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Prostaglandin E_2 is a key factor for monocyte-derived dendritic cell maturation: enhanced T cell stimulatory capacity despite IDO

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Abstract: The exclusive ability of dendritic cells (DCs) to stimulate primary and secondary immune responses favors the use of antigen-loaded human monocyte-derived DCs (MoDCs) in vaccinations against tumors. Previous studies demonstrated that PGE_2 is fundamental during MoDC maturation to facilitate migration toward lymph node-derived chemokines. A recent study challenged the use of PGE_2, as PGE_2 induced IDO in mature MoDCs. In MoDCs compatible for clinical use, we now demonstrate that PGE_2 is responsible for IDO induction if matured by soluble CD40 ligand, LPS, or cytokines. In contrast, IDO expression in MoDCs matured by TLR3 triggering occurs independently of PGE_2. It is surprising that despite active IDO protein, MoDCs matured with PGE_2 display a greater potential to stimulate naïve $CD4^+$ and $CD8^+$ T cell proliferation, which is not increased further by IDO inhibition. Moreover, we found elevated levels of tryptophanyl-tRNA-synthetase (TTS) in T cells cultured with PGE_2-matured MoDCs. Our data demonstrate that PGE_2 induces IDO in MoDCs but that T cell-stimulating capacities of PGE_2-matured MoDCs overcome IDO activity, probably through TTS induction. As PGE_2 is critical for DC migration and enhances the capability of MoDCs to induce T cell proliferation, we highly recommend supplementing DC maturation stimuli with PGE_2 for use in clinical trials. *J. Leukoc. Biol.* 82: 1106–1114; 2007.

Key Words: *cell proliferation · chemotaxis · vaccination*

INTRODUCTION

Dendritic cells (DCs) are key regulators of the immune system and have the unique ability to initiate naïve T cell responses. Immature DCs are present in peripheral tissues, where they are poised to capture antigens from incoming pathogens. Uptake and processing of antigens in conjunction with stimulation with inflammatory cytokines lead to the maturation of DCs. One characteristic of this differentiation process is the change of the chemokine receptor expression profile, resulting in the up-regulation of the chemokine receptor CCR7 [1, 2], and CCR7 expression renders DCs responsive to the chemokines CCL19 (EBI1-ligand chemokine, Exodus-3, MIP-3β, CKβ11) and CCL21 (secondary lymphoid-tissue chemokine, Exodus-2, 6Ckine, TCA-4), which direct their homing to the T cell zone of draining lymphoid organs [3]. There, DCs present their peptide antigens to naïve T cells, thereby inducing an immune response involving cytotoxic T cells, Th cells, B cells, as well as NK cells [4, 5].

The exclusive ability to stimulate primary and secondary immune responses prompted the use of antigen-loaded, human monocyte-derived DCs (MoDCs) in vaccinations against tumors in numerous clinical studies [6–8]. However, DC-based immunotherapy, as currently applied, has not yet proven to be clinically successful [9]. Nevertheless, the question is not whether immunotherapies using DCs work but how to refine the immunological and clinical parameters of vaccination with DCs to improve the efficacy [8]. One major problem was that antigen-loaded DCs failed to leave the injection site [10]. This drawback was solved by the discovery that addition of the proinflammatory mediator PGE_2 to any classical maturation stimulus of MoDCs facilitates migration toward CCR7 ligands [11–14]. Indeed, PGE_2 is a general and mandatory factor, as human immature and mature MoDCs as well as ex vivo DCs require PGE_2 during maturation to migrate in response to chemokines and chemoattractants [14]. It is interesting that the PGE_2 signal facilitating migration can be mediated by E-prostanoid 2 (EP2) or EP4 receptor triggering on human polyinosinic:polycytidylic acid [poly(I:C)]-matured MoDCs [14] but seems to be restricted to EP4 in mouse Langerhans cells [15]. Furthermore, PGE_2 was also shown to enhance DC maturation and their stimulatory capacity to prime naïve T cells and to modulate chemokine and cytokine production of DCs through a yet-unknown mechanism [11, 16–19].

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A recent study by Braun et al. [20] challenges the use of PGE_2 for DC maturation, describing that the addition of PGE_2 to maturation stimuli strongly up-regulated IDO in MoDCs. IDO is an enzyme, which degrades tryptophan, and expressed by DCs, can suppress T cell proliferation and survival and may promote tolerance [21, 22]. It is interesting that PGE_2 was shown to induce IDO on mRNA and protein level via EP2 receptor activation, but a second signal through TNFRs or TLRs was crucial for the enzyme activity [20]. In their hands, induction of IDO depended strictly on PGE_2, as DC maturation stimuli alone did not induce IDO expression [20].

As ex vivo-generated MoDCs are widely used in clinical trials, and a comprehensive study about IDO expression, cell migration, and T cell priming by MoDCs, which are generated under clinically approved conditions, is missing, we decided to investigate recent concerns about the use of PGE_2 and its effects on MoDCs under clinically relevant, serum-free conditions. Thus, we used different stimuli in conjunction with PGE_2 and specific PGE_2 receptor agonists for MoDC maturation and investigated IDO expression, activity, and cell migration and examined the capacity of MoDCs to stimulate T cell proliferation in relation to IDO expression.

MATERIALS AND METHODS

Generation of human MoDCs

Monocytes were isolated from PBMCs as described previously [13, 14]. Briefly, PBMCs were isolated from whole blood of healthy donors by density gradient centrifugation on Ficoll Paque Plus (Amersham Biosciences, Uppsala Sweden). Monocytes were positively selected from PBMCs using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured at 1×10^6 cells/ml in AIM-V medium supplemented with 50 ng/ml GM-CSF (Leukomax®, Norvatis, Basel, Switzerland) and IL-4 (supernatant of an IL-4-producing J558 cell line). Immature DCs were harvested after 5–6 days, and maturation was induced for 2 days by adding 0.5 μg/ml trimeric soluble CD40 ligand (sCD40L; PromoCell, Heidelberg, Germany), 20 μg/ml poly(I:C) (LPS-free, Sigma Chemical Co., St. Louis, MO, USA), 1 μg/ml LPS (*Salmonella abortus equi*, Sigma Chemical Co.), or a cocktail of proinflammatory cytokines (20 ng/ml TNF-α, 10 ng/ml IL-1β, and 20 ng/ml IL-6, all purchased from PromoCell). Where indicated, 1 μg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden), 1 μg/ml specific agonists for EP2 (butaprost, Cayman Chemicals, Ann Arbor, MI, USA), or EP4 (PGE_1 alcohol, Cayman Chemicals; ONO-AE1-329, ONO Pharmaceutical Co., Ltd., Osaka, Japan) was added.

