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# Autoregulation of Human Monocyte-Derived Dendritic Cell Maturation and IL-12 Production by Cyclooxygenase-2-Mediated Prostanoid Production[1]

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**PG added to cell culture profoundly affect the in vitro maturation and function of monocyte-derived dendritic cells (MDC). Because unstimulated monocytes express cyclooxygenase (COX)-1, and COX-2 when activated, we examined whether MDC express these enzymes and produce prostanoids that autoregulate maturation and IL-12 production. Immature MDC (I-MDC) and mature MDC express COX-1, but, unlike monocytes, both MDC populations constitutively express COX-2. However, COX-2 regulation in both MDC populations differs from monocytes, as IL-4 does not suppress enzyme expression. COX-2 is functional in MDC as a specific inhibitor, NS-398, significantly reduces $PGE_2$ production. I-MDC undergoing maturation with soluble CD40 ligand (sCD40L) increase $PGE_2$ synthesis, but prostanoid synthesis is switched to COX-1. However, with IFN-$\gamma$ present, sCD40L-stimulated PG metabolism is redirected to COX-2, and $PGE_2$ synthesis increases severalfold. Endogenous PG production by MDC does not regulate CD40, CD80, CD86, or HLA DR expression; however, it does promote MDC maturation, as NS-398 significantly reduces CD83 expression in I-MDC matured with sCD40L/IFN-$\gamma$. PG produced through COX-2 also autoregulate IL-12, but the effects are dependent on the MDC maturation state. Blocking COX-2 reduces I-MDC secretion of IL-12p40, whereas it increases IL-12p40 and p70 production by maturing MDC. COX-2-mediated PG production impacts MDC function as maturing these cells in the presence of NS-398 yields MDC that stimulate significantly more IFN-$\gamma$ in an allogeneic mixed lymphocyte response than MDC matured without this inhibitor. These studies demonstrate that MDC express both COX isoforms constitutively and produce prostanoids, which autoregulate their maturation and function.** ***The Journal of Immunology*****, 2000, 165: 4298–4304.**

Prostaglandins are important lipid mediators for a wide variety of physiological cellular functions (1–4). PG synthesis is regulated by a series of steps involving the release of endogenous arachidonic acid (AA)[3] by phospholipase $A_2$ ($PLA_2$), and the subsequent conversion of AA to $PGH_2$. Conversion of AA to $PGH_2$, the first and rate limiting step in PG biosynthesis, is mediated through two isoenzymes, cyclooxygenase 1 (COX-1, also referred to as PG synthase 1) and COX-2 (also known as PG synthase 2). Constitutively expressed COX-1 is primarily responsible for cellular homeostasis, whereas COX-2 is inducible and is responsible for high-level production of prostanoids that modulate inflammation and mitogensis (1). Monocyte expression of COX-2 is induced by a variety of stimuli, including LPS, PMA, and IL-1$\beta$. In monocytes, LPS enhances and stabilizes COX-2 transcripts (5–7). In several cells types, including monocytes, COX-2 expression is suppressed by IL-4, IL-10, and IL-13 via transcriptional and postranscriptional regulation (8).

Expression of COX enzymes, prostanoid production, and the autocrine effects of these molecules have not been reported for monocyte-derived dendritic cells (MDC). However, previous studies described the effects of exogenous PG on MDC maturation and function. Kalinsky et al. (9) demonstrated that high concentration ($10^{-6}$ M) of exogenous $PGE_2$ added to monocytes in the presence of GM-CSF and IL-4 profoundly modulated MDC development, as these cells did not lose CD14, expressed low levels of CD1a, and produced significantly less IL-12p70 and higher levels of IL-10 (9). Additionally, MDC derived under these conditions stimulated Th2 responses, whereas MDC cultured without exogenous $PGE_2$ stimulated Th1 responses. Other studies demonstrated that $PGE_2$ ($10^{-6}$ M), when added to cultures following monocyte differentiation into immature MDC (I-MDC), synergized with TNF-$\alpha$ or TNF-$\alpha$/IL-1/IL-6 at $10^{-8}$ M to induce maturation, immunostimulatory capacity, and IL-12 production (10, 11). These studies demonstrate that exogenous prostanoids markedly affect MDC maturation and function and that the effect is highly dependent on the developmental stage of the MDC.

Given the profound effects of prostanoids on MDC maturation and function, and because myeloid-derived cells produce large quantities of these lipid molecules, we assessed COX expression and prostanoid production by these cells. Our studies demonstrate that MDC, unlike monocytes, constitutively express both COX-1 and COX-2 and produce prostaglandins in an autocrine manner that regulate MDC maturation and function.

## Materials and Methods

### *Isolation of monocytes and dendritic cell culture conditions*

PBMC were isolated from buffy coats from one unit of whole blood using Histopaque Ficoll (1.077, endotoxin tested; Sigma, St. Louis, MO). Cells were washed two times with Dulbecco's PBS, $Ca^{2+}$, and $Mg^{2+}$ free (endotoxin tested; Cellgro, Herndon, VA) and resuspended in RPMI 1640 media with L-glutamine (Life Technologies, Grand Island, NY) supplemented with 10% FCS (endotoxin tested; HyClone, Logan, UT), and 1% streptomycin, penicillin, and neomycin (Sigma). PBMC were allowed to

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[3] Abbreviations used in this paper: AA, arachidonic acid; $PLA_2$, phospholipase $A_2$; MDC, moncyte-derived dendritic cells; I-MDC, immature MDC; M-MDC, mature MDC; COX, cyclooxygenase; sCD40L, soluble CD40 ligand; TBX, thromboxane.



