alyzed by an Amersham ECL and Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

### Quantification of released AA

Samples (DC and LPS-stimulated DC) with 10 million cells (pellet) were used for the fatty acid analysis; heptadecanoyl acid (C17; 10 mg/sample) was added as an internal standard. Lipids were extracted according to the method of Folch et al. (40). The organic phase was evaporated to dryness under nitrogen, and transesterification was conducted with 2.5% H<sub>2</sub>SO<sub>4</sub> in methanol for 1 h at 80°C. Fatty acid methyl esters were then extracted with 1 vol hexane/H<sub>2</sub>O (1/1, v/v) and analyzed in Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a Carbowax capillary column and a flame ionization detector. Fatty acid methyl esters were identified by comparison of relative retention times with standards and were quantified by comparison with the internal standard.

### Effects of LPS on PGE<sub>2</sub> production

Immature BM-DC were seeded at 1 × 10<sup>6</sup> cells/ml into six-well plates (Costar, Cambridge, MA) for an additional 48 h at 37°C in complete medium with increasing concentrations of LPS (*Escherichia coli*, serotype E26:B6; Sigma-Aldrich). At the end of the culture period some cultures were supplemented with AA (Calbiochem, Meudon, France) at a final concentration of 1 µM for 30 min. Supernatants were collected by centrifugation at 2500 × g for 15 min, and PGE<sub>2</sub> production was measured by ELISA (Cayman Chemicals). The detection limit was 15 pg/ml.

### Effects of selective COX inhibitors on eicosanoid production

To address the relative contribution of each isoform of COX to PGE<sub>2</sub> production, 1 × 10<sup>6</sup> immature DC/ml were cultured for 48 h in complete medium with and without LPS (1 µg/ml) and graded concentrations of a selective COX-1 inhibitor, SC-560, or a selective COX-2 inhibitor, NS-398, without exogenous AA. To study the effect of endogenous PGE<sub>2</sub> on LTB<sub>4</sub> production, parallel cultures were treated with both COX selective inhibitors (4 µM indomethacin, 20 nM SC-560, and 10 µM NS-398) or NDGA (1 µM). Controls included DC stimulated with LPS without inhibitors. PGE<sub>2</sub> and LTB<sub>4</sub> production was measured by ELISA in the presence and the absence of exogenous AA in all supernatants. The detection limit for LTB<sub>4</sub> was 4 pg/ml.

### Nitrite assay

The release of NO was measured in the absence of exogenous AA by the assessment of NO<sub>2</sub><sup>-</sup>, a stable metabolite, determined by colorimetric reaction (41). After cell stimulations, 50 µl culture supernatant was transferred in triplicate into 96-well plates, to which 50 µl 0.1% (v/v) *N*-1-naphthylethylene diamine HCl in distilled H<sub>2</sub>O and 50 µl 1% (v/v) sulfanilic acid in 5% (v/v) phosphoric acid were added. Results were determined with a microplate reader at 540 nm. Standard curves were generated with dilutions of NaNO<sub>2</sub> (0–100 µM). The detection limit was 0.1 mM/10<sup>6</sup> cells.

### IL-10 and IL-12p70 measurement

Cytokines were measured by ELISA (R&D Systems, Abingdon, U.K.) in the supernatants of BM-DC matured in the presence of PGE<sub>2</sub> or LPS with and without COX inhibitors. Controls included cells matured in complete medium alone. IL-12p70 and IL-10 were also measured in supernatant from cells treated with graded concentrations of LTB<sub>4</sub> or with NDGA after LPS stimulation. To investigate whether PGE<sub>2</sub>-induced IL-10 mediated inhibition of IL-12 production, LPS-treated DC cultures were treated with increasing concentrations of anti-IL-10 (purified rat anti-mouse IL-10 mAb, clone JES5-16E3; BD PharMingen) or an isotype control (purified Rat IgG2b; BD PharMingen). The effects of NO on IL-12 production by LPS-stimulated cells were also tested by the use of 0.5 mM *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase, or 0.25 mM *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), a donor of NO. The detection limits were 4 pg/ml for IL-10 and 2.5 pg/ml for IL-12p70.

### Effects of exogenous IL-10 on IL-12p70 production

To evaluate the effects of exogenous IL-10 on IL-12 production, LPS-treated DC were exposed to complete medium containing 10 µM NS-398 with increasing concentrations of exogenous IL-10 (mouse rIL-10; PeproTech). Controls included LPS-stimulated DC treated with NS-398 alone. After 48 h supernatants were collected for the assessment of IL-12p70 production. The percentages of suppression of IL-12-p70 production were calculated and compared with control values.

### Preparation of C57BL/6 lymphocytes and allogeneic MLR

Spleen C57BL/6 lymphocytes used for allogeneic MLR as responder cells were obtained as previously reported (42). Briefly, 8-wk-old female C57BL/6 (H-2D<sup>b</sup>) mice (Iffa Credo, Lyon, France) were killed by cervical dislocation. Splenocytes were isolated and separated by flotation on Ficoll (Sigma-Aldrich). Mononuclear splenocytes were allowed to stick to plastic petri dishes (Costar) for 4 h in RPMI 1640 complete medium at 37°C in a regular incubator. Then nonadherent cells were gently removed, washed twice, and kept in RPMI 1640 complete medium, and adherent cells were discarded. Nonadherent cells contained <0.5% CD14<sup>+</sup> cells and at least 80% CD4<sup>+</sup> lymphocytes. The allostimulatory capacity of DCs was tested in MLR. DCs were stimulated for 48 h with 1  $\mu$ M exogenous PGE<sub>2</sub> or 1  $\mu$ g/ml LPS in the presence and the absence of COX inhibitors. After extensive washing with PBS (BioWhittaker, Walkersville, MD), DC were counted and incubated with 10  $\mu$ g/ml (final concentration) mitomycin C (Sigma-Aldrich) for 35 min at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>, then washed four times with PBS. To test the effects of PGE<sub>2</sub> and IL-10 on the cell stimulatory function of DC in allogeneic MLR, T lymphocytes ( $2 \times 10^5$ /ml) were seeded into 96-well flat-bottom plates (Costar) together with  $5 \times 10^4$ /ml mitomycin C-treated DC. Controls included DC treated with mitomycin without lymphocytes and lymphocytes cultured alone. Some LPS-treated DC were incubated with T cells in the presence of anti-IL-10 (100 ng/ml). Plates were incubated for 4 days at 37°C in 5% CO<sub>2</sub>. All experiments were performed in triplicate. On day 4 of the MLR, cell cultures were pulsed with 1  $\mu$ Ci/well [methyl-<sup>3</sup>H]thymidine (sp. act., 2 Ci/mmol; Amersham) for 6 h. The plates were harvested onto glass-fiber filters with an IH-10 harvester (Ionetech, Dottiikon, Switzerland), and the filters were counted for 1 min in a 1450 Millicounter (Wallac, Turku, Finland).