Cell migration assay

To measure chemotaxis, 1×10^5 DCs were placed on a polycarbonate filter with a pore size of 5 μm in a 24-well Transwell™ plate (Corning Costar, NY, USA). Cells were allowed to migrate toward 250 ng/ml CCL21 (PromoCell) for 3 h at 37°C/5% CO_2. Migrated cells were counted by flow cytometry (LSRII, BD Biosciences, Erembodegen, Belgium), and specific migration was calculated as percentage of the number of input cells after subtraction of spontaneous migration toward AIM-V medium without chemokine.

MoDC induced T cell proliferation

Human peripheral blood T cells were isolated from PBMCs of healthy donors using the pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer's protocol. Naïve T cells were negatively sorted using anti-CD45RO-conjugated magnetic microbeads (Miltenyi Biotec), resulting in a pure population of $CD45RA^+$-expressing cells. Naïve T cells were separated further into $CD4^+$ and $CD8^+$ populations using anti-CD4-conjugated magnetic microbeads (Miltenyi Biotec). Naïve $CD4^+$ and naïve $CD8^+$ cells, respectively, were cocultured with mature 10,000 MoDCs in graded ratios in RPMI-1640 medium containing 10% FCS. Where indicated, 20 μM 1-methyltryptophan (1-MT; Sigma Chemical Co.) was added. T cell proliferation was measured after 5 days of coculture using a BrdU cell proliferation ELISA kit (Roche, Indianapolis, IN, USA), according to the manufacturer's protocol.

IDO mRNA and protein expression

Total RNA of mature MoDCs was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and transcribed into cDNA using the Taqman® RT reagent (Applied Biosystems, Rotkreuz, Switzerland) with random hexamer primers, according to the manufacturer's instructions. For amplification of IDO mRNA, 1 μl cDNA was added to QuantiTect SYBR Green PCR Master Mix (Qiagen) containing 200 nM forward primer 5′-TGTCCGTAAGGTCTTGCCAAGA and 200 nM reverse primer 5′-CACCAATAGAGAGACCAGGAAGAATC. Real-time PCR was performed with an initial step of denaturation at 95°C for 15 min followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C using the Taqman 7700 (Applied Biosystems). IDO mRNA expression was normalized to two housekeeping genes, β-2 microglobulin (β2M) and ubiquitin C (UBC), using the primers 5′-GCTATCCAGCGTACTCCAAAGATTC and 5′-CAACTTCAATGTCGGATGGATGA for β2M and 5′-ATTTGGGTCGCGGTTCTTG and 5′-TGCCTTGACATTCTCGATGGT for UBC, respectively, and SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM forward as well as reverse primer, according to the manufacturer's instructions. Relative mRNA expression was calculated with the ΔΔ comparative threshold cycle method.

IDO protein expression was analyzed by Western blotting using an anti-IDO-specific antibody (Upstate, Charlottesville, VA, USA). The blot was reprobed with a β-actin antibody (Abcam, Cambridge, UK) to ensure equal protein loading.

Detection of active IDO protein in MoDCs

Mature MoDCs were harvested and washed three times in HBSS. Cells (2×10^6) were resuspended in 1 ml HBSS containing 100 μM L-tryptophan (Sigma Chemical Co.) and incubated for 4 h at 37°C/5% CO_2. Supernatants were collected and subjected to HPLC analysis after addition of 200 mM H_2SO_4. Tryptophan and kynurenine were analyzed using a HPLC system consisting of two high-pressure pumps (Shimadzu LC-10ATvp), an autoinjector (Gilson 234), a C18 reversed-phase column (Grom-Sil 120 ODS-5ST, 5 μm, 150×4.6 mm, Grom, Herrenberg, Germany), and a UV-VIS diode array detector (SPD-M 10, Shimadzu, Japan). Analysis was performed at room temperature using a gradient program with a mobile phase comprising a mixture of 100 mM ammonium phosphate buffer, pH 2.6, and methanol at a flow rate of 1 ml/min with a detection wavelength of 227 nm. The solvent phase [5% (v/v) methanol] was held initially for 1 min, and then the concentration was increased to 45% over a period of 6 min, lowered to 5% within 0.5 min, and held for an additional 7 min. Amounts of tryptophan and kynurenine in MoDC supernatants were quantified with the CLASS-VP software (Shimadzu) on the basis of standard solutions of 20 μM, 50 μM, and 100 μM tryptophan and kynurenine (Sigma Chemical Co.), respectively.

Quantification of tryptophanyl-tRNA-synthetase (TTS)

MoDCs, matured in the presence or absence of PGE_2, were cocultured with naïve, allogeneic $CD4^+$ T cells as described above. After 4 days, T cells from the coculture were positively selected using an anti-CD3 antibody and magnetic nanoparticles (StemCell Technologies, Vancouver, BC, Canada). RNA was extracted and transcribed into cDNA. TTS mRNA was quantified by real-time PCR as described previously [23]. TTS expression was normalized to two housekeeping genes (β2M and UBC) and calculated relative to unstimulated $CD4^+$ T cells from the same donor.