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adhere for 2 h at 37°C, 5% $CO_2$, 100% humidity, and nonadherent cells were washed away with Dulbecco's PBS. Complete RPMI 1640 tested negative for endotoxin (<2.0 EU/ml) (E-Toxate Kit, Sigma). Adherent cells were cultured for 6 days in complete RPMI supplemented with 500 U (50 ng/ml) GM-CSF (Endogen, Woburn, MA) and 500-1000 U IL-4 (R&D Systems, Minneapolis, MN) to generate I-MDC (12). To generate mature MDC (M-MDC), day 6 I-MDC were harvested, washed, replated at $3.0 \times 10^5$ cells/ml, and supplemented with 1 $\mu$g/ml sCD40L (gift from Immunex, Seattle, WA) and/or 1000 U of IFN-$\gamma$ (human recombinant, Endogen). Some cultures were supplemented with 1 $\mu$g/ml NS-398 (Cayman Chemical, Ann Arbor, MI), a specific COX-2 inhibitor, or 10 $\mu$g/ml indomethacin (Sigma), and a COX-1 and COX-2 inhibitor.

### *Surface and internal protein analysis*

The following mAbs directed against surface or internal proteins were used: CD14, HLA-DR (Becton Dickinson, San Jose, CA), CD1a, CD86, CD80, CD40 (PharMingen, San Diego, CA), CD83 (Coulter-Immunotech, Miami, FL), and COX-2 (FITC, Cayman Chemical). Appropriate fluorochrome-labeled isotype control Abs were used. Cells were suspended in PBS with 1% BSA (reagent grade, Sigma) and 0.1% sodium azide (Sigma). For surface marker labeling, cells were incubated with 1 $\mu$g of fluorochrome-conjugated Ab/$1 \times 10^6$ cells for 20 min at room temperature, then washed one time with 2.0 ml PBS and resuspended in 500 $\mu$l of 1% formaldehyde in PBS. Intracellular labeling of COX-2 was performed as previously described (13). All cells were analyzed on Becton Dickinson FACScalibur or FACSort. Flow cytometry data was analyzed and median fluorescent intensity calculated with WinMidi (Version 2.7, Joseph Trotter).

Cultured MDC were washed with PBS supplemented with protease inhibitors (1 $\mu$g/ml of each leupeptin, pepstatin, and aprotinin; Sigma) and 5 $\mu$g/ml indomethacin and frozen at −70°C. Lysates were thawed, sonicated, and centrifuged for 10 min at 14,000 rpm. Equal quantities of protein were separated by SDS-PAGE with a 10% Tris-HCl gel (Bio-Rad), and transferred to nitrocellulose (Optitran; Schleicher & Schull, Keene, NH.) Nitrocellose was probed with mAbs directed against COX-1 and COX-2 (Cayman Chemical) and secondary Abs (anti-mouse IgG-HRP; Amersham, Arlington Heights, IL). Peroxidase activity was detected by chemiluminescence (ECL Western blotting detection system; Amersham).

### *$PGE_2$ and cytokine assays*

Supernatants from cultures of MDC were harvested for analysis of PGE2 and IL-12. I-MDC were cultured for 6 days, washed from the plate, counted, and replated at $3 \times 10^5$ cells/ml in media containing GM-CSF and IL-4. I-MDC were cultured for an additional 48 h before supernatants were harvested for analysis. Supernatants from maturing M-MDC, were prepared by harvesting I-MDC on day 6, replating these cells at the same density in media containing GM-CSF, IL-4, and maturation stimuli. Cells were cultured for an additional 48 h and then supernatants were harvested. MDC culture supernatants from various conditions were analyzed for IL-12p70 and IL-12p40 (gift from Dr. Maurice Gately, Hoffman Roche, Nutley, NJ) by ELISA in duplicate as previously described (14). The lower limit of IL-12p40 and IL-12p70 detection in this assay is 15.6 pg/ml. Supernatants for IL-10 were measured by ELISA (Endogen, capture Ab clone 9D7 and detection Ab, clone 12G8 biotinylated). The lower limit of detection for IL-10 is 20.5 pg/ml. Measurement of $PGE_2$ was performed using a competitive enzyme immunoassay (Cayman Chemical). To correct for $PGE_2$ contained in the sera added to our media, we assayed in duplicate media alone (baseline). The final $PGE_2$ concentration was calculated by subtracting the baseline from the assayed supernatant value. The limit of detection for $PGE_2$ assay is 30 pg/ml. Final $PGE_2$, IL-10, and IL-12 were standardized to quantity/ml/$1 \times 10^6$ cells.

### *Allogeneic MLR*

I-MDC and MDC matured with sCD40L and IFN-$\gamma$ in the presence and absence of NS-398 were generated according to the protocol described above. The three MDC population were washed twice, counted and placed in 48-well flat-bottom wells (Costar, Cambridge, MA; $2.5 \times 10^4$ cells/well) along with nylon wool-purified allogeneic responder T cells ($2.5 \times 10^5$ cells/well) in the presence or absence of NS-398. On day 5 of the mixed lymphocyte reaction, supernatants were harvested and analyzed for IFN-$\gamma$ and IL-4 production. Human IFN-$\gamma$ was measured by specific sandwich ELISA using purified NIB42 as capture Ab and biotinylated 4S.B3 as detection Ab (PharMingen). The lower limit of detection for this assay is 15.6 pg/ml. Human IL-4 was also measured by specific sandwich ELISA using 8D4−8 as capture Ab and biotinylated MP4−25D2 as detection Ab (PharMingen) The lower limit of detection for this assay is 7.8 pg/ml.