### Statistical analysis

The results are expressed as the mean  $\pm$  SEM. Analysis of data was performed using Student's *t* test; *p* < 0.01 was considered significant.

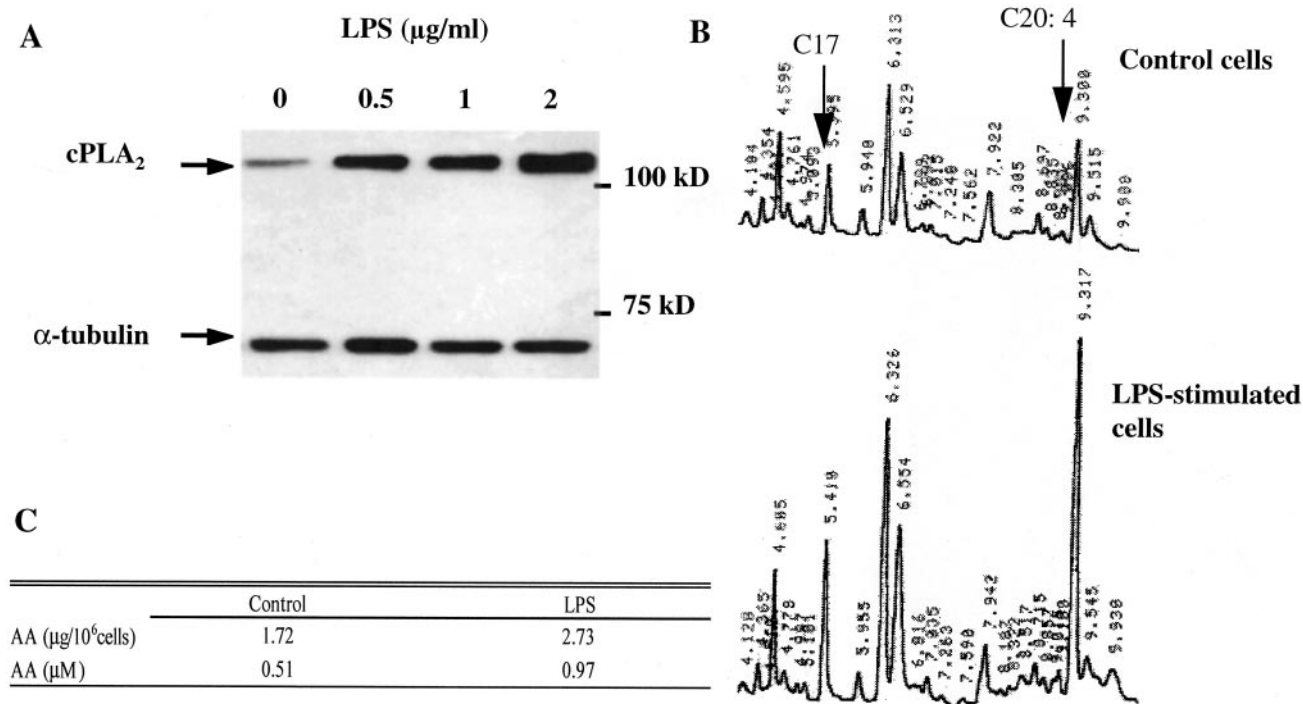
## Results

### Cytosolic PLA<sub>2</sub> expression and AA release by BM-DC

AA release and the generation of lipid mediators require the expression and the activation of PLA<sub>2</sub>. Western blot analyses show that although cPLA<sub>2</sub> is constitutively expressed in BM-DC, it is also inducible by LPS. Fig. 1A shows that LPS dose-dependently up-regulates cPLA<sub>2</sub> expression. In terms of AA release, LPS-treated BM-DC released substantial amounts of AA (2.73  $\mu$ g/10<sup>6</sup> cells) compared with unstimulated cells (Fig. 1, B and C).

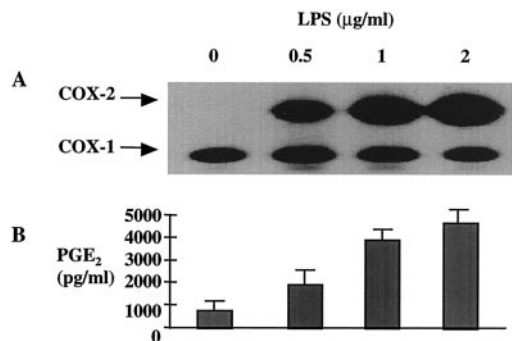
### COX expression and eicosanoid production in BM-DC

Western blot analyses were performed to evaluate COX protein expression in mature BM-DC. Fig. 2A shows that resting BM-DC constitutively express COX-1 but not COX-2. COX-2 protein was detectable after LPS exposure, and its expression was dose-dependently up-regulated by LPS. No changes were observed in COX-1 protein expression under the same conditions. When we assessed eicosanoid production from BM-DC, we found that PGE<sub>2</sub> was the predominant PG produced by BM-DC, while production of other COX-issued prostanoids was quite weak. As shown in Table I, BM-DC are able to produce PGE<sub>2</sub> in the absence and the presence of exogenous AA. These results show that the addition of exogenous AA is not required for the generation of measurable amounts of PGE<sub>2</sub>. Treatment of BM-DC with 1  $\mu$ g/ml LPS resulted in the production of high quantities of PGE<sub>2</sub> (>3 ng/ml/1  $\times 10^6$  cells; Table I), and LPS enhances PGE<sub>2</sub> production in dose-dependent fashion (Fig. 2B). When we assessed COX-1- and COX-2-mediated PGE<sub>2</sub> production, COX-2 appeared to be the predominant isoform of COX involved in the production of endogenous PGE<sub>2</sub> by LPS-stimulated DCs, because NS-398 dose-dependently decreased PGE<sub>2</sub> production (Table II). A dose of 10  $\mu$ M NS-398



