1-Methyl-Tryptophan Can Interfere with TLR Signaling in Dendritic Cells Independently of IDO Activity

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The compound 1-methyl-tryptophan (1-MT) is a competitive inhibitor of IDO, which is a rate-liming enzyme in the catabolism of tryptophan (Trp). IDO converts the amino acid l-Trp to kynurenine and further catabolites (1, 2). Trp metabolism plays a major role in the control of propagation of various intracellular pathogens like Toxoplasma gondii, Chlamydia psittaci, human CMV, and different cocci. It is an essential amino acid for the growth of pathogens, and Trp shortage induced by IDO activity can inhibit their replication (3–7). Trp depletion can block the proliferation of various cell types including tumor cells (8–10).

The inhibitor 1-MT has been initially used to block the immune privilege of placenta (11). Treatment of pregnant mice with 1-MT has induced the rejection of the allogeneic fetus by breaking the tolerance of maternal T lymphocytes for the fetus. Maternal T cell tolerance appeared to rely on IDO-expressing cells at the maternal-fetal interface that deprive the local microenvironment in Trp and inhibit T cell proliferation. It is believed that 1-MT restores the fetal interface that deprive the local microenvironment in Trp and tolerance appeared to rely on IDO-expressing cells at the maternal-fetal interface that deprive the local microenvironment in Trp and inhibit T cell proliferation. It is believed that 1-MT restores the fetal interface that deprive the local microenvironment in Trp and inhibit T cell proliferation. It is believed that 1-MT restores the fetal interface that deprive the local microenvironment in Trp and inhibit T cell proliferation.

The inhibition of IDO activity in tumor cells leads to an enhanced CTL activity against tumor cells and a reduced tumor growth. It has also been shown that long-term survival of pancreatic islet allografts induced by CTLA4-Ig is abrogated by 1-MT treatment of recipient mice (12). Overall, in vivo, 1-MT seems to enhance T cell alloreactivity and T cell responses against tumor Ag, allograft Ag, and autoantigens (13–17). From these in vivo studies, it was proposed that IDO could have an immunoregulatory function. In vitro studies have focused primarily on the main activators of the immune system, dendritic cells (DC) and macrophages that can express IDO. The addition of 1-MT in cocultures of T or NK cells with APC or tumor cells maintains high concentrations of Trp and improve T cell and NK cell proliferation. Moreover, by blocking IDO, 1-MT inhibits the production of Trp catabolites like kynurenine that have been shown to reduce T cell and NK cell proliferation (18–24).

The maturation of DC can be induced by many bacterial components, which are recognized by different TLR. LPS from Gram-negative bacteria are recognized by TLR4, components of Gram-positive bacteria and bacterial lipopeptides are recognized by TLR2/TLR1 or TLR2/TLR6, and poly(I:C) (pIC) (pIC) mimicking dsRNA is recognized by TLR3. TLR use various adaptors activating different signaling pathways. Briefly, all TLR except TLR3 can use the MyD88 adaptor leading to NF-κB activation. TLR3 uses the Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) adaptor that activates the transcription factors IFN regulatory factor-3 and NF-κB. TLR4 can also activate a MyD88-independent pathway involving the adaptors TRIF-related adapter molecule and TRIF, triggering IFN regulatory factor-3 activation and NF-κB stimulation (25).

The polarization of T cells is dependent on various factors including the origin of DC, their degree of maturation, and kinetics of activation (26–30). Myeloid DC, like monocyte derived-DC, prime mainly Th1 cells secreting large amounts of IFN-γ but little IL-4, IL-5, and IL-10, whereas plasmacytoid DC can activate Th1 or Th2 cells that secrete large amounts of IL-4, IL-5, and IL-10 but little IFN-γ, depending on the activation signal and the infectious
agent. At least three distinct functional subsets of DC have been reported according to the final outcome of maturation. Cells with high costimulatory capacity and IL-12 production would promote Th1 responses, cells with high costimulatory capacity but low IL-12 production would drive Th2 differentiation, and cells with low costimulatory capacity and IL-12 production would give rise to tolerogenic Th cells. Semimature DC with high expression of costimulatory molecules and low secretion of proinflammatory cytokines (IFN-γ) have also been described and may stimulate regulatory T cells (31). Lanzavecchia and colleagues (28) also proposed the DC exhaustion model where Th1-polarized DC are first generated, whereas the same cells analyzed at later time points of maturation stop secreting IL-12 and prime Th2 and nonpolarized cells.