## Results

### *MDC express COX-1 and COX-2*

To determine whether MDC express COX-2, we employed an established protocol employing GM-CSF and IL-4 to generate I-MDC from peripheral blood monocytes (12). After 6 days in culture, I-MDC were harvested and washed, then replated and cultured for an additional 48 h in new media containing GM-CSF and IL-4. MDC maturation was stimulated by culturing I-MDC with either soluble trimeric CD40L (sCD40L) in the presence or absence of human recombinant IFN-$\gamma$ for the same 48-h period. Cells and culture supernatants were harvested at the 48-h time point for analysis.

We first analyzed the MDC from these cultures for COX-1 and COX-2 expression by intracellular flow cytometry (13) and immunoblotting. As seen in Fig. 1, *A* and *B*, I-MDC stimulated with IFN-$\gamma$ only, sCD40L only, and sCD40L/IFN-$\gamma$ constitutively express COX-1 and COX-2. We were also able to detect intracellular COX-2 expression by flow cytometry (see Fig. 1*C*) and further establish expression in MDC. This is in marked contrast to monocytes that express COX-1 constitutively (data not shown) but require LPS induction for COX-2 expression (Fig. 1*D*). Of interest, whereas monocyte COX-2 is readily suppressed by 500 U/ml of IL-4 (Fig. 1*D*, *lane 3*), the same concentration of IL-4 present in MDC cultures does not regulate COX-2 in either I- or M-MDC (Fig. 1, *A* and *B*). We also find that IL-10 does not suppress COX-2 (data not shown). These findings with MDC are in marked contrast to several studies demonstrating that LPS-induced monocyte COX-2 expression is readily down-regulated by antiinflammatory cytokines IL-4, IL-10, and IL-13 (8). However, our results are similar to findings by Maloney et al. (15) that showed COX-2 induced by LPS or GM-CSF in neutrophils was not down-regulated by IL-4 or IL-10. These data suggest that GM-CSF, IL-4, or factors produced in culture by monocytes or the differentiating process induces COX-2 in manner that provides resistance to cytokine regulation.

### *Prostanoid production by I-MDC and M-MDC*

Next, we assessed COX-1- and COX-2-mediated prostanoid production by MDC populations. We analyzed the supernatants of I- and maturing M-MDC cultured in the presence and absence of NS-398, a specific COX-2 inhibitor, or indomethacin, a COX-1 and -2 inhibitor, added during the last 48 h of cell culture. We find that I-MDC spontaneously produce thromboxane (TBX) >

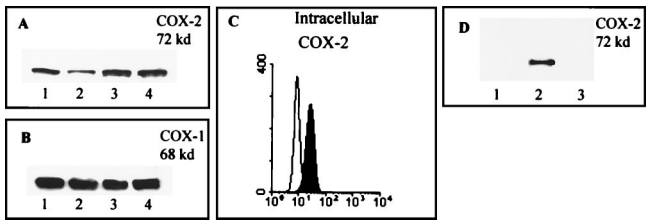


**FIGURE 1.** I-MDC and M-MDC express COX-1 and COX-2. SDS-PAGE electrophoresis with a 10% Tris-HCl gel loaded with 30 $\mu$g of protein from MDC cell lysates and 10 $\mu$g of protein from monocytes cell lysates. COX-2 (*A*) and COX-1 (*B*) expression in *lane 1*, I-MDC (GM-CSF and IL-4 for 8 days); *lane 2*, M-MDC matured with 1 $\mu$g/ml sCD40L only; *lane 3*, M-MDC matured with 1 $\mu$g/ml sCD40L and 1000 U/ml IFN-$\gamma$; and *lane 4*, I-MDC with 1000 U/ml IFN-$\gamma$. *C*, Intracellular staining of COX-2 with FITC-conjugated mAb (filled histogram is anti-COX-2 and open histogram is isotype control) in I- MDC (M-MDC not shown). *D*, COX-2 expression in monocytes cultured for 24 h. *Lane 1*, Complete RPMI; *lane 2*, 1 $\mu$g/ml LPS; *lane 3*, 1 $\mu$g/ml LPS with 500 U/ml of IL-4.

$PGE_2$ > prostacyclin but no $PGD_2$ (data not shown). NS-398 and indomethacin significantly reduced $PGE_2$ production to a similar degree, suggesting that prostanoid synthesis occurs predominantly through COX-2 in I-MDC (Fig. 2). It is possible that small numbers of residual monocytes, ~1% of our cultures, produced large quantities of prostanoids and accounted for COX-2-mediated PG. Although this possibility exists, monocytes do not express COX-2 during culture without activation. Furthermore, the expression of this enzyme is readily suppressed in monocytes by the presence of IL-4 in the culture (see Fig. 1*D*).