**FIGURE 1.** Cytosolic PLA<sub>2</sub> expression and AA release by BM-DC. *A*, Western blot analyses with mAb to cPLA<sub>2</sub> and mouse  $\alpha$ -tubulin in BM-DC stimulated with increasing concentrations of LPS. A total of 15  $\mu$ g protein was loaded in all lanes. Similar results were obtained in four different experiments. The measurement of the released AA was performed by gas chromatography. *B*, A chromatogram of methyl esters of the fatty acids extracted from control cells and from LPS-stimulated cells is shown. The peaks corresponding to the internal standard, heptadecanoyl methyl ester (C17), and to the arachidonyl methyl ester (C20: 4) are indicated, and values are calculated (*C*).





**FIGURE 2.** LPS dose-dependently up-regulates COX-2 expression and PGE<sub>2</sub> production by BM-DC. BM-DC matured in the presence of graded concentrations of LPS were harvested, washed, and analyzed by FACS for their purity (>98%). A, For the expression of COXs, a total of 15 μg protein from the 1800 × g cytosolic fraction was analyzed by Western blot analysis as described in *Materials and Methods* using specific anti-COX-1 or anti-COX-2. B, The production of PGE<sub>2</sub> was measured by ELISA in the presence of 1 μM AA. The results are the mean ± SD of six different experiments.

caused an 81% decrease in PGE<sub>2</sub> production ( $p < 0.01$ ;  $n = 4$ ). The lack of effect of SC-560, a COX-1-selective inhibitor, suggests that COX-1 did not contribute significantly to the total pool of PGE<sub>2</sub> in LPS-stimulated cells. However, in resting BM-DC, SC-560 decreased PGE<sub>2</sub> production in dose-dependent fashion (Table II). Indomethacin (IC<sub>50</sub> (COX-1), 1.67 μM) used at 1 and 4 μM, which inhibited only COX-1, did not affect the higher production of PGE<sub>2</sub> following LPS exposure (Table I). Added together, COX-1 and COX-2 inhibitors caused >94% inhibition in PGE<sub>2</sub> production (Table I).

We also analyzed the production of LTB<sub>4</sub>, the major product of the 5-LO pathway, in the presence and the absence of exogenous AA. We found that LPS-stimulated BM-DC produce large amounts of LTB<sub>4</sub> compared with control cells (Table I). An 80% inhibition of LTB<sub>4</sub> synthesis was observed in the presence of 1 μM NDGA. In addition, production of LTB<sub>4</sub> was 2-fold higher when both COX pathways were blocked (Table I), with or without addition of exogenous AA. These results suggest that COX metabolites may inhibit LTB<sub>4</sub> synthesis. Compared with control cells, addition of exogenous AA to cells did not significantly modify BM-DC eicosanoid production, suggesting that PGE<sub>2</sub>, the major PG formed, did not inhibit the production of LTB<sub>4</sub> by limiting substrate availability in BM-DC. In addition, we found that exogenous PGE<sub>2</sub> inhibited the production of LTB<sub>4</sub> without any change in 5-LO expression (H. Harizi and N. Gualde, manuscript in preparation). It should be interesting to investigate the interaction between COXs and 5-LO pathways that might regulate tissue ho-

meostasis and contribute to the modulated production of eicosanoids.

#### LPS-stimulated BM-DC produce high level of NO

Among the other soluble mediators induced by LPS is NO. Compared with control cells, treatment of BM-DC with 1 μg/ml LPS resulted in the production of >50 μM NO (Table III). A 90% inhibition of NO production was observed after addition of the NO synthase inhibitor, L-NMMA. When we examined the effect of PGE<sub>2</sub> on NO production, we found that exogenous PGE<sub>2</sub> at 1 μM did not affect the production of NO by either BM-DC or T cells (Table III). In addition, COX-2-selective inhibitors did not modify the production of NO, suggesting that COX-2-produced PGE<sub>2</sub> did not affect the endogenous production of NO.

#### Phenotype of COX inhibitor-treated BM-DC

BM-DC express relevant surface Ags such as CD11c, CD40, CD80, CD86, and MHC class II (Fig. 3). To examine whether endogenous PGE<sub>2</sub> affects the BM-DC phenotype, we analyzed surface marker expression of BM-DC matured in the presence of inhibitors. We have recently shown that exogenous PGE<sub>2</sub> reduces MHC class II expression in BM-DC (12). In this study we observed that COX inhibitors did not modify the expression of CD11c and CD40 markers, but the COX-2 inhibitor NS-398 up-regulated the expression of the MHC class II, and costimulatory molecules (Fig. 3). We also found that indomethacin, SC-560 (a COX-1 inhibitor), and NDGA did not affect the expression of any marker (data not shown).

#### IL-12p70 and IL-10 production by BM-DC

In our study supernatants from various culture conditions (see *Materials and Methods*) were assayed by ELISA to determine the production of cytokines in the absence and the presence of LPS, COX inhibitors, or exogenous PGE<sub>2</sub>. As shown in Fig. 4, which summarizes the average of six experiments, BM-DC did not spontaneously produce a significant quantity of IL-10. However, they secreted >1.5 ng/ml/10<sup>6</sup> cells of IL-12p70. Addition of 1 μM exogenous PGE<sub>2</sub> enhanced the generation of IL-10 and simultaneously reduced the production of IL-12p70 (–95%). LPS alone (1 μg/ml) induced a similar effect as exogenous PGE<sub>2</sub> (Fig. 4). In fact, the effects of the PG were mimicked by LPS (which stimulates COX-2) and reversed by the addition of 10 μM NS-398, a COX-2-specific inhibitor, which up-regulates IL-12p70 and down-regulates IL-10 production. These results suggest that COX-2-mediated PGE<sub>2</sub> plays a central role in the reciprocal regulation of IL-10 and IL-12, because addition of indomethacin or SC-560, a COX-1 selective inhibitor, did not significantly affect the production of IL-12p70 after LPS exposure (Fig. 4). When we analyzed the effects of LTB<sub>4</sub> on BM-DC cytokine production, we found that