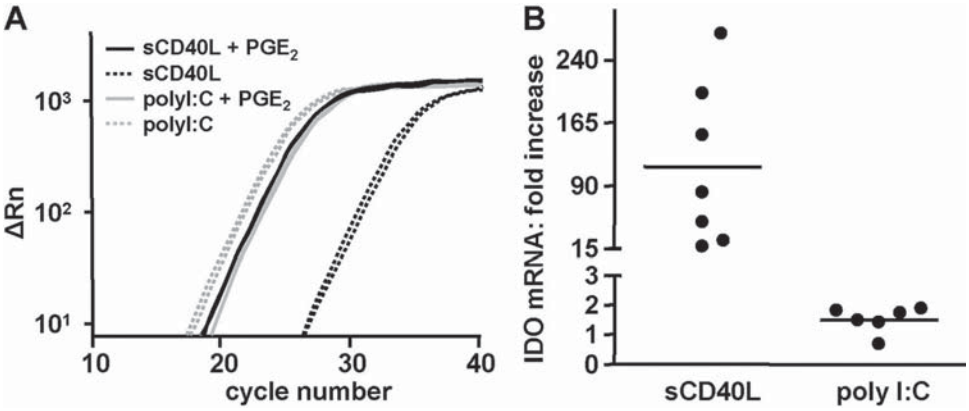

Fig. 1. PGE_2 enhances IDO mRNA expression in human MoDCs matured with sCD40L but not with poly(I:C). MoDCs, generated under serum-free, clinically relevant conditions, were matured for 48 h with trimeric sCD40L or poly(I:C) in the presence or absence of PGE_2. Expression of IDO mRNA was analyzed by real-time RT-PCR. (A) Amplification plots of IDO mRNA expression in MoDCs of a representative donor in duplicates matured with sCD40L (black lines) and poly(I:C) (gray lines), respectively, are shown. Dashed lines represent MoDCs matured in the absence of PGE_2, whereas solid lines correspond to MoDCs matured in the presence of PGE_2. ΔRn, normalized reporter signal minus baseline signal. (B) Induction of IDO mRNA expression by PGE_2 in mature MoDCs of multiple donors was quantified by real-time RT-PCR and expressed as fold increase induced by PGE_2. (●) The mean of a duplicate analysis of a single donor [$n=7$ for sCD40L; $n=6$ for poly(I:C)].

RESULTS

Influence of PGE_2 on IDO expression in human mature MoDCs

Several protocols exist to generate mature DCs ex vivo. One of which, using TNF-α, together with PGE_2 as maturation stimuli, was recently shown to induce the expression of IDO [20], an enzyme involved in the degradation of tryptophan and thought to induce immune tolerance (reviewed in refs. [21, 22]). To test whether IDO mRNA is expressed on human MoDCs generated under serum-free conditions compatible for clinical trials, we isolated human monocytes from peripheral blood of healthy donors by positive selection using CD14 microbeads. Monocytes were differentiated into immature MoDCs by incubation for 5–6 days in serum-free medium containing IL-4 and GM-CSF. Immature MoDCs were then matured for 2 days with poly(I:C) or trimeric sCD40L in the absence or presence of PGE_2, which is essential to generate a general migratory DC phenotype [14]. IDO mRNA from mature MoDCs was quantified by real-time RT-PCR. As shown in **Figure 1A**, PGE_2 provoked a dramatic increase of IDO mRNA in MoDCs matured with sCD40L, similar to MoDCs matured with TNF-α in combination with PGE_2 [20]. In striking contrast, MoDCs matured by poly(I:C) constitutively expressed high levels of IDO mRNA, independently of PGE_2 (Fig. 1A). The induction of IDO mRNA on sCD40L-matured MoDCs by PGE_2 was on average 113-fold, ranging from 17- to 270-fold, depending on the donor ($n=7$), whereas PGE_2 had virtually no effect (less than 1.5-fold, $n=6$) on IDO expression in poly(I:C)-matured MoDCs (Fig. 1B).

Next, we investigated IDO expression on protein level. Again, we found substantial IDO expression in poly(I:C)-matured MoDCs independently of PGE_2 supplementation (**Fig. 2A**). In contrast, IDO expression was only detected in sCD40L-matured MoDCs if PGE_2 were present during the maturation process (Fig. 2A). In the latter case, the level of IDO was similar to poly(I:C)-matured MoDCs, confirming our data obtained about the mRNA level. Furthermore, we also matured MoDCs with LPS or a cocktail of cytokines including TNF-α, IL-1β, and IL-6 in the presence or absence of PGE_2. Under these conditions, IDO expression was restricted to MoDCs matured in the presence of PGE_2 (Fig. 2A). These data clearly suggest that PGE_2 is responsible for the induction of IDO on MoDCs matured with sCD40L, LPS, or cytokine cocktail, whereas TLR3 triggering alone was sufficient for a high expression of the tryptophan-degrading enzyme. We have demonstrated recently that the addition of PGE_2 to DC maturation cocktail has to occur at the beginning of the maturation period to induce a migratory phenotype but can be omitted at later time-points [14]. Thus, we investigated whether IDO up-regulation in maturing MoDCs also happens at early time-points and whether short-term incubation of DCs with PGE_2 may

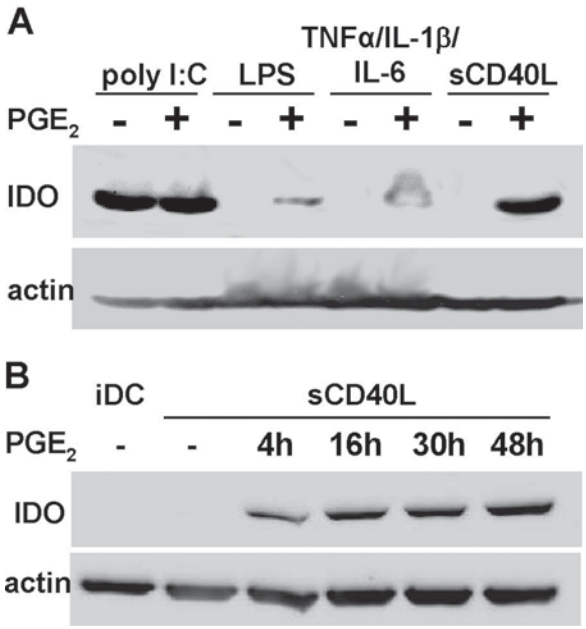

Fig. 2. PGE_2 is not generally required for IDO protein expression in human MoDCs. IDO protein expression was analyzed by Western blotting using an IDO-specific antibody in total cell lysates of MoDCs matured with sCD40L, poly(I:C), LPS, or a combination of IL-1β, IL-6, and TNF-α in the presence or absence of PGE_2 (A). The blot was reprobed with β-actin antibody to ensure equal protein loading. (B) Expression of IDO protein was analyzed in immature DCs (iDC) or sCD40L-matured MoDCs, which were cultured in the absence or presence of PGE_2 for the initial 4, 16, or 30 h of maturation or for the full maturation period (48 h).