We next evaluated the production of PG by I-MDC undergoing maturation when stimulated for 48 h with sCD40L (Fig. 2). MDC cultured in these conditions synthesize 2-fold more $PGE_2$ but use primarily COX-1 as indomethacin, but not NS-398, markedly reduced PG production. We also evaluated the effects of IFN-$\gamma$ on sCD40L mediated maturation as this cytokine in combination with sCD40L strongly influenced MDC function and development, especially secretion of IL-12p70 (16). When MDC were matured with sCD40L in combination with IFN-$\gamma$, a 3- to 4-fold increase in COX-2-mediated $PGE_2$ production occurred, which was reduced to I-MDC levels in the presence of NS-398 (Fig. 2). IFN-$\gamma$ also stimulated a 2-fold increase in COX-2-dependent $PGE_2$ production from I-MDC (data not shown). The effects of IFN-$\gamma$ on COX-2-mediated PG production by in I-MDC and maturing MDC may be related to the increased access of COX-2 to substrate as this cytokine readily stimulates AA release through G-protein-mediated activation of $PLA_2$ (17). We also analyzed the production of TBX and prostacyclin in MDC undergoing maturation with the sCD40L/IFN-$\gamma$ stimulus. We found that the production of these prostanoids increased in proportion to $PGE_2$ with this stimulus and were reduced to the same degree with NS-398 (data not shown). These data suggest that the synthesis of prostanoids through COX-2 is the primary pathway for I-MDC, whereas stimulation of I-MDC by sCD40L in the absence of IFN-$\gamma$ switches AA metabolism to COX-1-dependent pathway. However, when inflammatory stimuli such as IFN-$\gamma$ or LPS and TNF-$\alpha$ (data not shown) are present, COX-2-mediated PG synthesis again predominates.

The quantity of $PGE_2$ produced by MDC ($10^{-9}$ M) is relatively small in comparison to LPS-activated monocytes, which produce micromolar quantities of $PGE_2$. It is not readily evident why quantitative differences in PG metabolism exist between these two types of myeloid cells. Based on the Western blots, we do not find that monocytes express a greater mass of COX-2 than MDC (data not shown). Therefore, it may be that the presence of IL-4 in MDC cultures limits $PLA_2$ activity and substrate availability (18). However, culturing MDC in the absence of IL-4 for 24 h increased $PGE_2$ production, but the prostaglandin levels remained in the nanomolar range (data not shown). Alternatively, higher levels of AA may be liberated when monocytes are stimulated with LPS. However, stimulation of maturing MDC with LPS leads to only nanomolar quantities of $PGE_2$ (data not shown). Thus the quantitative set point for production of prostanoids by MDC appears to be substantially lower than that of macrophages or monocytes.

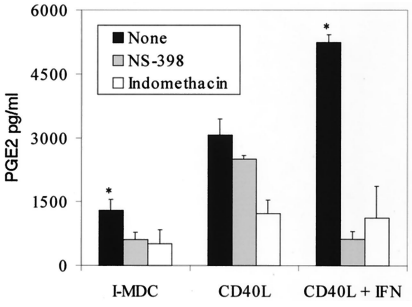


**FIGURE 2.** $PGE_2$ production is mediated by COX-2 in I-MDC and sCD40L/IFN-$\gamma$ matured MDC but is mediated by COX-1 in MDC matured with sCD40L alone. $PGE_2$ was measured by competitive immunoassay in the presence and absence of indomethacin, a COX-1 and -2 inhibitor or NS398, a COX-2-specific inhibitor. $PGE_2$ levels are expressed as pg/ml/$1 \times 10^6$ cells. Data represent the mean and SEM of at least four independent experiments. *, $p \leq 0.05$ as calculated by one-way ANOVA.

*COX-2 mediated PG synthesis promotes I-MDC maturation*

To establish whether endogenous PG affect differentiation of I-MDC from monocytes, we analyzed surface Ag expression of CD1a, CD14, CD40, CD80, CD86, CD83, and HLA-DR on these cells cultured in the presence and absence of NS-398 (Fig. 3). Our data demonstrate that blocking endogenous COX-2-mediated prostanoid production did not affect expression of CD1a, HLA-DR, or the expression of the costimulatory molecules during differentiation from monocytes to I-MDC (Fig. 3). These data are consistent with Kalinski et al. (9) who reported that MDC exposed to $10^{-9}$ M exogenous $PGE_2$, equivalent to levels produced by I-MDC, did not affect MDC differentiation from monocytes.

We also analyzed the same series of Ag markers on I-MDC matured with sCD40L and sCD40L with IFN-$\gamma$ in the presence of either NS-398 or indomethacin. Again, we found that blocking COX-2 in MDC stimulated with sCD40L/IFN-$\gamma$ did not modify expression of CD40, CD80, CD86, or HLA-DR (Fig. 4). We also find that indomethacin used to block COX-1, the predominant enzyme metabolizing arachidonate in MDC matured with sCD40L alone, likewise did not affect expression of these same Ags (data not shown). Previous studies demonstrated that micromolar concentrations of $PGE_2$ enhanced I-MDC maturation when used in cell culture in combination with LPS, TNF-$\alpha$, or a mixture of inflammatory cytokines (10, 19). However, in the present studies, we did not find that reducing PG limited MDC maturation based on the expression of these Ags. It appears that large quantities of $PGE_2$, such as that produced by macrophages, are required to modulate surface molecules such as CD86.