Table I. Eicosanoid production by BM-DC<sup>a</sup>

	BM-DC Treated with LPS (1 μg/ml)							
	Unstimulated Cells	None	Indo (4 μM)	SC-560 (20 nM)	NS-398 (10 μM)	Indo + NS	SC + NS	NDGA (1 μM)
PGE <sub>2</sub>	819 ± 65	3670 ± 154 <sup>b</sup>	2833 ± 112	2973 ± 152	697 ± 56 <sup>b</sup>	256 ± 31 <sup>b</sup>	198 ± 24 <sup>b</sup>	3679 ± 171
PGE <sub>2</sub> (+ AA)	906 ± 62	3790 ± 152 <sup>b</sup>	2850 ± 173	2956 ± 123	755 ± 95 <sup>b</sup>	265 ± 20 <sup>b</sup>	260 ± 30 <sup>b</sup>	3755 ± 167
LTB <sub>4</sub>	680 ± 56	1224 ± 112	1380 ± 088	1466 ± 135	2033 ± 147	2433 ± 175	2451 ± 177	204 ± 27 <sup>b</sup>
LTB <sub>4</sub> (+ AA)	702 ± 32	1240 ± 102	1450 ± 125	1500 ± 140	2145 ± 154	2450 ± 195	2650 ± 187	248 ± 21 <sup>b</sup>

<sup>a</sup> Formation of PGE<sub>2</sub> and LTB<sub>4</sub> was measured by ELISA in supernatants from control cells and LPS-stimulated cells, with and without addition of 1 μM exogenous AA. Some LPS-stimulated BM-DC were treated with COX inhibitors (4 μM indomethacin, 20 nM SC-560, 10 μM NS-398) or 5-LO inhibitor (NDGA at 1 μM). Controls included BM-DC stimulated with LPS (1 μg/ml) and BM-DC cultured alone. Results (expressed in picograms per milliliter per 1 × 10<sup>6</sup> cells) represent the mean ± SEM of six different experiments.

<sup>b</sup> Values of  $p < 0.01$  by Student's *t* test when compared to controls.

Table II. Selective COX inhibitors dose-dependently inhibit PGE<sub>2</sub> production from BM-DC in the absence of exogenous AA<sup>a</sup>

	BM-DC Treated with LPS (1 $\mu$ g/ml)											
	SC-560 (nM)				SC-560 (nM)				NS-398 ( $\mu$ M)			
	0	10	20	40	0	10	20	40	0	10	20	40
PGE <sub>2</sub> (pg/ml)	819 $\pm$ 65 (100)	450 $\pm$ 47 (55)	41 $\pm$ 9 (5)	25 $\pm$ 4 (3)	3670 $\pm$ 154 (100)	3266 $\pm$ 191 (89)	2973 $\pm$ 152 (81)	2936 $\pm$ 187 (80)	3670 $\pm$ 191 (100)	697 $\pm$ 56 (19)	624 $\pm$ 59 (17)	588 $\pm$ 43 (16)

<sup>a</sup> Cells matured in the presence of LPS (1  $\mu$ g/ml) were treated with graded concentrations of selective COX-2 inhibitor, NS-398, or selective COX-1 inhibitor, SC-560 for 48 h. PGE<sub>2</sub> production (expressed in picograms per milliliter per 10<sup>6</sup> cells) was measured by ELISA, and the percentage of PGE<sub>2</sub> production was calculated. The effect of COX-1 selective inhibitor was also tested in the absence of LPS stimulation. Results are the mean  $\pm$  SEM of four independent experiments.

treatment of BM-DC with increasing concentrations of exogenous LTB<sub>4</sub> did not modify the production of either IL-10 or IL-12. Compared with controls (LPS alone and LPS plus NS-398), the combination of LPS and NDGA (which caused 80% inhibition of endogenous LTB<sub>4</sub> production) produced no significant decrease in cytokine release (Fig. 5). These results suggest that LTB<sub>4</sub> had no effect on cytokine production from BM-DC.

*Exogenous IL-10 down-regulates IL-12-p70 production by BM-DC, while neutralization of either endogenous PGE<sub>2</sub> or IL-10 enhances IL-12p70 production*

We studied the production of IL-12-p70 by BM-DC treated with exogenous IL-10. As shown in Fig. 6A, addition of exogenous recombinant mouse IL-10 to DC in the presence of NS-398, a COX-2-selective inhibitor, dose-dependently decreased the production of IL-12p70. A dose of 2 ng/ml IL-10 caused a 90% decrease in IL-12 release ( $p < 0.01$ ;  $n = 6$ ). In view of the potential of PGE<sub>2</sub> to stimulate IL-10 production, we investigated whether IL-10 acted as an intermediate in the suppression of IL-12p70 by PGE<sub>2</sub>. For that purpose we used an anti-IL-10 neutralizing mAb. As shown in Fig. 6B, anti-IL-10 dose-dependently neutralizes endogenous IL-10, and a dose of 100 ng/ml resulted in the neutralization of >95% of the endogenously induced IL-10 and a >88% increase in IL-12p70 production, whereas the isotype-matched control mAb had no effect (Fig. 7). IL-12p70 production increased as the concentration of IL-10 neutralizing mAb increased (data not shown) and was maximal at a concentration exceeding 100 ng/ml. These results suggest that the endogenously produced IL-10 was a potent inhibitor of IL-12p70 production. Our results also show that treatment of DC with NS-398 enhances the synthesis of IL-12p70 (Fig. 7), as in anti-IL-10-treated DC. However, SC-560 and indomethacin had no effect on IL-12 (Fig. 4), suggesting that COX-2, but not COX-1, was involved in the modulation of IL-12 production by DC.