IDO can be induced in vitro or in vivo by various agents like cytokines (IFN-γ, TNF-α), CD40L, CTLA4-Ig, influenza virus, or bacterial LPS (32–37). Several subsets of IDO-expressing DC have been described. CD11c⁺ murine DC express IDO protein but enzyme activity is only detected in the CD8⁺ subset (38). In mice, plasmacytoid DC that express IDO can inhibit T cell responses (39, 40). A particular subset of human myeloid DC expressing CCR6, CD123, and a constitutively active form of IDO is deficient for T cell stimulation and may thus play a central role in tolerance (21). Another group has shown that human monocye-derived DC expressing active IDO after IFN-γ stimulation do not suppress T cell proliferation (41). Therefore, further studies will be necessary to describe the different functions of human DC expressing IDO (42). Moreover, some studies suggest that IDO is necessary for DC activation (43).

In previous in vitro experiments, 1-MT was added in cocultures of DC with T cells to study the role of IDO on T cell proliferation and activation (18, 19, 24). Because DC maturation is a crucial step to control immune responses, we analyzed the direct effect of 1-MT on the maturation of human monocyte-derived DC. It is shown that 1-MT affected differentially the function of DC depending on the quality of the maturation signal. In the presence of 1-MT, pIC-stimulated DC maintained their capacity to induce a Th1 response, whereas DC stimulated with TLR2 ligands had an increased ability to stimulate IFN-γ secretion by T cells. In contrast, 1-MT on TLR4-stimulated DC reoriented DC toward a Th2 function, a process involving both ERK and p38-MAPK. Interestingly, all of these effects of 1-MT were not correlated to the inhibition of IDO activity.

### Materials and Methods

#### Generation and treatment of DC

PBMC were isolated from human peripheral blood of healthy donors by standard density gradient centrifugation on Ficoll-Hypaque. Mononuclear cells were separated from PBL by centrifugation on a 50% Percoll solution (Amersham Biosciences). Monocytes were purified by immunomagnetic depletion (Dynal) using a mixture of mAb anti-CD19 (4G7 hybridoma), anti-CD3 (OKT3; American Type Culture Collection), and anti-CD56 (NKHI; Beckman Coulter). Monocytes (purity, >90%) were differentiated to immature DC (iDC) during 7 days with 40 ng/ml human rGM-CSF and 250 U/ml human rIL-4 in RPMI 1640 (Abcys) supplemented with 2 mM glutamine, 10 mM HEPES, 40 ng/ml gentamicin (Invitrogen Life Technologies), and 10% FCS. Differentiating monocytes were treated at day 5 with 1 mM 1-methyl-tryptophan or 2.5 mM Trp or 60 μM kynurenine (Sigma-Aldrich) and at day 6 with 1 μg/ml LPS (Escherichia coli, serotype 0127:B8; Sigma-Aldrich), 10 μg/ml pIC (Amersham Biosciences), 10 μg/ml peptidoglycan (PGN) of Staphylococcus aureus (Sigma-Aldrich), or 10 μg/ml Pam3CSK4 (Pam) (Axxora). All cells and supernatants were collected at day 7. Control mature DC (mDC) were obtained by adding TLR ligands at day 6 for 24 h. When indicated, 40 μM PD98059 (PD), an inhibitor of MEK1/2 (Bioworld), or 25 μM SB203580 (SB), an inhibitor of p38-MAPK (Bioworld), were added 30 min before 1-MT treatment. All DC were >95% pure as assessed by CD14 and CD1a labeling.

#### Phenotype

Phenotype was analyzed on a FACSCalibur (BD Biosciences) using FITC-conjugated anti-CD14, HLA-DR, –CD80, and –CD54, and PE-conjugated anti-CD1a, –CD86, –CD83, and –CD40 (Beckman Coulter).