However, prostanoids produced by MDC are not without affect on MDC maturation. Blocking COX-2 with NS-398 profoundly inhibited CD83 expression following sCD40L/IFN-$\gamma$ stimulation (Fig. 4). The predominant effect of prostanoids appears to be mediated through COX-2 as the addition of indomethacin did not enhance this effect (data not shown). When MDC were stimulated with sCD40L alone, substantially lower levels of CD83 expression were achieved. As MDC stimulated in this manner produce $PGE_2$ primarily through COX-1 we blocked PG production with indomethacin and evaluated CD83 expression. Unlike I-MDC matured with sCD40L and IFN-$\gamma$, the COX inhibitor did little to affect CD83 expression in these conditions. These data are consistent with the previous reports suggesting that $PGE_2$ increases CD83 expression on MDC. However, these studies employed micromolar concentrations of $PGE_2$ to enhance CD83 expression (10). Although lower doses of $PGE_2$ equivalent to that made by MDC were not tested in these reports, it may be that sCD40L provides a qualitatively different stimulus than LPS, TNF-$\alpha$, or a combination of inflammatory cytokines such that nanomolar levels of PG are effective. Based on the present findings, it appears that lower levels of endogenous PG uniquely stimulate expression of CD83 in contrast to other maturation Ags, e.g., CD86.

*Endogenous prostanoid production affects secretion of IL-12*

MDC secretion of the Th1-polarizing cytokine, IL-12, has been extensively studied (9, 11, 16, 20, 21). To examine the effect of

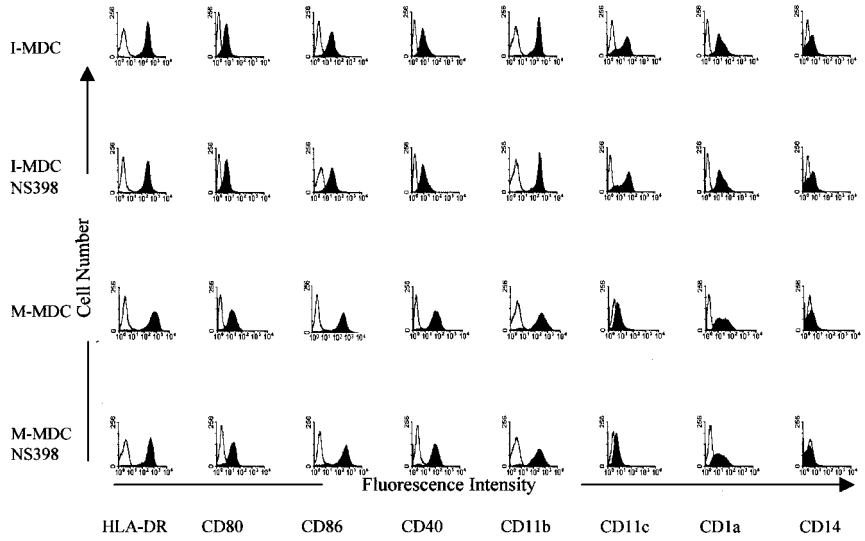


**FIGURE 3.** Blocking COX activity does not affect expression of HLA-DR and costimulatory molecules on I-MDC or MDC. I-MDC were cultured with GM-CSF and IL-4 in presence and absence of COX-2 inhibitor, NS-398. M-MDC cultured for 6 days with GM-CSF and IL-4 then matured in the presence or absence of NS-398 with soluble trimeric CD40L and IFN-γ. MDC were stained with Abs to cell surface markers conjugated to fluorochromes listed in *Materials and Methods*. Filled histogram indicates cell surface staining and open histogram represents isotype Ab staining.

endogenous PG on secretion of IL-12p40 and IL-12p70, we prepared MDC and assayed both forms of this cytokine in the supernatants in the presence and absence of COX inhibitors. We chose to study IL-12 production during maturation of MDC using sCD40L alone and in combination with IFN-γ, the latter combination stimulating production of biologically active IL-12p70 (16). Consistent with previous reports, we found that I-MDC produced only IL-12p40 and did not produce IL-12p70 (19, 20, 22). When I-MDC were cultured in the presence of NS-398 for 48 h, IL-12p40 was significantly reduced (Fig. 5). The inhibition of IL-12 by indomethacin was not different from that of NS-398, suggesting the effects of prostanoids on this cytokine are predominantly mediated by the COX-2 isoform (data not shown). These results are consistent with those of Rieser et al. (11) who showed an increase in total IL-12 when I-MDC were exposed to $PGE_2$ or other compounds which increase intracellular cAMP. In marked contrast, I-MDC undergoing maturation for 48 h with sCD40L and IFN-γ in the presence of COX-2 inhibitor, significantly increased IL-12p40 production ($p = 0.007$) and increased, but not significantly, IL-12p70 production ($p = 0.068$; Fig. 5). These findings mirror previous studies that showed addition of $PGE_2$ to cell culture suppressed IL-12p70 production by maturing MDC (20, 23). Preliminary studies in our laboratory showed $PGE_2$ to be the predominant prostanoid suppressing IL-12 production. Prostacyclin had similar but lesser effects than $PGE_2$ on secretion of IL-12, whereas TBX and metabolites of $PGD_2$ had little to no effect (manuscript in preparation). Collectively, these data further demonstrate that prostanoids produced via COX-2 modulate MDC function and markedly affect the secretion of IL-12. However, the effect is dependent on the state of differentiation of these cells.