*PGE<sub>2</sub>-induced suppression of IL-12-p70 production is due to enhanced endogenous production of IL-10 but not NO*

As shown in Fig. 7, LPS- and PGE<sub>2</sub>-treated DC produce low levels of IL-12p70 compared with control cells ( $p < 0.01$ ). In addition,

the inhibitory effect of PGE<sub>2</sub> (either exogenous or LPS-induced) was prevented by anti-IL-10. Our results confirm the involvement of IL-10 in IL-12 suppression by PGE<sub>2</sub>. We also found that addition of L-NMMA, an inhibitor of NO synthase, or SNAP, an NO donor, did not affect the suppression of IL-12 production by PGE<sub>2</sub> or LPS in BM-DC (Fig. 7), suggesting that endogenous NO does not mediate the suppressive effect of PGE<sub>2</sub>. Taken together with the influence of exogenous IL-10 (Fig. 6A), we demonstrate that COX-2 PGE<sub>2</sub>-induced IL-10 suppresses the production of IL-12p70 in BM-DC.

*BM-DC stimulatory capacity*

We therefore tested the capacity of DC treated with COX inhibitors, exogenous PGE<sub>2</sub>, or anti-IL-10 to induce the proliferation of allogeneic T cells. We observed that the proliferative response of T lymphocytes was significantly diminished (50% inhibition) when DC were exposed to 1  $\mu$ M exogenous PGE<sub>2</sub> (Fig. 8;  $p < 0.01$  compared with unstimulated cells). Compared with control cells (LPS alone), LPS-stimulated DC treated with NS-398 or anti-IL-10 induced a significantly higher proliferation of allogeneic T cells (Fig. 8;  $p < 0.01$ ), while thymidine uptake by the allo-stimulated lymphocytes was not increased by indomethacin alone.

## Discussion

Several lines of evidence show that production of PGE<sub>2</sub> is enhanced during inflammation, and this lipid mediator can dramatically modulate the immune response (43). Given these observations and because myeloid-derived cells produce large amounts of proinflammatory lipid mediators, we investigated COX expression, PGE<sub>2</sub> synthesis, and cytokine production by BM-DC. We reported that production of AA-derived metabolites did not require the addition of exogenous substrate, because BM-DC are able to produce PGE<sub>2</sub> and LTB<sub>4</sub> under the two experimental conditions, and these APC express cytosolic PLA<sub>2</sub>, which catalyzes the release of endogenous AA from the cell membrane. The expression of cPLA<sub>2</sub> is up-regulated by LPS in a dose-dependent fashion, and an important liberation of AA was observed in LPS-treated BM-DC

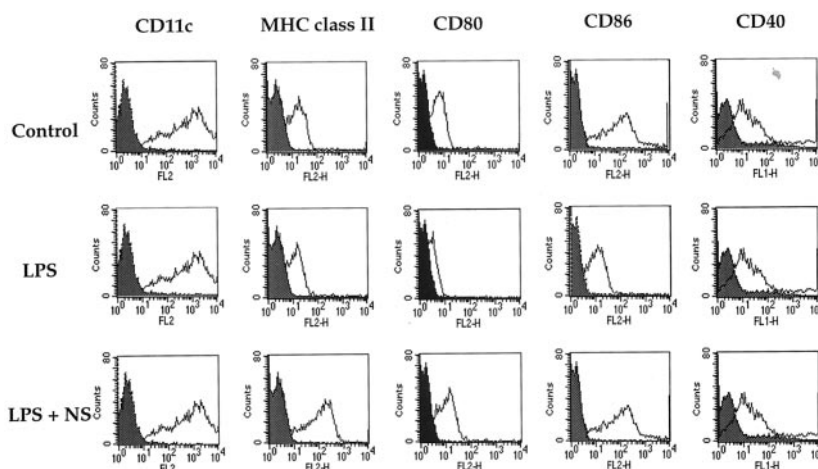
Table III. NO production by BM-DC<sup>a</sup>

	NO <sub>2</sub> <sup>-</sup> ( $\mu$ M)				
	—	PGE <sub>2</sub>	LPS (1 $\mu$ g/ml)	LPS (1 $\mu$ g/ml) + NS-398	LPS (1 $\mu$ g/ml) + L-NMMA
BM-DC	1.04 $\pm$ 0.12	1.50 $\pm$ 0.20	58.10 $\pm$ 02 <sup>b</sup>	55.50 $\pm$ 03	5.65 $\pm$ 0.6 <sup>b</sup>
T cells	0.80 $\pm$ 0.10	0.60 $\pm$ 0.20	0.500 $\pm$ 0.1	0.7000 $\pm$ 0.4	0.90 $\pm$ 0.30

<sup>a</sup> A total of 10<sup>6</sup> BM-DC/ml or an equivalent number of T cells were incubated for 48 h in the presence of 1  $\mu$ g/ml LPS or 1  $\mu$ M PGE<sub>2</sub>. Parallel cultures were run with NS-398 (10  $\mu$ M) or L-NMMA (0.5 mM). Supernatants were collected and analyzed for NO<sub>2</sub><sup>-</sup> release. Results are the mean  $\pm$  SD obtained from triplicate cultures and are representative of six separate experiments.

<sup>b</sup> Values of  $p < 0.01$ .

**FIGURE 3.** Phenotypic analysis of BM-DC matured in the presence or the absence of COX-2 inhibitor. Immature BM-DCs were treated with LPS (1  $\mu$ g/ml) with or without NS-398 (10  $\mu$ M), a COX-2 specific inhibitor, for 48 h. Controls included untreated-BM-DC. Cells were stained with Abs to cell surface markers conjugated to fluorochromes listed in *Materials and Methods*, and FACS analysis was performed. Filled histograms represent isotype Ab staining; open histograms represent cell surface staining. Results are representative of eight different experiments.

