A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation

Deborah Braun, Randy S. Longman, and Matthew L. Albert

Prostaglandins, a family of lipid molecules released during inflammation, display immunomodulatory properties in several models. One use includes exposure of monocyte-derived dendritic cells (DCs) to a cocktail of cytokines that contains prostaglandin E2 (PGE2) for purposes of maturation; such cells are currently being used for cancer immunotherapy trials. Our analysis of the transcription profile of DCs matured in the presence of tumor necrosis factor α (TNFα) and PGE2 revealed a strong up-regulation of indoleamine 2-3 dioxygenase (IDO), an enzyme involved in tryptophan catabolism and implicated in both maternal and T-cell tolerance. Using quantitative assays to monitor levels of IDO mRNA, protein expression, and enzyme activity, we report that PGE2 induces mRNA expression of IDO; however, a second signal through TNF receptor (TNF-R) or a Toll-like receptor (TLR) is necessary to activate the enzyme. Interestingly, use of TNFα, lipopolysaccharide, or Staphylococcus aureus Cowan I strain (SAC) alone does not induce IDO. The effect of PGE2 is mediated by activation of adenylate cyclase via the Gs-protein–coupled receptor E prostanoid-2 (EP2). A better understanding of these regulatory mechanisms and the crosstalk between TNF-R/TLR and EP2 signaling pathways will provide insight into the regulation of T-cell activation by DCs and may help to improve existing immunotherapy protocols. (Blood. 2005;106:2375-2381)

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cells, or the stimulation of antigen-specific CD4+ and CD8+ T-cell responses. In prior studies, we have used TNFα and PGE2 to mature DCs. Such cells possess the ability to both prime and tolerate CD8+ T cells, depending on the immune context and the route by which antigen is processed and presented.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme involved in tryptophan catabolism. Its role in antimicrobial resistance is well described; by actively depleting tryptophan, essential for the growth of microorganisms, both within the infected cell and in the surrounding milieu, IDO serves to suppress growth of invasive bacteria. More recently, it has been established that IDO regulates maternal tolerance and possibly more general aspects of T-cell tolerance. IDO expression has been reported in placental trophoblasts and interferon γ (IFNγ)-activated APCs (including macrophages and DCs), reflecting its counter-inflammatory role.

Herein, we characterize the mechanism by which IDO is up-regulated in monocyte-derived DCs. We report that that PGE2 induces mRNA expression of IDO; however, the enzyme remains inactive. Only in response to a second signal via TNF receptor (TNF-R) or Toll-like receptor (TLR) do we observe bioactive IDO. Surprisingly, use of TNFα, lipopolysaccharide, or Staphylococcus aureus Cowan I strain (SAC) alone does not induce IDO. This study offers new insights into the immune-modulatory effects of PGE2 as well as provides a better understanding as to how IDO expression is achieved in inflammatory situations. Importantly, this work will impact the design of future DC-based immunotherapy trials.

Materials and methods

Human subject materials

Human blood components were obtained from healthy donors (Etablissement Français du Sang [EFS], Rungis, France). Materials were stripped of patient identifiers and shipped to Institut Pasteur in accordance with institutional policy (no. HS2003-5720) and the tenets of the Helsinki protocol.

Reagents

TNFα (Endogen, Boston, MA) was used at a concentration of 100 ng/mL and PGE2 (Endogen, Boston, MA) was used at a concentration of 100 ng/mL, unless otherwise stated. Lipopolysaccharide (LPS), serotype 055:B5 (Sigma) was sonicated and used at 50 ng/mL, SAC was used at 0.0025% wt/vol (Pansorbin, Calbiochem-Behring, La Jolla, CA), 1-tryptophan, 1-kynurenine, and forskolin (Sigma) were used as described in “Results”; prostaglandin E receptor subtype (EP1), EP3, and EP4 agonists (L-335677, L-826266, and L-161982, respectively) were provided by Merck Frost & Co (Quebec, Canada) and used at a concentration of 50 μM; butaprost and 19(R)-OH PGE2 (EP2 agonists), sulprostone (EP1>EP3 agonist) were obtained from Cayman Chemicals, Ann Arbor, MI, and used at 0.5 to 250 μM. The adenylate cyclase inhibitor SQ22536 (Biomol International, LP, Plymouth Meeting, PA) was titrated, and the optimal concentration to inhibit forskolin was found to be 1 μM. H-89 (Sigma) was used at 10 to 50 μM for the inhibition of protein kinase A (PKA).