### *IL-10 production by MDC is not regulated by endogenous PG synthesis*

Previous studies in murine macrophages demonstrated that IL-10 production in LPS-stimulated macrophages occurred through a cAMP/$PGE_2$-dependent mechanism (24). We therefore evaluated the production of IL-10 in I-MDC and maturing MDC. We find that I-MDC do not produce detectable levels of IL-10, whereas M-MDC matured with soluble sCD40L alone or with and IFN-γ produce low levels that are not significantly reduced with NS-398 or indomethacin (Fig. 6). These experiments do not suggest that PG produced by MDC stimulate IL-10 production. Furthermore, they demonstrate that PG-mediated suppression of IL-12 in maturing MDC is mediated directly by endogenous $PGE_2$ directly and not through its effect on IL-10.

### *Blocking COX-2 in maturing MDC enhances stimulation of Th1 responses*

Blocking COX-2-mediated prostanoid production in maturing MDC increased IL-12p70 secretion (Fig. 5), and leads to a sustained capacity of M-MDC to produce higher levels of IL-12p70 with subsequent stimulation (data not shown). Because of these effects, we questioned whether M-MDC generated in the presence of NS-398 would promote Th1 responses to a greater degree than M-MDC matured in the absence of this specific inhibitor. To address this question we evaluated the ability of I-MDC and M-MDC matured with sCD40L and IFN-γ in the presence or absence of NS-398 to activate allogeneic T cell IFN-γ or IL-4 production. As shown in Fig. 7, MDC matured with sCD40L and IFN-γ in the presence of NS-398 stimulated significantly more IFN-γ in the allogeneic MLR than did MDC matured without NS-398 ($p < 0.0048$). We did not detect IL-4 in any of the culture supernatants (lower limit of detection is 7.8 pg/ml). These data suggest that COX-2 mediated PG production by MDC during the maturation process determines their subsequent capacity to produce IL-12 and to promote Th1 responses. Another possibility may be that blocking prostanoid production during maturation affects M-MDC factors other than IL-12 that promote Th1 responses. We attempted but were unable to detect IL-12p70 in these cell cultures. This may be due to the low numbers of MDC ($2.5 \times 10^4$ cells) in each condition or because of cytokine consumption. Finally, the addition of NS-398 at the beginning of the mixed lymphocyte response had no effect on IFN-γ production stimulated by I-MDC or either M-MDC population and suggests IL-12 and IFN-γ production by M-MDC and T cells, respectively, are not affected by prostanoids produced during the allogeneic mixed lymphocyte response. Alternatively, it may be that the levels of prostanoids produced by MDC or T cells are too low to affect the response.

## Discussion

This is the first report demonstrating that I-MDC and M-MDC constitutively express both COXs, COX-1, and the normally inducible COX-2 and synthesize nanomolar quantities of PG, e.g., $PGE_2$. The predominant isoform of COX used to produce prostanoids by I-MDC is COX-2. An interesting finding of this study was that when I-MDC undergo maturation with sCD40L alone, $PGE_2$

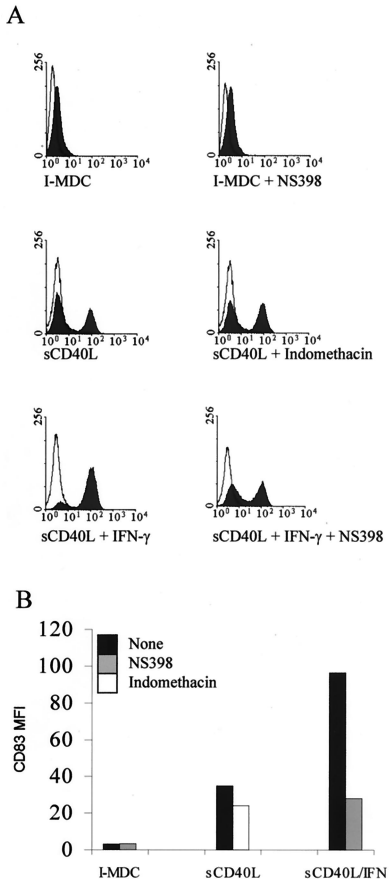


**FIGURE 4.** Endogenous PG regulate CD83 in M-MDC matured with sCD40L and IFN-$\gamma$. *A*, Flow cytometric analysis of CD83 in I-MDC and M-MDC matured with sCD40L alone with our without Indomethacin, and with sCD40L/IFN-$\gamma$ in the presence or absence of NS398. *B*, Bar graph displays comparison of the median fluorescence intensity (MFI) in I-MDC and M-MDC matured with sCD40L alone and sCD40L/IFN-$\gamma$ in the presence or absence of NS398 or Indomethacin. These results are representative of four independent experiments.