#### Cytokine assay

Culture supernatants were stored at −80°C. IL-6, IL-10, IL-1β, TNF-α, and IL-13 levels were determined using cytokine-specific ELISA kits (Endogen). IL-12 p40 and p70 were assayed using ELISA kits from Biosource. IL-2, IL-4, IL-5, IL-10, and IFN-γ were determined using the human Th1-Th2 cytokine cytometric bead array kit 1 (BD Biosciences).

#### Mixed lymphocyte reaction

T lymphocytes were purified after Ficoll-Hypaque and Percoll gradient centrifugation by immunomagnetic depletion using a mixture of mAb anti-CD19 (4G7), anti-CD56 (NKHI1), anti-CD16 (3G8), anti-CD14 (RMO52), and anti-glycopherin A (11E4B7.6) (Beckman Coulter). T lymphocytes were purified after Ficoll-Hypaque and Percoll gradient centrifugation by immunomagnetic depletion using a mixture of mAb anti-CD19 (4G7), anti-CD56 (NKHI1), anti-CD16 (3G8), anti-CD14 (RMO52), and anti-glycopherin A (11E4B7.6) (Beckman Coulter). T lymphocytes were >95% pure as assessed by CD3 labeling. Primary MLR were conducted in 96-well flat-bottom culture plates. DC recovered at day 7 were extensively washed and resuspended in complete RPMI 1640/10% FCS. Cells were cocultured in triplicate with 2 × 10⁵ allogeneic T cells in 200 μl at DC:T cell ratios ranging from 1:10 to 1:40. Supernatants were recovered at indicated time points for IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ measurement.

### Table I. Secretion of cytokines in MLR supernatants

<table>
<thead>
<tr>
<th></th>
<th>IL-2⁺</th>
<th>IFN-γ⁺</th>
<th>IL-5⁺</th>
<th>IL-13⁺</th>
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<tr>
<td>IDC</td>
<td>44 ± 14</td>
<td>441 ± 141</td>
<td>10 ± 0.2</td>
<td>47 ± 10</td>
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<tr>
<td>1-MT</td>
<td>75 ± 9</td>
<td>236 ± 134</td>
<td>1 ± 0.4</td>
<td>31 ± 6</td>
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<tr>
<td>LPS</td>
<td>168 ± 56</td>
<td>36,126 ± 376</td>
<td>31 ± 5</td>
<td>345 ± 94</td>
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<tr>
<td>LPS + 1-MT</td>
<td>308 ± 19</td>
<td>7,547 ± 1,720</td>
<td>112 ± 48</td>
<td>982 ± 102</td>
</tr>
<tr>
<td>pIC</td>
<td>106 ± 36</td>
<td>2,019 ± 947</td>
<td>58 ± 23</td>
<td>542 ± 32</td>
</tr>
<tr>
<td>pIC + 1-MT</td>
<td>246 ± 0.2</td>
<td>2,088 ± 1,067</td>
<td>23 ± 4</td>
<td>585 ± 36</td>
</tr>
<tr>
<td>PGN</td>
<td>187 ± 74</td>
<td>2,657 ± 626</td>
<td>121 ± 21</td>
<td>1,024 ± 72</td>
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<tr>
<td>PGN + 1-MT</td>
<td>470 ± 109</td>
<td>10,011 ± 2,939</td>
<td>114 ± 61</td>
<td>1,553 ± 35</td>
</tr>
<tr>
<td>Pam</td>
<td>82 ± 23</td>
<td>21,001 ± 4,204</td>
<td>23 ± 5</td>
<td>698 ± 87</td>
</tr>
<tr>
<td>Pam + 1-MT</td>
<td>179 ± 44</td>
<td>43,186 ± 12,048</td>
<td>46 ± 5</td>
<td>1,314 ± 57</td>
</tr>
</tbody>
</table>

a Maximum values of secretions quantified in MLR supernatants at day 2 of coculture.

b Maximum values of secretions quantified in MLR supernatants at day 5 of coculture. Secretions were determined by cytoketric bead array and are expressed in picograms per milliliter. Means ± SD from five independent experiments are shown.
**T cell response against tetanus toxin**

DC were treated as for MLR and autologous CD3 T cells were purified as described above from frozen PBL. DC recovered at day 7 were extensively washed and resuspended in complete RPMI 1640/10% FCS. Cells were cocultured in triplicate with $2 \times 10^5$ allogeneic T cells in 200 µl at 1:20 DC/T cell ratio. Purified tetanus neurotoxin (25 µg/ml) (provided by Dr. C. Villiers, Institut National de la Sante´ et de la Recherche Me´dicale Unite´ 548, Grenoble, France) was then added to cocultures. Supernatants were recovered after 5 days of coculture for IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ measurement. Tetanus neurotoxin has no effect on DC or T cells alone (data not shown).