Isolation and preparation of dendritic cells

Monocyte-derived DCs were prepared as described. Briefly, buffy coats were obtained from healthy donors, and peripheral blood mononuclear cells (PBMCs) were isolated by sedimentation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). CD14-enriched and CD14-depleted fractions were prepared using CD14 Miltenyi microbeads followed by magnetic cell sorting according to the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). Immature DCs were prepared from the CD14+ fraction by culturing cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; Berlix, Seattle, WA) and IL-4 (R&D Systems, Minneapolis, MN) for 7 days. GM-CSF (1000 U/mL) and IL-4 (500 U/mL) were added to the cultures on days 0, 2, and 4. To generate mature DCs, the cultures were transferred to fresh wells on day 6 or 7, and the indicated maturation stimulus was added for an additional 1 to 2 days. At days 6 to 7, greater than 95% of the cells were CD14+CD83+HLA-DR+ DCs. After maturation, on days 8 to 9, 70% to 95% of the cells were of the mature CD14+CD83+HLA-DR+ phenotype (data not shown).

Quantitative analysis of IDO mRNA expression

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1 to 2 μg RNA using oligo-dtt (Roche, Indianapolis, IN) and SuperScript reverse transcriptase (Invitrogen) according to manufacturers’ instructions. IDO-specific mRNA is quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TATA box binding protein (TBP) using the following primers: IDO forward, 5′-AGAGTCAATCC-CTCCATGTC-3′; IDO reverse, 5′-AAATCAGTGCTCCTAGTCC-3′; GAPDH forward, 5′-ACTCCACGACGTACTCAGG-3′; GAPDH reverse, 5′-GGTCCGAGTCAACGGATTTG-3′; TBP forward, 5′-GCCAGAGGCCCCAAGTGG-3′; and TBP reverse, 5′-TCACAGCTTCCCACCATATT-3′. Primers for EP receptors are described in Kamphuis et al. Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using the SYBR Green JumpStart Taq ReadyMix (Sigma) according to the manufacturer’s instructions. The reactions were run on a PTC200 equipped with a Chromo4 detector (MJ Research, Boston, MA). The analyses were performed with the Opticon Monitor software version 2.03 (MJ Research). All the measures were performed in duplicate and validated when the difference in threshold cycle (CT) between the 2 measures was less than 0.3. Amplification and dissociation curves of increasing amounts of total PBMC cDNA allowed validation of our assay; dissociation curves displayed a single peak, ruling out the presence of primer dimers or parasitic products (Supplemental Figure S1A, available on the Blood website; see the Supplemental Figures link at the top of the online article). The ratio of gene of interest–housekeeping gene was calculated according to the formula: ratio = 2-ΔΔCT (ΔCT = mean Ct gene − mean Ct housekeeping). GAPDH and TBP were used to normalize for IDO and EP-receptor mRNA expression, respectively.

Detection of IDO protein expression

Cell lysates were prepared from 106 DCs using RIPA buffer (20 mM Tris [tris(hydroxymethyl)methanemethane] pH 7.5, 150 mM NaCl, 1% glycerol, 1% Nonidet P-40 ([Octylphenoxypolyethoxethanol], Complete [Roche; protease inhibitor cocktail]). One third of the total protein lysate was separated on 12% or 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to polyvinylidene difluoride (PVDF) membranes, protein loading was monitored using Ponceau Red staining. IDO protein was detected using a rabbit polyclonal antibody (Ab) preparation (generous gift of David Munn, Medical College of Georgia, Augusta, GA) and anti–rabbit IgG–horseradish peroxidase (HRP; Santa Cruz Biototechnology, Santa Cruz, CA), and visualized by chemiluminescence (ECL; Amersham, Piscataway, NJ).

Determination of IDO enzymatic activity

To monitor enzyme activity, DCs were washed and resuspended in Hanks Buffer (HBSS) containing 100 μM tryptophan (Life Technologies, Grand Island, NY) and incubated for 4 hours. Supernatants were harvested and assayed for the presence of kynurenine, the first stable catabolite downstream of IDO. Kynurenine was detected by either high-pressure liquid chromatography (HPLC) or using a modified spectrophotometric assay.
HPLC was performed according to Yong and Lau with minor modifications. Briefly, 40 μL clarified sample was injected into an Amersham reverse phase C2/C18 column and eluted with KH2PO4 buffer (0.01 M KH3PO4 and 0.15 mM EDTA [ethylenediaminetetraacetic acid], pH 5.0) containing 10% methanol at a flow rate of 1.0 mL/min. The spectrophotometer was set at 254 nm to detect both kynurenine and tryptophan. Retention time was determined empirically using standard solutions of kynurenine and tryptophan. IDO activity is reported as the concentration of kynurenine produced. Alternatively, kynurenine were measured spectrophotometrically. The amount of 50 μL of 30% trichloroacetic acid was added to 100 μL culture supernatant, vortexed, and centrifuged at 8000g (10,000 rpm) for 5 minutes. Volume (75 μL) of the supernatant was then added to an equal volume of Ehrlich reagent (100 mg P-dimethylbenzaldehyde, 5 mL glacial acetic acid) in a microtiter plate well (96-well format). Optic density was measured at 492 nm, using a Multiskan MS (Labsystems, Helsinki, Finland) microplate reader. A standard curve of defined kynurenine concentration (0-100 μM) permitted analysis of unknowns.