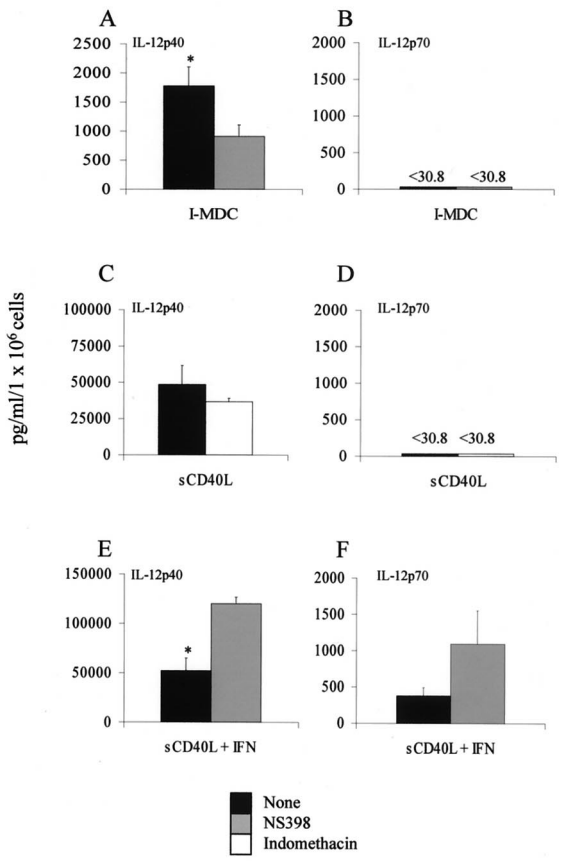


**FIGURE 5.** Endogenous PG autoregulate IL-12p40 and IL-12p70 production by I-MDC and M-MDC. Supernatants from I-MDC and M-MDC in the presence or absence of NS-398 or indomethacin were analyzed for IL-12p40, IL-12p70 by ELISA. *A*, IL-12p40 in I-MDC, $*$, $p = 0.045$. *B*, IL-12p70 in I-MDC. *C*, IL-12p40 in MDC undergoing maturation with sCD40L. *D*, IL-12p70 in MDC undergoing maturation with sCD40L. *E*, IL-12p40 in sCD40L/IFN-$\gamma$ matured MDC, $*$, $p = 0.007$. *F*, IL-12p70 in sCD40L/IFN-$\gamma$ matured MDC, $*$, $p = 0.06$.

synthesis proceeds through COX-1. In contrast, MDC stimulated with sCD40L in combination with IFN-$\gamma$ leads to higher levels of $PGE_2$, but production reverts back to the COX-2 pathway. The observation that PG synthesis fluctuates from one COX isoform to the other is not a novel finding. Previous studies demonstrated that this phenomenon occurs as a consequence of the coupling of COX isoforms to distinct $PLA_2$ isoenzymes, e.g., cytoplasmic $PlA_2$ to COX-2, and linkage of apparently discrete pools of AA to either COX-1 or COX-2 (25). Supporting these published studies, we find that mouse macrophages expressing both COX-1 and COX-2 produce $PGE_2$ only through COX-1 when AA added to cultures, whereas IFN-$\gamma$ stimulates only COX-2-mediated PG synthesis (manuscript in preparation).

The regulation of COX-2 expression in MDC is unlike that of the precursor monocyte population, as MDC are highly resistant to suppression by the anti-inflammatory cytokines IL-4 and IL-10. The reason for the marked alteration in COX-2 regulation is not apparent but may be related to the continuous presence of GM-CSF in vitro. Another possibility is that long-term culture or long-term exposure to IL-4 may also diminish the MDC response to this cytokine. Alternatively, studies by Hart et al. (15) demonstrated that freshly isolated monocytes were more responsive to IL-4 induced TNF-$\alpha$ suppression than macrophages cultured for 7 days. These authors concluded that the monocytes' responses to immunoregulatory cytokines such as IL-4 may not mirror responses by their differentiated or activated counterparts (15). This suggestion is also supported by the studies of Maloney et al. (26) that demonstrated neutrophil expression of COX-2 was likewise resistant to IL-4, IL-10, and IL-13. Therefore, the pathway of myeloid differentiation or maturation may dictate the responsiveness of COX-2 to anti-inflammatory cytokines.

The production of PG appears to autoregulate some aspects of MDC maturation (e.g., CD83) and function (e.g., IL-12 production by MDC). Our finding that endogenous prostanoids generated through COX-2 in vitro did not interfere with the expression of HLA-DR and costimulatory molecules on I-MDC was expected because previous studies showed that less that $10^{-9}$ M $PGE_2$ had little effect on these differentiation Ags for MDC. Although we find that endogenous production of prostanoids does not modify HLA-DR or the costimulatory molecules CD40, CD80, and CD86; however, COX-2 PG markedly modulate the expression of the maturation Ag, CD83, in sCD40L/IFN-$\gamma$ stimulated MDC. It appears that the threshold for prostanoid regulation of CD83 differs markedly from that of HLA-DR and costimulatory molecules. In the case of costimulatory molecules, cells producing higher levels of $PGE_2$ than MDC, perhaps macrophages, within the local environment may be required to affect the up-regulation of these molecules as previously described (10).

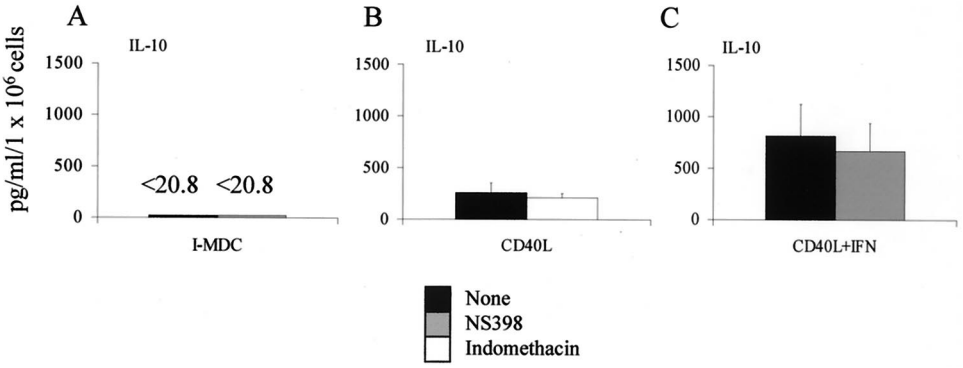


**FIGURE 6.** Endogenous PG do not significantly affect IL-10 production in MDC. IL-10 production was measured by direct ELISA in the presence or absence of COX inhibitor (*A*) I-MDC, presence and absence of NS398, (*B*) sCD40L matured MDC with and without Indomethacin, and (*C*) sCD40L/ IFN-γ matured MDC with and without NS398.