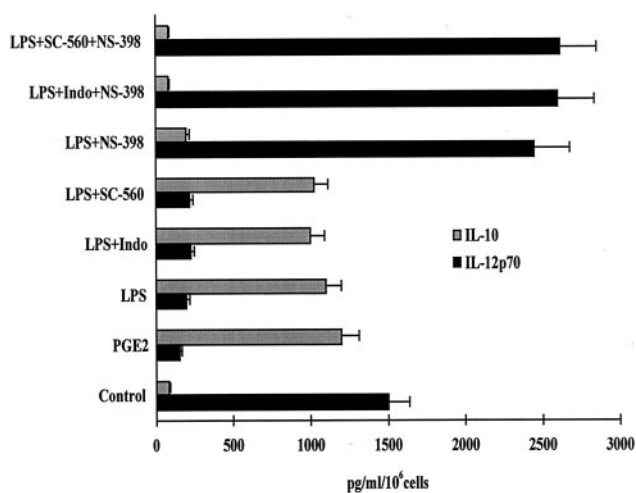


compared with control cells. These data are consistent with previous studies, which reported induction of cPLA<sub>2</sub> by LPS in human leukocytes (44, 45). Analysis of COX expression shows that resting BM-DC express only COX-1 and produce low levels of PGE<sub>2</sub>. After LPS stimulation, COX-2 appears to be the predominant COX isoform in terms of protein expression and PGE<sub>2</sub> production. Selective COX-1 and COX-2 inhibitors were used to demonstrate that production of PGE<sub>2</sub>, the predominant PG formed, derives largely from COX-2.

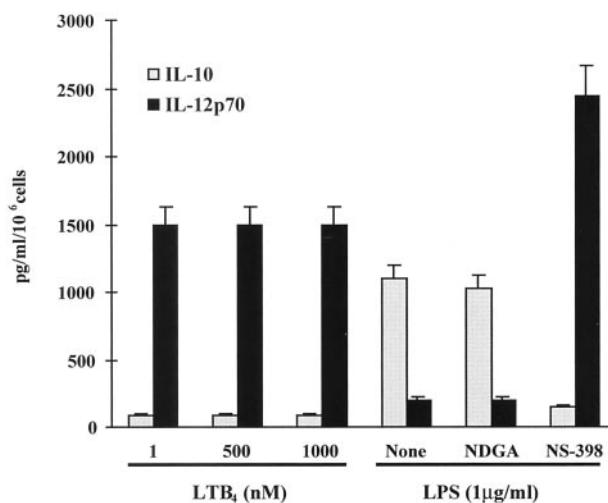
Because we found no significant differences in the profile of the relative amounts of eicosanoids (PGE<sub>2</sub> and LTB<sub>4</sub>) in the presence and the absence of exogenous AA, and addition of exogenous AA is not required for the formation of measurable amounts of PGE<sub>2</sub>, we performed all experiments in the absence of exogenous AA. Selective COX-1 or COX-2 inhibitors decreased PGE<sub>2</sub> production from endogenous AA.

PGE<sub>2</sub> is accepted as a potent modulator of APC function and plays a prominent role in regulating Th1- and Th2-type responses (10, 13). We have recently reported that in our BM-DC model exogenous PGE<sub>2</sub> alters Ag presentation by inhibiting the expres-

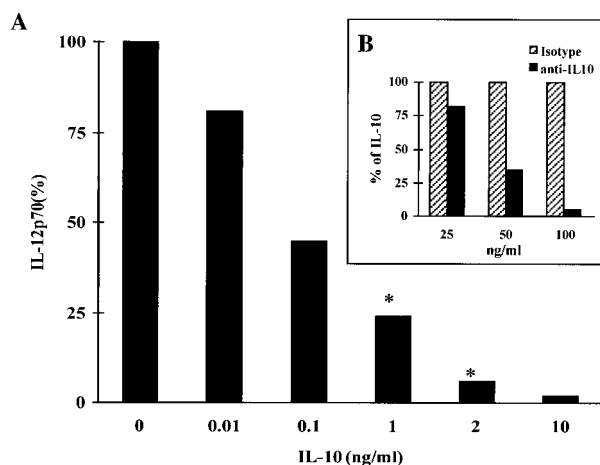
sion of MHC class II protein (12). These data are quite similar to those published by Snyder et al. (46), who reported that PGE<sub>2</sub> diminished the expression of both MHC molecules and the accessory effect of macrophages. With regard to the effects of endogenous PGE<sub>2</sub> on DC, we analyzed the phenotype and function of COX inhibitor-treated DC. For this purpose the in vitro-generated BM-DC were matured in the presence of COX-selective inhibitors and examined for their 1) expression of relevant surface markers, 2) production of IL-10 and IL-12, and 3) APC stimulatory function. To investigate whether endogenous PGE<sub>2</sub> affects the maturation of murine DC, we analyzed the expression of CD11c, MHC class II, CD40, CD80, and CD86 in DC matured in the presence of inhibitors. We found that indomethacin, SC-560, and NDGA did not affect the expression of these markers (data not shown). However, although NS-398 did not modify CD11c and CD40 expression, it up-regulated the expression of CD80, CD86, and the MHC class II molecules. These data suggest that the predominant effects of PGE<sub>2</sub> appear to be mediated through the COX-2 pathway, which affects the expression of molecules involved in the APC function of DC. Our data are not in agreement with a report by



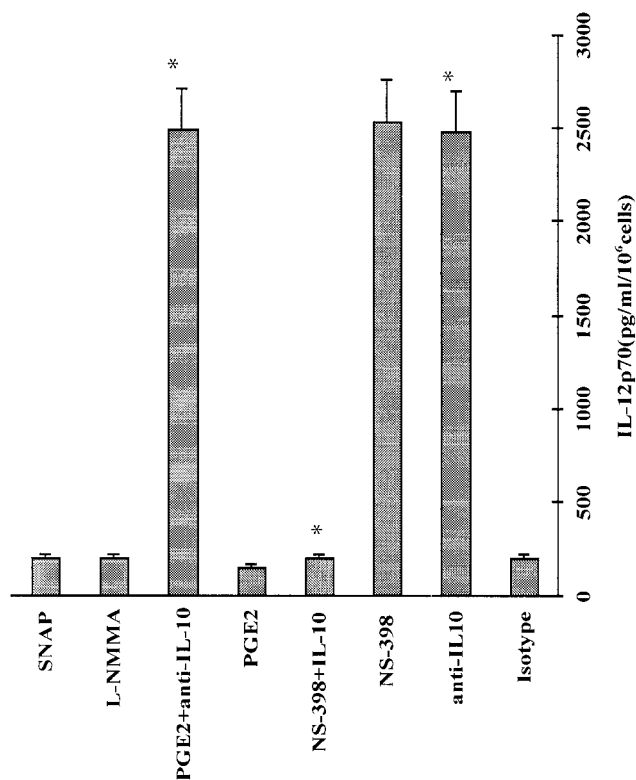
**FIGURE 4.** COX-2-mediated PGE<sub>2</sub> up-regulates IL-10 and down-regulates IL-12p70 production by LPS-stimulated BM-DC. Supernatants from LPS-stimulated BM-DC (1  $\mu$ g/ml) in the presence and the absence of COX inhibitors were analyzed by ELISA (R&D Systems) for cytokine production. Controls included unstimulated BM-DC and BM-DC treated with 1  $\mu$ M exogenous PGE<sub>2</sub>. Cytokine levels are expressed as picograms per milliliter per 1  $\times 10^6$  cells. Data are the mean  $\pm$  SEM of six different experiments.