Results

IDO expression and activity are up-regulated during DC maturation

The phenotypic and functional changes that occur during DC maturation are critical for generating MHC/peptide complexes and engaging T cells; however, the molecular definition of distinct maturation programs has only recently been considered. As previously reported by others, we analyzed the transcriptional profile engaged during DC maturation. This has been reproduced in 4 of 4 donors, and the 100-fold increase in expression of IDO as a result of DC maturation. This was reproduced in 4 of 4 donors, and the relative signal intensity of IDO mRNA expression is shown, using GAPDH expression as an internal reference (Figure 1).

On the basis of IDO’s proposed role in immune tolerance, we evaluated the effect of different DC maturation stimuli on IDO expression. Despite some published data in this area, a thorough assessment of the expression and activity of IDO as influenced by DC maturation had yet to be performed. We first established assays to monitor IDO mRNA expression using real-time quantitative RT-PCR (q-PCR), and Western blot. RNA was extracted from iDCs exposed to distinct maturation stimuli, and IDO expression was quantified as described in “Materials and methods.” Consistent with our gene array studies, iDCs matured with TNFα and PGE2, but no evidence of tryptophan catabolism was detected in iDCs or in LPS- or SAC-matured DCs (Figure 2C). In our initial studies, IFNγ-exposed DCs served as a positive control for IDO expression and activity. DCs matured from multiple individuals permitted evaluation of donor variability (Table 1). Because of our interest in monitoring IDO functional activity in several conditions for DC stimulation, we took advantage of a medium-throughput (96-well assay) colorimetric assay for monitoring kynurenine.

To validate this approach, we established a standard curve for quantifying kynurenine and compared the experimental values obtained using the colorimetric assay with those from the HPLC analysis. A strong correlation was observed (Supplemental Figure S2B-D), thus allowing us to use the colorimetric assay for monitoring IDO activity.

Prostaglandin E2 is responsible for the expression of IDO mRNA

In an attempt to define the stimuli responsible for IDO expression, we revealed a surprising result. Similar to treatment of iDCs with LPS or SAC, when used alone TNFα did not up-regulate IDO expression. Instead, it was exposure of PGE2 that accounted for expression of IDO mRNA (Figure 3A-B). Although we observed a 50- to 200-fold increase in transcription of IDO, PGE2-treated DCs lacked measurable IDO activity (Figure 3C).

This finding suggests that, although PGE2 induces transcription of the IDO gene, a second signal, such as exposure to TNFα, is required to achieve active IDO enzyme. Similar to TNF-R engagement, TLR ligation also induced IDO activity when used in combination with PGE2 (Figure 4A-B). Characterization of the pAb used suggests that it may be selective for active enzyme (data
not shown); consequently, we are unable to determine whether TNF-R/TLR regulates a transcriptional or posttranscriptional event. What is clear from our data, based on Figures 2 to 4, is that there exists a two-step regulation of IDO activity during DC maturation. Signaling via EP2 triggers IDO expression

Eight human prostanoid receptors have been described, of which 4 bind PGE2 with high affinity (dissociation constant ($K_d$) in the low-nM range). Of these 4 receptors, EP1 and EP3 are coupled to inhibitory G proteins; whereas EP2 and EP4 signal via stimulatory G proteins. On the basis of our microarray data and published studies, only EP2 and EP4 are expressed by monocyte-derived DCs (data not shown). We validated these findings in our culture system using quantitative RT-PCR. As shown, iDCs express higher levels of EP2 and low levels of EP4. After exposure to TNF and

Table 1. IDO expression and activity

<table>
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<th>Conditions</th>
<th>IDO mRNA (qPCR)*</th>
<th>IDO activity (µM kynurenine, HPLC)</th>
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<td>Average</td>
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<tr>
<td>TNFα + PGE2 + IFN-γ</td>
<td>1</td>
<td>5.1</td>
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</tbody>
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N/A indicates not applicable.