**Intracellular staining of cytokines**

MLR were conducted for 5 days and T cells were expanded for 7 days with 25 U/ml recombinant human IL-2 (BioSource International), washed, and restimulated with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (VWR International) for 5 h. Brefeldin A (10 ng/ml; Sigma-Aldrich) was added during the last 2 h. Cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences). Intracellular staining was performed using FITC-labeled anti-IFN-γ mAb and PE-labeled anti-IL-5 and IL-13 mAb (BD Biosciences).

**IDO expression and activity**

Total RNA was extracted from cells collected at day 7 using RNeasy mini-kit (Qiagen). A total of 100 ng of total RNA was reverse transcribed using the thermoscript RT-PCR system (Invitrogen Life Technologies). Primers used for PCR amplification are as follows: 5'-GCTTTCACACAGGTCATA-3' and 5'-GGTCATGGAGATGTCCGTAA-3' for IDO, and 5'-GGAGGTGTAATGGACGTTA-3' and 5'-CTGAGACTCCTTGCTAGATG-3' for S12. The amplified products were analyzed by gel electrophoresis (691 bp for IDO and 311 bp for S12).

Trp is converted by IDO to N-formylkynurenine, which is further catalyzed to kynurenine. Quantification of kynurenine in supernatants thus reflects IDO activity. Kynurenine was measured in fresh supernatants of DC collected at day 7 as described previously (44). Briefly, 100 µl of 30% TCA was added to 200 µl of supernatant and vortexed. After centrifugation, 125 µl of supernatant was incubated with 125 µl of Ehrlich reagent ($p$-dimethylaminobenzaldehyde; Sigma-Aldrich) in a microtiter plate for 10 min at room temperature. OD490 was measured. Values were referred to a standard curve with defined kynurenine concentrations (0–120 µM; Sigma-Aldrich) and normalized to $10^6$ cells.

**Phosphorylation of p38-MAPK, ERK, and c-Fos**

For studies on ERK and p38 phosphorylation, $2 \times 10^6$ differentiating monocytes were treated at day 5 with 1 mM 1-MT and collected at day 6. Cells were extensively washed and starved for 2 h in complete RPMI 1640 medium without serum. Cells were treated with the different TLR ligands for 5, 10, 15, 30, or 45 min. Cells were washed twice with cold PBS, and pellets were lyzed in radioimmunoprecipitation assay buffer containing 1 mM PMSF and 1% protease inhibitors. Phosphorylated and total ERK and p38 MAPK were quantified by specific ELISA (Assay Designs).

For studies on c-Fos phosphorylation, $2 \times 10^6$ differentiating monocytes were treated at day 5 with 1 mM 1-MT and collected at day 6. Cells were extensively washed and resuspended in RPMI 1640/0.3% delipidated BSA. Cells were treated with the different TLR ligands for 1, 2, 4, or 6 h. Cells were then washed twice with cold PBS and pellets were lyzed in radioimmunoprecipitation assay buffer containing 1 mM PMSF and 1% protease inhibitors. Phosphorylated and total ERK and p38 MAPK were quantified by specific ELISA (Assay Designs).

**FIGURE 1.** 1-MT induces a Th2 function of DC stimulated with LPS. A, Kinetics of secretion of IFN-γ, IL-5, and IL-13 in MLR supernatants. MLR were conducted with control iDC (▫), LPS-stimulated DC (○), and LPS-stimulated DC pretreated with 1-MT (▲). Cytokines were measured in MLR supernatants at the indicated times. Mean ± SD of triplicates of one representative experiment of three. B, MLR were conducted for 5 days. After IL-2 expansion, T cells were stimulated with PMA and ionomycin in the presence of brefeldin A, and IL-5, IL-13, and IFN-γ expression was analyzed by intracellular staining. Data are shown for 1:20 DC/T cell ratio and were similar for other ratios. Data of one representative experiment of three are shown.
inhibitors. Phosphorylated c-Fos was quantified by a chemiluminescent ELISA (Endogen) and normalized to the amount of protein determined with the microBCA assay kit (Pierce).