In this study, we find a divergent regulation of IL-12 by COX-2-mediated prostanoid production. We find that endogenously produced PG increased the IL-12p40 in I-MDC but did not stimulate IL-12p70 production. This prostanoid-mediated enhancement of IL-12p40 production by I-MDC may serve to limit the Th1 immune response as IL-12 p40 homodimers function as a receptor antagonist (27, 28). As MDC mature in the presence of IFN-γ the level of COX-2-mediated prostanoid production increases which effectively suppresses IL-12p70 and p40. This is in agreement with studies of others demonstrating that addition of $PGE_2$ to cell culture reduces IL-12 production by M-MDC (23, 29). Thus, endogenous prostanoids appear to play an important role in limiting the capacity of M- MDC to become a potent Th1 promoting APCs by down regulating the production of biologically active IL-12p70 by these cells. The mechanism responsible for the interesting divergence in prostanoid-mediated regulation of IL-12 has not been defined. However, modulation of surface or nuclear receptors for $PGE_2$, e.g., EP1, EP2, EP3, or EP4 could be responsible for these changes in the response of MDC as they mature. Preliminary studies in the laboratory indeed suggest that maturation stimuli regulate EP receptor expression and this defines the response of MDC to $PGE_2$.

Previous reports have suggested that COX-2 is important for high level production of prostanoids by particular cells types and is the form of the enzyme associated with inflammation. It is of interest when MDC are exposed to IFN-γ in culture that $PGE_2$ production is enhanced and COX-2 is the predominant isoform of the enzyme used to produce prostanoids. These findings suggest that a default setting for I-MDC and maturing MDC is to increase COX-2-mediated $PGE_2$ production when involved in inflammation, when acting as an APC for established Th1 responses, or when encountering other IFN-γ producing cells (e.g., NK or NKT cells). Under these circumstances, MDC are thus programmed, via COX-2 expression, to suppress IL-12 production and thus autoregulate their capacity to further stimulate Th1 cells. Indeed, results from the allogeneic MLR support such a role for PG. These data suggest the production of endogenous PG during maturation directs MDC functional development such that their capacity to produce IL-12 and stimulate Th1 responses is significantly limited.

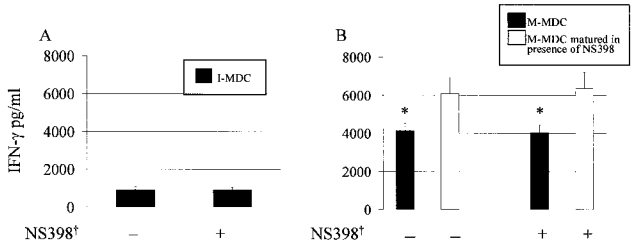


**FIGURE 7.** Enhanced IFN-γ production in T cells activated with M-MDC matured in the presence of a COX-2 inhibitor. Allogeneic T cells ($2.5 \times 10^5$ cells/well) from a single donor were cocultured $2.5 \times 10^4$ MDC/well. MDC populations were prepared from three separate donors as described. *A*, I-MDC (■). *B*, M-MDC (▨) and M-MDC matured in the presence of NS-398 (□). MDC were washed extensively before use and cultured with allogeneic T cells in either the absence (*left*) and presence (*right*) of NS-398. Each lymphocyte response was performed in duplicate cultures. Supernatants from each culture well were analyzed for IFN-γ by ELISA in duplicate and the data presented are the mean ± SE of three experiments. †, NS398 added at inception of MLR. ∗, $p < 0.001$.

Prostaglandin production by MDC appears to play an important and focused role in the function of MDC. From the findings of this study it appears that MDC tend to produce lower levels of $PGE_2$ than that produced by monocytes or macrophages. The lower level of PG produced by MDC may be of practical importance as these lipid molecules work in an autocrine fashion modulating MDC function, and perhaps in regulating T cells within their microenviroment in a paracrine fashion. Working in this manner, the effects of prostanoids would be contained and would limit the untoward effects of these molecules. In this context, determining regulation of prostanoid receptors on MDC and T cells thus becomes critical to understanding the effects of these lipid molecules on their target cells. Furthermore, the production of PG by MDC may provide these cells with a self-contained "signal 3" as proposed by Kalinski et al. (30), which would polarize the MDC away from stimulating Th1 responses, perhaps more toward a Th2 promoting APC. Our findings also have important implications regarding the effects of COX inhibitors, particularly the new class of COX-2-specific drugs, on the immune response. The potent anti-inflammatory action of these drugs may in part be limiting MDC maturation. These studies also raise a potential concern regarding the possibility that COX-2-specific drugs could potentiate Th1 responses. Indeed our in vitro experiments suggest that by removing the suppressive autocrine effects of PG on IL-12 production in maturing M-MDC these cells stimulate significantly higher levels of IFN-γ production by T cells. Further study in vivo is required to establish the effect of these drugs on the biology of MDC.

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