*Reported as a ratio of relative mRNA expression IDO/GAPDH. Non-normalized data are presented.
PGE2, the pattern of expression is reversed (Figure 5A). Of note, PGE2 seems to be responsible for the down-regulation of EP2, whereas both TNFα and PGE2 are required to observe up-regulation of EP4.

Next, we evaluated which EP receptor mediates IDO expression. This was done functionally, by exposing iDCs to agonists specific for EP1 to EP4 in the presence of TNFα, and IDO activity was measured as described above. In 3 of 3 individuals, only the EP2 agonist butaprost resulted in the induction of IDO activity (Figure 5B). As EP2 is a Gs-coupled receptor, we tested whether ectopic activation of adenylate cyclase would mimic the effects of a receptor agonist. When iDCs were treated with the adenylate cyclase activator forskolin in combination with TNFα, the IDO activity was equivalent to that in TNFα- and PGE2-matured DCs (Figure 6A). We next directly tested the effect of adding either an inhibitor of adenylate cyclase or the cAMP-triggered kinase PKA. As shown, both inhibitors decreased the PGE2- and forskolin-induced expression of IDO. Together, these results suggest that during DC maturation, PGE2 acts through the EP2 receptor expressed on iDC, which in turn activates adenylate cyclase leading to PKA activation and an increase in the transcription of the gene coding for IDO. The presence of a TNF-R or TLR agonist enhances expression, and, importantly, this second signal facilitates IDO enzymatic activity.

Discussion

A novel mechanism by which PGE2 modulates the immune system

Like many effectors of immune regulation, the actions of PGE2 are complex and in some instances at apparent odds. With respect to its effect on T cells, the data indicate that PGE2 inhibits proliferation and skew responses toward Th2 differentiation. And although inhibition of TNFα, IL-6, and IL-12 p35 in antigen-presenting cells would be consistent with a suppressive role for PGE2, recent studies indicate a proinflammatory activity of PGE2, based on the induction of bioactive IL-23. PGE2 has also been shown to act as a survival signal for thymocytes and may be critical for T-cell development. Furthermore, PGE2 is critical both for the generation of migratory DCs: monocyte-derived DCs demonstrate poor migration potential to CCR7 ligands unless matured in the presence of PGE2; in vivo, it has been demonstrated that mouse Langerhans-cell migration to the draining lymph node is dependent on PGE2 signaling via EP4. The stimulation of DC migration has been interpreted as proinflammatory, because migratory DCs are required for the trafficking of antigen to the T-cell area of lymph nodes, thus permitting the initiation of an immune response.

We report a novel mechanism by which PGE2 acts to counter inflammatory responses. Our study indicates that PGE2 induces mRNA expression of IDO by signaling via the Gs-protein–coupled receptor EP2. This in turn activates adenylate cyclase, catalyzing the formation of cAMP and activating PKA. Evidence to support this mechanism is provided by the use of EP-receptor analogs and the demonstration that only the EP2 agonist, butaprost, was able to mimic the effects of PGE2 (Figure 5). Additionally, we demonstrate that direct stimulation of adenylate cyclase triggered IDO expression;
Induce IDO, they are required to trigger enzymatic activity in PGE2.

200-fold increase in IDO expression, to our surprise, PGE2-treated DCs
of IDO (Figure 6). Although this pathway accounted for a 50- to
effect of the PKA inhibitor H-89, were assessed on IDO mRNA levels.

Effect of inhibiting adenylate cyclase during this culture using SQ22536, as well as the
cultured in the presence of PGE2 or forskolin, in addition to TNF.
IDO activity was assessed spectrophotometrically
(H9251. Monocyte-derived DCs
of EP2/4 and induces IDO activity in the presence of TNF
to trigger IDO.

Figure 6. PGE2 signals via cyclic adenosine monophosphate (cAMP) and PKA
to trigger IDO. (A) The adenylate cyclase activator forskolin mimics PGE2 activation
of EP2/4 and induces IDO activity in the presence of TNF.
Monocyte-derived DCs (MoDCs) were matured during 48 hours in the presence of increasing doses of
forskolin with or without TNF.
IDO activity was assessed spectrophotometrically after 4 hours at the end of the culture. (B) The adenylate cyclase inhibitor SQ22536
and the PKA inhibitor H-89 block IDO expression. Monocyte-derived DCs
were cultured in the presence of PGE2 or forskolin, in addition to TNF, for 48 hours. The
effect of inhibiting adenylate cyclase during this culture using SQ22536, as well as the
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to trigger IDO.