Results

Effect of 1-MT on DC is dependent on the maturation signal

To test the action of 1-MT on DC maturation, DC were treated with 1-MT 24 h before TLR stimulation. LPS was used as a prototype of TLR4 ligand, pIC for TLR3 stimulation, and PGN or Pam as ligands of the heterodimers TLR2/TLR6 or TLR2/TLR1, respectively. DC were then analyzed for their ability to stimulate allogeneic T cells in MLR, and cytokines released were measured in supernatants after 2–5 days of coculture. Under these experimental conditions, 1-MT was present before and during the induction of DC maturation but not in the cocultures with allogeneic T cells.

Viability of DC was not affected by the various treatments and addition of 1-MT to iDC had no effect on cytokine secretion in MLR (data not shown and Table I). Treatment of DC with 1-MT 24 h before the addition of the maturation agent increases the capacity of DC to stimulate IL-2 secretion by T cells (Table I). DC stimulated with TLR ligands were good inducers of IFN-γ secretion, although DC treated with pIC or PGN were less efficient than DC treated with LPS or Pam (Table I). Pretreatment of pIC-activated DC with 1-MT had no effect on IFN-γ, IL-5, and IL-13 secretion by T cells. IFN-γ production in MLR was enhanced by 1-MT when DC were activated with either PGN or Pam, whereas IL-5 was not modified and IL-13 was slightly increased (Table I).

In contrast, 1-MT pretreatment of LPS-activated DC yielded cells with a reduced ability to stimulate IFN-γ secretion by T cells and 1-MT pretreatment of LPS-activated DC yielded cells with an increased ability to induce IL-5 and IL-13 production by T cells in MLR, compared with LPS-treated DC.

Thus the effect of 1-MT on functional maturation of DC is dependent on the TLR triggered on DC.

Th2 polarization of DC matured with LPS is induced by 1-MT

DC activated by LPS that were treated by 1-MT had a reduced ability to induce IFN-γ secretion by allogeneic T cells but stimulated IL-5 and IL-13 secretion (Table I). This could result from a shift in DC function or from a rapid exhaustion of the Th1 potential of DC. The kinetics of secretion of IFN-γ, IL-5, and IL-13 was thus analyzed. In MLR with control LPS-treated DC, a reasonable amount of IFN-γ could be detected at day 2 of coculture, whereas IL-5 and IL-13 began to be detectable at day 3. Cytokine concentration in the supernatants progressively increased until day 5 (Fig. IA). The kinetics of IFN-γ production was not modified by 1-MT pretreatment of DC, although the quantity of cytokine released was drastically reduced. The kinetics of IL-5 and IL-13 secretion was not modified either and confirmed the strong induction of these cytokines by 1-MT pretreatment. Although IL-5 and IL-13 were detected later than IFN-γ, these secretions did not follow a first peak of IFN-γ secretion, indicating that the Th2 function of T cells was not the result of a Th1 exhaustion.

T cell populations activated in these MLR were further analyzed by intracellular staining. As expected, control LPS-treated DC predominantly stimulated Th1 cells producing IFN-γ (58% of IFN-γ+ T cells) (Fig. IB). Nineteen percent of these T cells also produced IL-13. Only 5% of T cells presented a Th2 profile with production of IL-13 without IFN-γ. No IL-5-producing T cells were detected under these experimental conditions, DC pretreated with 1-MT before LPS activated an increased number of T cells producing IL-13 without IFN-γ (36 vs 5% in control MLR), whereas only 30% of T cells produced IFN-γ (58% in control MLR). Five percent of T cells synthesized IL-5. These data indicate that 1-MT treatment induced a shift in the function of LPS-stimulated DC toward a Th2-type function.