prevent IDO expression. Therefore, we coincubated MoDCs, which were matured with sCD40L for 48 h, with PGE_2 during the initial 4, 16, or 30 h and for the entire 48 h of maturation, respectively (Fig. 2B). In fact, IDO protein levels increased the longer PGE_2 was present during MoDC maturation but were already detectable when maturing DCs were treated with PGE_2 exclusively for the initial 4 h of maturation, followed by a further incubation of 36 h in the presence of sCD40L alone (Fig. 2B).

PGE_2 is not obligatory for IDO activity in MoDCs matured with TLR3 ligands

As IDO expression does not necessarily correlate with its activity [20–22], we analyzed PGE_2-induced IDO for its activity to degrade tryptophan to kynurenine. To this end, MoDCs were matured with sCD40L or poly(I:C) in the presence or absence of PGE_2 for 2 days, and mature cells were incubated with tryptophan. To determine tryptophan degradation, we quantified residual tryptophan concentration as well as generated kynurenine in culture supernatants by HPLC. As expected, MoDCs matured with sCD40L alone, showing undetectable IDO expression, did not degrade tryptophan (**Fig. 3C**). In contrast, IDO from the supernatant of MoDCs matured with sCD40L and PGE_2 readily degraded tryptophan to kynurenine (Fig. 3C). Correlating with IDO expression, MoDCs matured with TLR3 ligand poly(I:C) produced active IDO independently of PGE_2, as more than 70% of tryptophan was converted to kynurenine in the presence as well as absence of PGE_2 during the maturation process (Fig. 3B). PGE_2 has been suggested to be a mandatory factor for active IDO expression in mature MoDCs [20]. Surprisingly, we found this statement not to hold true in general, as certain maturation pathways, such as TLR3-mediated maturation, seem to circumvent the obligatory signal by PGE_2.

PGE_2-induced IDO activity is mediated primarily by EP4 signaling

The effect of PGE_2 on human MoDCs can be mediated by two receptors, namely EP2 and EP4. We have shown previously that activation of EP2 and EP4, alone or in combination, is mandatory to induce a migratory phenotype of DCs [14], which is essential to ensure homing of antigen-loaded MoDCs to secondary lymphoid organs and thereby, trigger antigen-specific T cell proliferation. As PGE_2 also induces active IDO production, which may inhibit T cell proliferation, it has been suggested to prevent IDO expression, but simultaneously, to induce a migratory phenotype by substituting PGE_2 with a specific EP4 agonist [20]. To analyze IDO induction through EP4 receptor signaling under conditions compatible for clinical trials, we matured MoDCs with sCD40L in the absence or presence of PGE_2 or two specific EP4 receptor agonists, ONO-AE1-329 and PGE_1 alcohol. It is surprising that under these conditions, IDO protein was induced and fully active if MoDCs were matured in the presence of either of the two EP4 agonists and were comparable with PGE_2 (**Figs.** 3C and **4A**). It is unexpected that EP4 receptor-induced IDO activity was even