Also, treatment with an adenylate cyclase inhibitor or a PKA
inhibitor blocked EP2 signaling and inhibited PGE2-mediated induction
of IDO (Figure 6). Although this pathway accounted for a 50- to
200-fold increase in IDO expression, to our surprise, PGE2-treated DCs
displayed no active IDO enzyme as monitored by the catabolism of
tryptophan. Only when combined with a second signal via TNF-R or a
TLR did we observe the production of kynurenine (Figures 2-4).

Although use of TNF, lipopolysaccharide, or SAC alone does not
induce IDO, they are required to trigger enzymatic activity in PGE2-
treated DCs. These findings offer the first innate effector pathway for the
activation of IDO and suggest a link between DC migration and the
acquisition of mechanisms for immune tolerance. Indeed, such a
connection pushes one to consider the possibility that migratory DCs are
programmed so that its default pathway is tolerance.

**Activation of indoleamine 2,3-dioxygenase**

The actions of IDO offers an intriguing mechanism for achieving
T-cell tolerance (reviewed in Mellor and Munn23); it catabolizes the
essential amino acid tryptophan, resulting in the generation of a
putative proapoptotic agent, kynurenine, as well as other down-
stream immunomodulatory metabolites.35,46 IDO expression by
human monocyte-derived DCs and macrophages potentiates the
ability of these cells to inhibit T-cell proliferation. Furthermore, a
role for IDO has been shown critical in achieving maternal
tolerance, suppression of T cells reactive to haplotype-mismatched
allografts, and the control of autoreactive T cells.23 More recently,
it was demonstrated that the ectopic expression of IDO in tumor
cells confers immune evasion.37

Although there is strong support for IDO’s role in mediating T-cell
tolerance, less is known about the regulation of IDO expression and
the mechanism of enzyme activation. The only characterized trigger of IDO
expression is IFNγ. It has been demonstrated in some cell types that
IFNγ-induced gene expression can be enhanced or antagonized when
added in combination with other cytokines. For example in eosinophils,
it has been demonstrated that GM-CSF synergizes with IFNγ, whereas
IL-3 antagonizes IFNγ-mediated IDO expression.48 With respect to
stimulators of IDO activity, they include cytotoxic T-lymphocyte
antigen 4 (CTLA-4) and CD28, which have been shown to transmit
signals to the DCs by crosslinking the costimulatory molecules B7-1/2.
7 Of note, IFNγ also appears capable of achieving active IDO
enzyme; however, its contribution may need to be reevaluated in light of
data for B7-reverse signaling.49 If we are to interpret the findings that
IFNγ and B7-reverse signaling are critical for IDO activation, it appears
that stimulation of the adaptive immune system is required to achieve
tolerance of the adaptive immune system. In identifying PGE2 as a
trigger for IDO expression, we offer a novel means by which the innate
immune system may regulate T-cell immunity.

Although previous studies have demonstrated the possibility of
posttranslational control for IDO,48,50 we are unable to conclude
whether our observation that TNF-R and TLR ligands are required
for achieving enzymatic activity is reflective of translational
control or a still undefined posttranslational event. Nonetheless, our
results help to clarify the signals responsible for IDO expression
and activity in monocyte-derived DCs. Given the interest in using
DCs for clinical immunotherapy and the sensitivity of IDO
expression to the culturing conditions,51,52 we hope that our
findings will help clarify some of the differences observed in the
various systems that are currently in use.

**Implications for DC-based immunotherapy**

With respect to DC-based immunotherapy protocols, it is important
to note that many use maturation cocktails containing PGE2. As
discussed above, this is in part due to its role in sensitizing DCs to
CCR7 ligands, thus enhancing DC migration to the draining lymph
nodes. Our finding that PGE2 is also sensitizing DCs to TNFα-mediated
IDO activation predicates caution, as we may be inducing the opposite
of what is intended. One consideration would be to segregate the distinct
effects of PGE2. Migration appears to be mediated by EP4, whereas
IDO gene expression occurs in response to EP2 stimulation. Use of
EP4-specific agonists may allow such a separation to be achieved.
Alternatively, it may be possible to include an IDO inhibitor in the DC
maturation cocktail, thus generating a mature, CCR7-responsive DC
with an inactive IDO enzyme.

In sum, our findings illustrate a novel role for PGE2 and
important insight into the regulation of IDO. With a better
understanding of these regulatory mechanisms and the crosstalk
between TNF-R/TLR and EP2 signaling pathways, we hope to
uncover new insight into the regulation of T-cell activation by DCs
and the interplay between tumors and the immune system.

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