A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation
Deborah Braun, Randy S. Longman, and Matthew L. Albert

Introduction

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 E$_2$ (PGE$_2$) 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 $\alpha$ (TNF$\alpha$) and PGE$_2$ 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 PGE$_2$ 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 TNF$\alpha$, lipopolysaccharide, or Staphylococcus aureus Cowan I strain (SAC) alone does not induce IDO. The effect of PGE$_2$ 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.19,20 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.21

Indoleamine 2,3-dioxygenase (IDO) is an enzyme involved in tryptophan catabolism. Its role in antimicrobial resistance is well described; by 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.22-26 IDO expression has been reported in placental trophoblasts and interferon γ (IFNγ)-activated APCs (including macrophages and DCs), reflecting its counter-inflammatory role. An exciting advance for this field has been the discovery that IDO is initially expressed as a proenzyme. Although little is known regarding the biochemical signals responsible for transcriptional activation of the IDO gene, it has been shown that reverse signaling via B7 (CD80/CD86), as well as engagement of CD200R, serve to regulate IDO enzyme activity.27-29

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 (Établissement 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 5 μM, unless otherwise stated. Lipopolysaccharide (LPS), serotype 055:B5 (Sigma) was sonicated and 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.

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'-AGAGTCAAATCC-GATTTG-3'; IDO reverse, 5'-GGTCGGAGTCAAACGATTG-3'; GAPDH forward, 5'-ACCTGGACGACTACAGGG-3'; GAPDH reverse, 5'-GTTGCGGATCAAGCGATTG-3'; TBP forward, 5'-GCCAG-GACGACAGGAGTT-3'; and TBP reverse, 5'-TCACACGCTCCCCAC-CATATT-3'. Primers for EP receptors are described in Kamphuis et al.30 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 10^6 DCs using RIPA buffer (20 mM Tris [tris(hydroxymethyl)methylammonemethane] pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40 ([Octylphenoxypolyethoxylate]), 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 Biotechnology, 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 down-stream of IDO. Kynurenine was detected by either high-pressure liquid chromatography (HPLC) or a modified spectrophotometric assay.
HPLC was performed according to Yong and Lau\textsuperscript{31} with minor modifications. Briefly, 40 \mu L clarified sample was injected into an Amersham reverse phase C2/C18 column and eluted with KH\textsubscript{2}PO\textsubscript{4} buffer (0.01 M KH\textsubscript{2}PO\textsubscript{4} 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\textsuperscript{32,33} The amount of 50 \mu L of 30\% trichloroacetic acid was added to 100 \mu L culture supernatant, vortexed, and centrifuged at 8000 g (10 000 rpm) for 5 minutes. Volume (75 \mu L) 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 \mu M) 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\textsuperscript{34} we analyzed the transcriptional profile of monocyte-derived DCs at the various stages of differentiation using Affymetrix gene array experiments (R.S.L. and M.L.A., unpublished data collected 2003-2004). Strikingly, we observed a greater than 100-fold increase in expression of IDO as a result of DC maturation. This has been 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\textsuperscript{35-37} 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 TNF\textalpha and PGE\textsubscript{2} up-regulated IDO mRNA (Figure 2A). In contrast, iDCs exposed to LPS or SAC did not express measurable levels of IDO mRNA. Evaluation of cell lysates using an IDO rabbit pAb (generously provided by Dr David Munn, University of Georgia) showed good correlation between protein expression and the mRNA levels (Figure 2B).

In addition, it was important to monitor IDO enzymatic activity, because there has been a reported discrepancy between IDO expression and activity, suggesting possible posttranslational regulation of the enzyme.\textsuperscript{35,38} IDO activity may be assayed by quantifying tryptophan catabolism as well as the generation of kynurenine (the first catabolite in the metabolic pathway). After 36 to 48 hours of exposure to the distinct maturation stimuli, the DCs were cultured in HBSS containing 100 \mu M tryptophan. After 4 hours, the concentration of tryptophan and kynurenine was determined by HPLC. As shown, the 2 products can be easily separated, and the area under the curve correlates with the amount of the respective analyte (Supplemental Figure S2A). Notably, we observed robust enzyme activity in DCs matured with TNF\textalpha and PGE\textsubscript{2}, but no evidence of tryptophan catabolism was detected in iDCs or in LPS- or SAC-matured DCs (Figure 2C). In our initial studies, IFN\textgamma-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.\textsuperscript{33} 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 \textsubscript{E\textsubscript{2}} 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 TNF\textalpha did not up-regulate IDO expression. Instead, it was exposure of PGE\textsubscript{2} that accounted for expression of IDO mRNA (Figure 3A-B). Although we observed a 50- to 200-fold increase in transcription of IDO, PGE\textsubscript{2}-treated DCs lacked measurable IDO activity (Figure 3C).

This finding suggests that, although PGE\textsubscript{2} induces transcription of the IDO gene, a second signal, such as exposure to TNF\textalpha, is required to achieve active IDO enzyme. Similar to TNF-R engagement, TLR ligation also induced IDO activity when used in combination with PGE\textsubscript{2} (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 (Kd) 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 TNFα and

![Table 1. IDO expression and activity](image)

<table>
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<th>IDO activity (µM kynurenine, HPLC)</th>
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*Reported as a ratio of relative mRNA expression IDO/GAPDH. Non-normalized data are presented.

N/A indicates not applicable.
PGE₂, the pattern of expression is reversed (Figure 5A). Of note, PGE₂ seems to be responsible for the down-regulation of EP₂, whereas both TNF/α 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 TNF/α, 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 TNF/α, the IDO activity was equivalent to that in TNF/α- 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 skews responses toward T₉₂ differentiation.

And although inhibition of TNF/α, IL-6, and IL-12p₃₅ 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 EP₂.

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.

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. It has been demonstrated in some cell types that IFN-γ-mediated IDO 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.

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

The actions of IDO offer 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 downstream immunomodulatory metabolites.55,46 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 B7-1/2,27 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 separate 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.

**Acknowledgments**

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