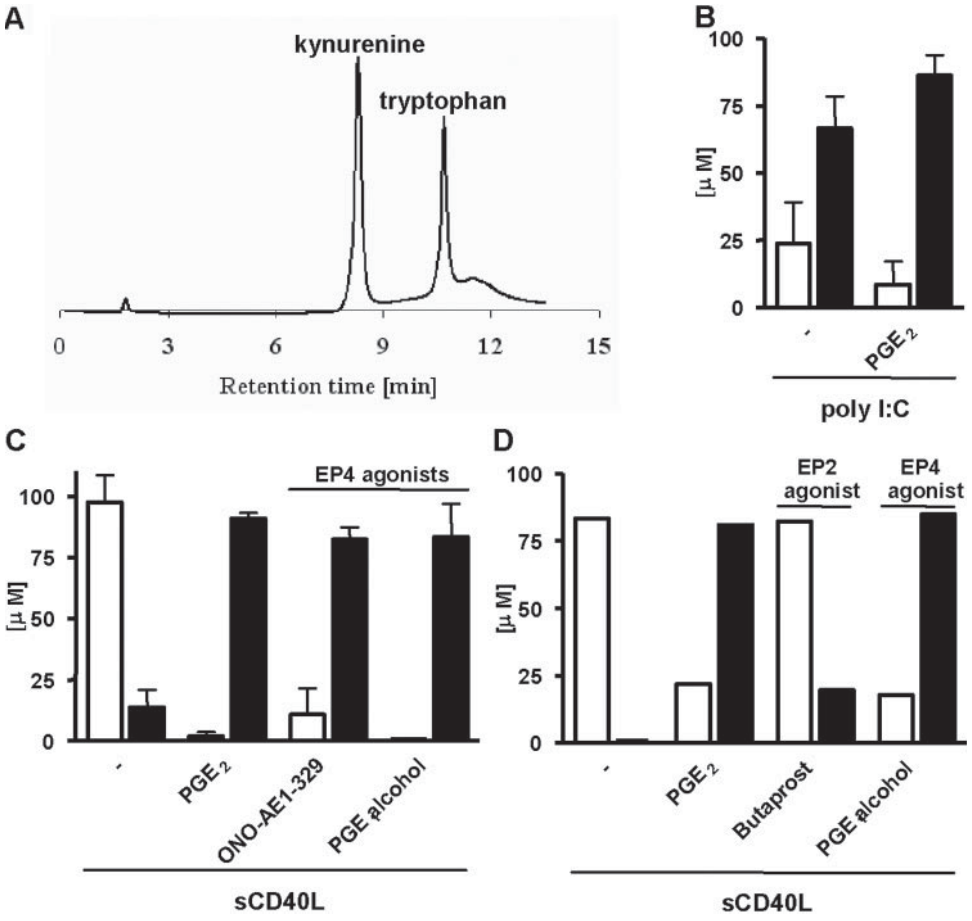

Fig. 3. PGE_2 induces active IDO protein, primarily through EP4 receptor signaling, but is not essential for IDO activity during maturation via TLR3. IDO activity was assessed by quantification of tryptophan degradation to kynurenine using HPLC analysis (A). MoDCs were matured for 48 h by poly(I:C) (B) or sCD40L (C and D) in the presence or absence of PGE_2, EP2 (butaprost)- or EP4 (ONO-AE1-329, PGE_1 alcohol)-specific agonists. Cells were washed and incubated in HBSS in the presence of 100 µM tryptophan for 4 h. Supernatants were subjected to HPLC analysis to assess IDO-mediated degradation of tryptophan (open bars) to kynurenine (solid bars). Mean values and SEM of seven independent experiments (B and C) of individual donors are shown.

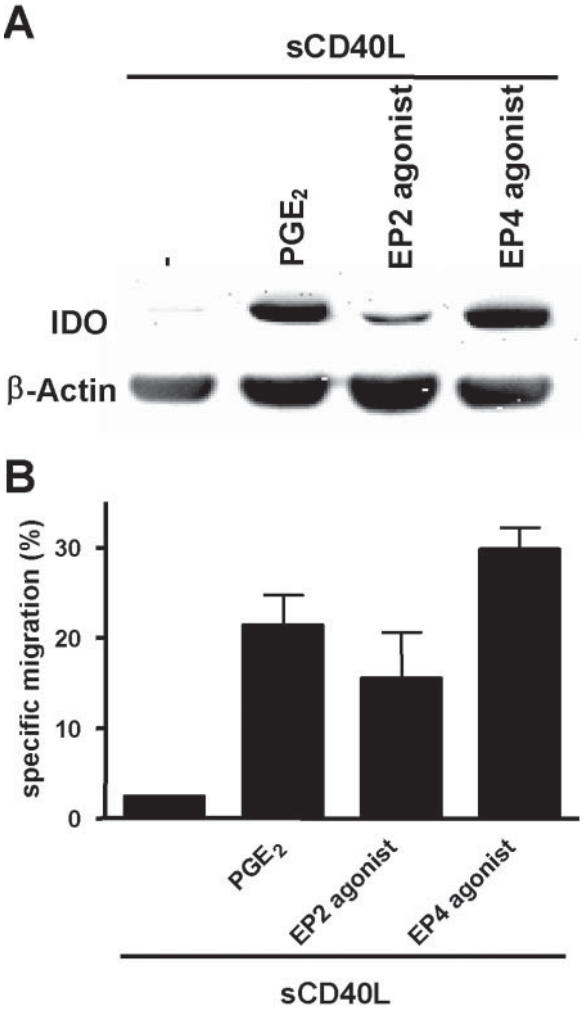

Fig. 4. PGE_2 induces IDO protein expression primarily via EP4. IDO protein expression (A) and migratory behavior (B) of MoDCs matured with sCD40L in the absence or presence of PGE_2, EP2 (butaprost), or EP4 (PGE_1 alcohol) receptor agonist was analyzed. (A) IDO protein expression was analyzed by Western blotting using an anti-IDO-specific antibody. The blot was reprobed with a β-actin antibody to ensure equal protein loading. One representative experiment out of eight is shown. (B) To prove functionality of EP receptor agonists, migratory capacity of MoDCs was analyzed in Transwell™ chemotaxis assay in response to CCL21. Mean values of two independent experiments with different donors are shown.

higher than EP2 receptor-mediated IDO induction by butaprost (Fig. 3D). The ineffective degradation of tryptophan by MoDCs matured with sCD40L in the presence of butaprost correlated with low induction of IDO protein expression (Fig. 4A). To ensure functionality of the EP2 agonist, we analyzed the migratory behavior of MoDCs. We matured MoDCs with sCD40L in the absence or presence of PGE_2, the EP2 agonist butaprost, or the EP4 agonist PGE_1 alcohol and subjected the cells to Transwell™ chemotaxis assays. As noted for poly(I:C) [14], sCD40L-matured MoDCs migrated in response to the homing chemokine CCL21, only if MoDCs were matured in the presence of PGE_2, EP2, or EP4 agonists, demonstrating that butaprost was biologically active (Fig. 4B). Although the EP2 agonist butaprost did not induce high levels of IDO, it provoked a migratory capacity similar to PGE_2 and EP4 agonist.

PGE_2 enhances the stimulatory capacity of MoDCs

Supplementation of PGE_2 to MoDC maturation stimuli [cytokine cocktail, sCD40L, or poly (I:C)] was shown to enhance their capacity to promote $CD4^+$ and $CD8^+$ T cell proliferation [11, 19]. To investigate the effect of PGE_2 on stimulatory properties of MoDCs compatible for clinical trials, we differentiated MoDCs from monocytes by addition of IL-4 and GM-CSF in serum-free medium. MoDCs were matured with sCD40L in the absence or presence of PGE_2 for 2 days and subsequently, cocultured with alloreactive, naïve $CD45RO^-CD4^+$ or $CD45RO^-CD8^+$ T cells. T cell proliferation was assessed after 5 days of stimulation using a BrdU cell proliferation ELISA. As depicted in **Figure 5**, IDO-positive MoDCs, matured in the presence of PGE_2, were significantly more efficient to induce T cell proliferation of $CD4^+$ and $CD8^+$ subpopulations.