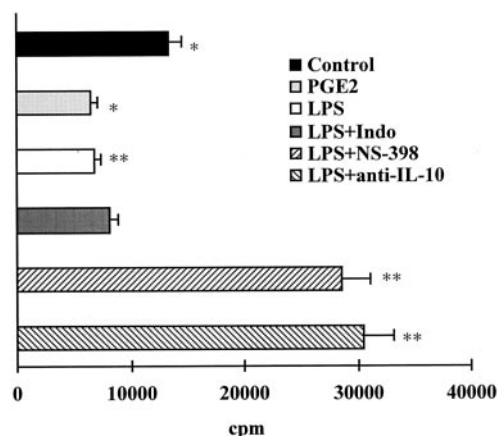
**FIGURE 5.** LTB<sub>4</sub> does not affect cytokine production in BM-DC. IL-12p70 and IL-10 production by BM-DC was measured by ELISA in the presence of graded concentrations of exogenous LTB<sub>4</sub>. This production was also measured after LPS stimulation in the presence and the absence of 1  $\mu$ M NDGA or 10  $\mu$ M NS-398. Results are expressed as picograms per milliliter per 1  $\times 10^6$  cells and are the mean  $\pm$  SEM of six different experiments.



**FIGURE 6.** Effects of IL-10 and anti-IL-10 on the production of cytokines. LPS-stimulated BM-DC ( $1 \mu\text{g/ml}$ ) were treated with increasing concentrations of anti-IL-10 (purified rat anti-mouse IL-10 mAb) or isotype-matched control (purified rat IgG2b; purchased from BD PharMingen Europe) for 48 h. The presence of IL-10 was analyzed by ELISA, and the results of six experiments are expressed as the percentage of IL-10 measured in the presence of isotype (B). Some LPS-treated BM-DC were stimulated with  $10 \mu\text{M}$  NS-398 in the presence of graded concentrations of IL-10 (A). The results of six different tests are expressed as the percentage of IL-12 levels measured in the absence of IL-10. \*,  $p < 0.01$  compared with NS-398 alone.



**FIGURE 7.** Anti-IL-10 totally reverses the inhibitory effect of COX-2-mediated PGE<sub>2</sub> on IL-12p70 production by BM-DC. Supernatants from PGE<sub>2</sub>-treated ( $1 \mu\text{M}$ ) or LPS-treated ( $1 \mu\text{g/ml}$ ) BM-DC in the presence or the absence of an anti-IL-10 ( $100 \text{ ng/ml}$ ), isotype ( $100 \text{ ng/ml}$ ), or NS-398 ( $10 \mu\text{M}$ ) were analyzed by ELISA for the production of IL-12p70. Effects of NO (endogenous and exogenous) on IL-12 production were examined in LPS-stimulated BM-DC treated with L-NMMA ( $0.5 \text{ mM}$ ) or SNAP ( $0.25 \text{ mM}$ ). Levels are expressed as picograms per milliliter per  $1 \times 10^6$  cells. Data are the mean  $\pm$  SEM of six different experiments. \*,  $p < 0.01$  compared with the respective controls.



**FIGURE 8.** Allostimulatory capacity of BM-DC. C57BL/6 T lymphocytes ( $2 \times 10^5$ ) were incubated with mitomycin C-treated BM-DC ( $5 \times 10^4$ ) obtained in vitro from bone marrow cells, and matured in the presence of  $1 \mu\text{M}$  exogenous PGE<sub>2</sub> or COX inhibitors ( $4 \mu\text{M}$  indomethacin,  $10 \mu\text{M}$  NS-398) for 48 h. Some LPS-stimulated BM-DC were incubated with T cells in the presence of  $100 \text{ ng/ml}$  anti-IL-10. Controls ( $<500 \text{ cpm}$ ; histograms not shown) included DCs treated with mitomycin without lymphocytes and lymphocytes cultured alone. Data are the mean  $\pm$  SD of triplicate culture from six representative experiments. \*,  $p < 0.01$  compared with unstimulated BM-DC; \*\*,  $p < 0.01$  compared with LPS-stimulated BM-DC treated with NS-398 or anti-IL-10.

Whittaker et al. (47), who reported that blocking COX-2 in monocyte-derived DC had no effect on CD40, CD80, CD86, and HLA-DR expression. However, the expression of CD83 appears to be regulated by COX-2 produced prostanoids. One can explain the discrepancy between the two reports because Whittaker et al. (47) adopted a quite different model from the one we used.

The ability of mature DC to act as a potent APC is also related to their production of cytokines, especially IL-12 (48, 50), which is a major Th1-driving cytokine, promoting cell-mediated immunity (51, 52). Resting BM-DC, which express only COX-1 and produce low levels of PGE<sub>2</sub>, produce IL-12p70, but not IL-10. However exogenous PGE<sub>2</sub> or LPS-mediated induction of COX-2 resulted in high levels of IL-10 and a potent suppression of IL-12 production. This is in agreement with earlier reports showing that IL-12 production by human monocytes and DC is suppressed after LPS exposure (53, 54). The synthesis of IL-12 is regulated by a positive feedback mechanism mediated by Th1 cells through IFN- $\gamma$  or by negative feedback through Th2 cells secreting IL-10 (55). IL-10 is known to be a potent suppressor of IL-12 gene expression at the transcriptional level (56). In accordance with other investigators (22, 23), we found that addition of IL-10 to DC profoundly inhibited the production of IL-12p70.