PGE_2-induced IDO does not limit the capacity of MoDCs to stimulate T cell proliferation

As addition of PGE_2 to sCD40L for maturation of MoDCs promotes the expression of active IDO, we investigated whether active IDO interfered with MoDC-induced T cell proliferation. To this end, we matured MoDCs with sCD40L in the absence and presence of PGE_2 and cocultured mature MoDCs with

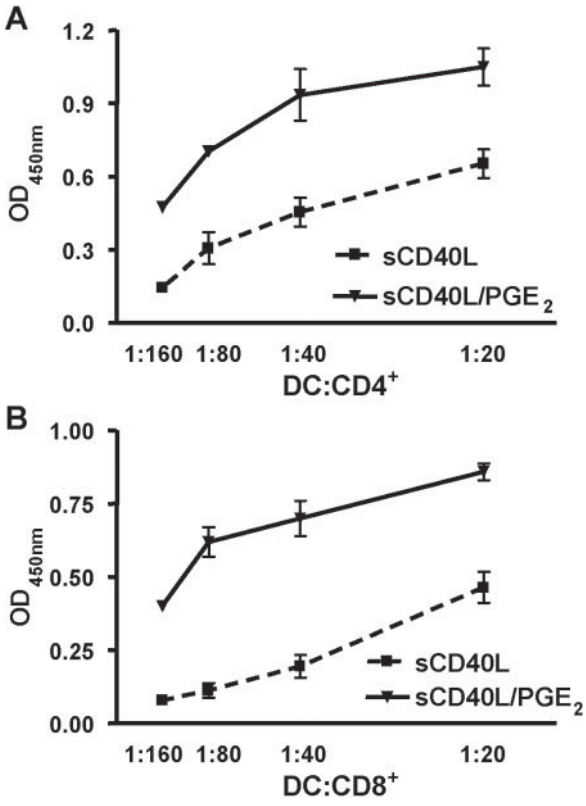

Fig. 5. PGE_2 enhances the stimulatory capacity of MoDCs. MoDCs were matured with sCD40L in the presence (solid lines) or absence (dashed lines) of PGE_2 and cocultured with alloreactive, naïve $CD4^+$ (A) or $CD8^+$ (B) T cells. After 5 days of stimulation, T cell proliferation was assessed by BrdU cell proliferation ELISA. Mean values and SEM of a representative experiment out of six are shown.

freshly isolated, alloreactive, naïve $CD45RO^{-}CD4^{+}$ or $CD45RO^{-}CD8^{+}$ T cells as above. IDO activity was inhibited by addition of 20 μM 1-MT, which is solved in HCl, and a solvent control was included to ensure specific IDO inhibition and exclude solvent-dependent effects. $CD4^{+}$ T cell proliferation was enhanced by PGE_2-matured MoDCs and could not be augmented further by addition of 1-MT (**Fig. 6A**). Even higher concentrations of 1-MT (200 μM, 2 mM) did not improve T cell proliferation (data not shown). As we were unable to measure the presence of IDO in the supernatant of the coculture, we cannot rule out that functional IDO may be lost during the T cell proliferation assay. However, in addition to CD4, IDO inhibition by 1-MT did not enhance alloreactive $CD8^{+}$ T cell proliferation stimulated by MoDCs matured in the presence of PGE_2 (Fig. 6B).

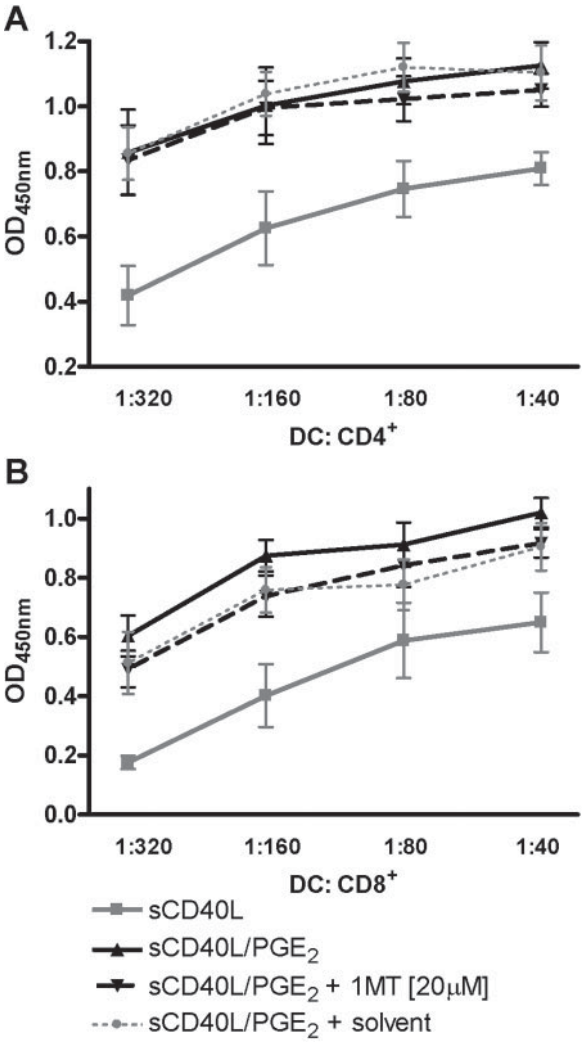

Fig. 6. PGE_2-induced IDO activity does not limit the enhanced stimulatory capacity of PGE_2-matured DCs. MoDCs were matured with sCD40L in the presence (black, solid lines) or absence (gray, solid lines) of PGE_2 and cocultured with alloreactive, naïve $CD4^{+}$ or $CD8^{+}$ T cells for 5 days. To inhibit IDO activity, 1-MT (black, dashed lines) was added for the entire coculture. To ensure specificity, HCl, the solvent of 1-MT, was added at the same concentration (gray, dotted line). Mean values and SEM of four to six independent experiments with different donors are shown.

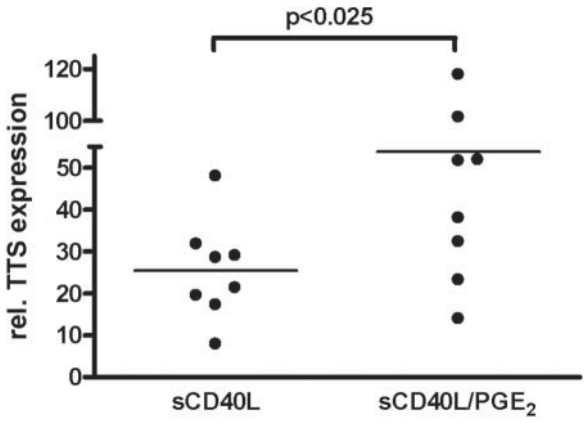

Fig. 7. Elevated expression of TTS in T cells cocultured with PGE_2-matured MoDCs. MoDCs matured with sCD40L in the presence or absence of PGE_2 were cocultured with naïve, allogeneic $CD4^{+}$ T cells. After 4 days, TTS mRNA of sorted T cells from the coculture was quantified by real-time RT PCR. Relative TTS expression was normalized to TTS mRNA in unstimulated T cells from the same donor. (●) The mean of a duplicate analysis of a single donor (n=8).

Elevated levels of TTS in T cells cocultured with PGE_2-matured MoDCs

TTS is another important enzyme of the tryptophan metabolism. TTS is responsible for the association of tryptophan with its specific tRNA. This tryptophan–tRNA complex generates a reservoir of tryptophan, which is available for protein de novo synthesis [24, 25]. Elevation of the TTS expression level can thus serve as a counter-mechanism to protect IDO-mediated tryptophan deprivation. To investigate whether this may explain why T cells can proliferate in the presence of active IDO, we cocultured naïve $CD4^{+}$ T cells with allogeneic MoDCs matured with sCD40L in the presence or absence of PGE_2. Four days after stimulation, T cells were isolated, and TTS mRNA was quantified by real-time RT-PCR. In fact, T cells derived from cocultures with sCD40L-matured MoDCs express, on average, 25-fold more TTS compared with naïve T cells from the same donor (**Fig. 7**). Moreover, the TTS levels increase further by a factor of two if PGE_2 was added to the MoDC maturation stimulus (Fig. 7). These data provide clear evidence that PGE_2-induced IDO expression in MoDCs is compensated and nullified with enhanced expression of TTS in the proliferating T cells.

DISCUSSION

The aim of efficient vaccines against cancer is to induce tumor-specific, effector T cells, which eliminate the cancerous tissue in conjunction with establishing a pool of tumor-specific memory T cells, controlling eventual tumor relapse [8]. DCs are key players in launching an efficient T cell-mediated immunity, owing to their unique capacity to acquire, process, and present antigens to all subsets of T cells. The first clinical study of a DC vaccine was published 10 years ago, in which follicular B cell lymphoma patients were treated with peripheral blood-derived DCs loaded with recombinant tumor-spe-

cific idiotype proteins [26]. This hallmark study initiated an entire series of experimental studies and clinical trials using antigen-loaded DCs as vaccines to augment tumor-specific T cell responses in cancer patients [6–8, 27, 28]. One important aspect was the development of a protocol to generate large amounts of MoDCs [29]. Most clinical studies nowadays use such MoDCs, in which monocytes are isolated from peripheral blood and cultured in the presence of IL-4 and GM-CSF for several days to differentiate into immature DCs. These cells can be matured further with different stimuli, such as poly(I:C), sCD40L, LPS, or a combination of the cytokines IL-1β, IL-6, and TNF-α. These matured MoDCs, when loaded with antigens, are capable of propagating antigen-specific T cells in vitro and show cytotoxic activity for antigen-bearing target cells in the case of $CD8^+$ T cells. However, despite a robust activation of the adaptive immune response against the targeted tumor antigen, DC-based immunotherapies often showed only a marginal clinical anti-tumor activity [9, 30]. This drawback is explained easily by the fact that in vitro-generated, antigen-loaded, radioactively labeled, mature DCs could not be detected in lymph nodes of the patients [10]. Migration of mature DCs to the T cell zone of secondary lymphoid organs is guided by the homing chemokines CCL19 and CCL21 [3, 31]. We and others [11–14] have realized that CCR7 expression, the chemokine receptor for CCL19 and CCL21, alone is not sufficient to facilitate migration of clinical-grade MoDCs. In fact, whatever stimuli may be used to mature clinical-grade MoDCs, supplementation of the maturation cocktail with the proinflammatory mediator PGE_2 is fundamental for the development of a migratory MoDC phenotype [11–14, 32]. Moreover, also ex vivo peripheral blood DCs rely on PGE_2 addition for efficient migration [14]. The crucial role of PGE_2 for DC migration has been substantiated further by the finding that skin-derived Langerhans cells derived from *ptger4* null mice, which lack the PGE_2 receptor EP4, showed impaired homing to draining lymph nodes, whereas $EP2^{-/-}$ animals had no such phenotype [15]. In contrast, in humans, PGE_2 triggering can be mediated through EP2 or EP4 to give rise to migratory DCs (Fig. 4B and ref. [14]).

A recent study by Braun and co-workers [20] now challenged the use of PGE_2 supplementation for MoDC maturation, as they found that PGE_2 was responsible for the induction of IDO, which is the initial and rate-limiting enzyme converting tryptophan to kynurenine; and tryptophan is an essential amino acid important for protein synthesis, cell survival, and proliferation [33, 34]. Hallmark studies by Munn et al. [35] and Mellor et al. [36] discovered that IDO was able to prevent rejection of the fetus during pregnancy. IDO expression in DCs depends on IFN-γ stimulation and correlates with the inhibition of T cell proliferation, which can be prevented with the IDO inhibitor 1-MT [37–39]. Moreover, the tryptophan-derived catabolites kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid can induce activation-induced T cell death [40, 41]. Braun and collaborators [20] now described that the presence of PGE_2 during TNF-α- or LPS-induced maturation of human MoDCs induces active IDO protein. In the absence of PGE_2, however, they found no IDO protein. The authors therefore suggested reconsidering the use of PGE_2 in DC-based immunotherapy protocols. Using human MoDCs compatible for clinical trials, we now describe that the addition of PGE_2 to LPS, sCD40L, or a cocktail of cytokines for MoDC maturation provoked up-regulation of IDO on mRNA and protein level (Figs. 1 and 2A), confirming and extending the observations by Braun et al. [20]. However, we demonstrated that TLR3-mediated MoDC maturation using poly(I:C) induced IDO expression, independently of PGE_2 (Figs. 1 and 2A), and poly(I:C)-induced IDO protein was fully active (Fig. 3B). In our hands, IDO protein expression strictly correlated with the enzyme's activity. Thus, PGE_2 is not a general prerequisite for IDO expression in mature MoDCs, as implied previously [20].