Confirming a recent study (57), we found that LPS-treated DC secreted high levels of IL-10, which appears to be an important regulator of DC biology and function. In contrast to Whittaker et al. (47), who showed that prostanoids did not modulate IL-10 production by human monocyte-derived DC, we demonstrate that COX-2-mediated PGE<sub>2</sub> enhances the production of IL-10, and that COX-2-produced PGE<sub>2</sub> might be a key point in the reciprocal regulation of IL-10 and IL-12 production, because it enhances IL-10 and spontaneously inhibits IL-12 production in LPS-treated BM-DC. This is in accordance with published data (14, 16).

Our data demonstrate that the inhibitory effect of PGE<sub>2</sub> on IL-12 production was completely reversed by an anti-IL-10 mAb. Based on the influence of exogenous IL-10, PGE<sub>2</sub>, and NS-398 on BM-DC, it is likely that PGE<sub>2</sub>, IL-10, and IL-12, produced by murine DC might interact, hence regulating APC function and therefore



the immune response. Together with the results obtained with anti-IL-10, we conclude that PGE<sub>2</sub> inhibits IL-12 production in an IL-10-dependent mechanism. Our finding is not in agreement with studies using human macrophages or DC; when considering the effects of IL-10 on DC phenotype and function, one should keep in mind that mouse and human DC populations are obviously not the same. Our results could be related to the high sensitivity of that lineage to the suppressive effect of PGE<sub>2</sub>, as was reported by Kuroda et al. (58). However, in the data published by Kuroda, the distinctions between lineages exist only in terms of sensitivity, but there were no differences in terms of general orientation of the effects of PGE<sub>2</sub> on the immune response among the different mice.

The biological actions of PGE<sub>2</sub>, including its effects on immunity, are mediated by G protein-coupled receptors, designated E prostanoid receptors (59). The existence of this complex family of E prostanoid receptors coupled to distinct intracellular signals provides a molecular basis for the diverse physiological actions of PGE<sub>2</sub>. It is clear that the reports on PGE<sub>2</sub> effects on DC are sometimes confusing (12, 47). That could be related to the heterogeneity of the APC, the species used for the experiments, the signaling pathways affected in the maturation of the DC, etc.

Among the panel of AA metabolites produced by BM-DC, we noticed that LTB<sub>4</sub> is released, but in a lesser quantity compared with PGE<sub>2</sub>. Because it was reported before that LTB<sub>4</sub> up-regulates IL-1, IL-2, and IFN- $\gamma$  production and enhances NK cell activity (45), effects quite opposite to what is usually known concerning the immunomodulation induced by PGE<sub>2</sub>, we investigated the effects of LTB<sub>4</sub> on BM-DC phenotype and functions. We found that neither the exogenously added nor the endogenously produced LTB<sub>4</sub> affected the production of cytokines by BM-DC.

It is well known that LPS activates macrophages to release proinflammatory mediators. Among the other soluble mediators stimulated by LPS is NO. In murine macrophages NO regulates IL-12 gene expression (61). Using L-NMMA, an NO synthase inhibitor, or SNAP, a donor of NO, we were able to show that in our experimental setting NO produced by DC did not affect the production of IL-12. This is in accordance with published data (53).

Next, we examined the ability of COX inhibitor-treated BM-DC to stimulate T cells in allogeneic MLR. In contrast to Kalinski et al. (62), we found that blocking COX-2 with NS-398 enhances the stimulatory potential of BM-DC compared with the control. Because it is reported that the stimulatory capacity of APC is inhibited by IL-10 (19, 25), and that COX-2-mediated PGE<sub>2</sub> up-regulates the production of IL-10 by DC, we performed allogeneic MLRs in the presence of anti-IL-10. We found that the neutralization of the endogenously produced IL-10 markedly increased the stimulatory potential of BM-DC. These results suggest that PGE<sub>2</sub> inhibits the APC function of DC via IL-10. This inhibitory influence of IL-10 may be due to the down-regulation of MHC class II and costimulatory molecules as was previously reported (20, 63, 64).

In the present paper we provide evidence that PGE<sub>2</sub> enhances IL-10, which down-regulates IL-12p70 production, and DC stimulatory capacity. Our study confirms that inflammatory effects of PGE<sub>2</sub> on DC functions are mediated by an autocrine feedback mechanism involving endogenous IL-10 and expands the concept that molecules produced during inflammation may influence IL-12 production by LPS-stimulated APC, which are highly susceptible to the induction of LPS tolerance induced by endogenous PGE<sub>2</sub> from the COX-2 pathway. It should be also underlined that our assessment of eicosanoids was limited to PGE<sub>2</sub> and LTB<sub>4</sub>. One cannot be sure that blocking COX-2 by NS-398 inhibits only PGE<sub>2</sub> synthesis and does not weakly affect other PG production. However, our former assessment using gas chromatography and mass

spectrometry showed that immune cells, among them APC, produce mostly PGE<sub>2</sub> and that synthesis of other COX-issued eicosanoids (PGs, prostacyclin, thromboxane) is quite weak (65).

PGE<sub>2</sub> belongs to a family of short-lived chemical paracrine messengers that has been studied over the past 20 years for its immunomodulatory properties (66). However, the precise role of this eicosanoid has remained more or less unknown. One can expect that, by reaching an upstream step in the immune response, it may be easier to better evaluate the different roles of PGE<sub>2</sub> for suppression and/or organization of the direction of the immune response (as, for instance, Th1 vs Th2 or vice versa). Because DC, which were originally identified by Steinman (reviewed in Ref. 1), represent the pacemakers of the immune response, one could accept that PGE<sub>2</sub>, which contributes widely to that major function of the professional APC, might be considered a pacemaker mediator of the immune response.

## Acknowledgments

We are grateful to Prof. James S. Goodwin (University of Texas Medical Branch, Galveston, TX) for reading the manuscript.

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