In humans, the PGE_2-promoted, enhanced migratory capacity of MoDCs is mediated through the PGE_2 receptors EP2 and EP4 [14], in contrast to mice, where the effect is accredited to EP4 alone [15]. Braun and colleagues [20] demonstrated that PGE_2-induced, active IDO in human MoDCs is mediated exclusively through EP2 triggering. As a consequence, they suggested replacing PGE_2 with a specific EP4 agonist in maturation protocols for DC-based immunotherapies to induce a migratory phenotype but to prevent expression of active IDO. In striking contrast, using two independent, specific agonists, we clearly demonstrate that under serum-free, clinically relevant conditions, PGE_2-induced IDO protein expression and activity are mediated primarily by the PGE_2 receptor EP4 (Figs. 3C and 4A). EP2 triggering also induced IDO activity but to a much lower level (Fig. 3D), which correlated with lower IDO protein induction (Fig. 4A). The addition of PGE_2, EP2, or EP4 agonists during MoDC maturation is not only essential for the development of a migratory phenotype but also induces active IDO protein. It is interesting that IDO may even be critical for DC activation and chemotaxis, as incubation of maturing DCs with the IDO inhibitor 1-MT impaired maturation-induced regulation of chemokine receptor expression [42]. With respect to DC-based immunotherapy, antigen-loaded DCs must acquire a migratory phenotype to reach the draining lymph node to initiate an efficient immune response. Initial studies using MoDCs, which were matured in the absence of PGE_2, showed a clear deficit in migration [10]. These data clearly argue for the use of PGE_2 (or receptor agonists) for the generation of clinical-grade DCs. The induction of IDO, which may inhibit antigen-specific T cell proliferation within the lymph node, now challenges the use of PGE_2. It is unexpected that despite active IDO expression, PGE_2-matured MoDCs showed an enhanced capacity to induce allogeneic $CD4^+$ and $CD8^+$ (Figs. 5 and 6), as well as antigen-specific [19] T cell proliferation compared with MoDCs matured in the absence of PGE_2. To test whether the augmented capacity of PGE_2-matured MoDCs was nevertheless limited by active IDO expression, we inhibited IDO activity with 1-MT. It is striking that we were unable to increase the number of proliferating T cells by PGE_2-matured MoDCs when IDO activity was blocked (Fig. 6). The robust T cell activation of Th as well as cytotoxic T cell subpopulations induced by PGE_2-matured MoDCs seems not to be restrained by IDO expression. However, we cannot rule out that IDO-expressing MoDCs may lose functional IDO expression during the coculture with T cells. The fact that efficient T cell proliferation can be induced by IDO-positive DCs was discovered recently. Terness and colleagues [39] found that the proliferation of neither OKT3-stimulated human T cells of

healthy donors nor myelin basic protein-specific T cells of patients with multiple sclerosis was impaired by autologous MoDCs expressing IDO. Moreover, resistance of synovial T cells of rheumatoid arthritis patients to IDO-mediated tryptophan deprivation was shown to be associated with enhanced expression of the enzyme TTS [23], which protects T cells from IDO activity in two ways [23, 43]: One includes the formation of TTS complexes, which act as reservoirs of tryptophan for protein synthesis, thus overcoming the tryptophan-deprivation effect of IDO. The other way is the reduction of tryptophan catabolites, which can induce cell death. We discovered that T cells, which are cocultured with MoDCs, expressed a substantial amount of TTS. Interestingly, we found even higher amounts of TTS in proliferating T cells, which were engaged by MoDCs, matured in the presence of PGE_2 (Fig. 7). This novel finding can explain easily why MoDCs matured with PGE_2 can still prime T cells, despite IDO expression. Alternatively, we cannot exclude that the presence of T cells mediated a down-modulation/inactivation of IDO in MoDCs. It is interesting enough that IDO as well as TTS are induced by IFN-γ [24]. Thus, it is conceivable that under pathological conditions, where IFN-γ is produced, TTS activity of T cells, which are recruited along with IDO-positive DCs to the lymph node at an early phase, is capable of silencing the tolerogenic potential of IDO. However, to regulate the immune response, T cell proliferation within the lymph node needs to be stopped at later time-points, e.g., when IFN-γ is no longer produced to induce TTS, whereas the homed DC may still express functional IDO.

In summary, we demonstrate that PGE_2 induces active IDO expression in DCs generated under clinically relevant conditions early during maturation. However, PGE_2 is not fundamental for IDO induction, as functional IDO expression is observed in TLR3-mediated MoDC maturation in the absence of PGE_2. As under serum-free conditions, IDO expression by PGE_2 is mediated primarily through the EP4 receptor, the proposed replacement of PGE_2 by a specific EP4 agonist [20] for generation of migratory and IDO-negative MoDCs can no longer be recommended. Although PGE_2 induces functional IDO in MoDCs, the T cell-stimulating capacity is enhanced. In addition, we demonstrate that T cells, activated by PGE_2-matured MoDCs, express high levels of TTS, which protect them from tryptophan deprivation by IDO-expressing DCs. Overall, PGE_2 is not only fundamental for the development of a migratory phenotype but enhances T cell stimulatory capacities of mature MoDCs.

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