Effect of 1-MT can also be observed in a recall response

Because allogeneic MLR does not reflect an Ag-specific response, we examined the effect of 1-MT treatment of DC in an autologous response to tetanus neurotoxin. As shown in Fig. 2, the results found in allogeneic MLR can be observed in an Ag-specific response. Without Ag, DC induced basal secretions of cytokines by T cells and 1-MT pretreatment of LPS-activated DC yielded cells with a reduced ability to stimulate IFN-γ secretion by T cells, but...
with increased capacity to induce IL-2 and IL-5. These basal secretions are due to the presentation of FCS Ags to T cells. When the recall Ag is added, all secretions are increased and the impact of 1-MT pretreatment on LPS-stimulated DC is more striking, inducing a strong reduction of DC ability to stimulate IFN-γ secretion by T cells. This treatment also tends to increase the ability of DC to induce IL-13 secretion by T cells, but this is not as obvious as for IL-5 or IL-2.

These results indicate that 1-MT modulates the capacity of mDC to stimulate memory responses, suggesting that this molecule could have an impact in vivo on immune responses to pathogens.

**Effect of 1-MT on phenotypic maturation and cytokine secretion by DC**

DC stimulated by LPS, pIC, or PGN showed a classic phenotype of mDC with a strong induction of CD86 and CD40 (Fig. 3) as well as CD80, CD83, and HLA-DR (data not shown). Pam was a weaker inducer of phenotypic maturation of DC. Pretreatment with 1-MT had no effect on phenotypic maturation induced by pIC or Pam. Obvious effects of 1-MT were observed when LPS maturation was induced by PGN and LPS. Indeed, for PGN- or LPS-matured DC, the expression of maturation markers was reduced by 1-MT pretreatment, although these markers were still expressed at high level (Fig. 3). An intermediate expression was also observed for CD80, CD83, CD54, and HLA-DR (data not shown). All cells were CD14+CD1a+ whatever the treatment and 1-MT alone did not affect the phenotype of iDC (Fig. 3E and data not shown).

Cytokine secretion of DC treated or not with 1-MT and stimulated with the different TLR ligands was then examined. pIC and Pam induced weak cytokine secretions and 1-MT pretreatment had only minor effects on these secretions (Table II). As expected, LPS and PGN were strong inducers of all the cytokines tested, except for IL-12p70, which was only induced by LPS. Pretreatment of LPS or PGN-stimulated DC with 1-MT was characterized by a strong reduction in secretion of IL-6, IL-10, IL-12p70, and TNF-α (Table II). In all conditions, cells remained negative for IFN-γ and IL-1β secretion, and DC viability was not modified (data not shown). The basal level of cytokine secretion by iDC was not affected by 1-MT alone (Table II).

The overall data indicate that 1-MT pretreatment of DC can interfere with the phenotypic maturation and the cytokine secretion depending on TLR signaling.

**Inhibition of IDO activity is not sufficient to change DC polarization**

IDO mRNA was weakly detected in iDC, whereas LPS, pIC, and PGN strongly increased its transcription. Pam was a weak inducer of IDO transcription (Fig. 4A). Treating DC with 1-MT did not affect the induction of mRNA by the different TLR ligands (data not shown). Increased expression of IDO by LPS and pIC treatment correlated with an enhanced enzymatic activity that was measured by the production of the Trp catabolite kynurenine in culture supernatants (Fig. 4B). Pretreatment of DC with 1-MT before activation with LPS or pIC blocked the induction of IDO activity, which remained at basal level. Induction of IDO activity by Pam and PGN was weak and may not be significant, especially with respect to the lack of inhibition by 1-MT following PGN stimulation. No IFN-γ secretion was detected in DC culture supernatants, confirming that IDO induction can be IFN-γ independent.

Kynurenine is the main catabolite of Trp and is involved in the inhibition of T cell proliferation (18, 22). Because its production is inhibited by 1-MT, we asked whether addition of kynurenine during the pretreatment of DC with 1-MT could restore a normal phenotypic and functional maturation induced by LPS. Fig. 4C shows that kynurenine had no effect on the allostimulatory function of LPS-stimulated DC and could not inhibit the Th2 shift induced by 1-MT. Kynurenine had no effect on phenotypic maturation and cytokine secretion of LPS-stimulated DC (Fig. 4C and data not shown). IDO activity also results in Trp deprivation. An excess of Trp did not mimic the effect of 1-MT on DC function, indicating that Trp concentration did not regulate DC maturation mediated by TLR4 signaling (Fig. 4C). The compound 1-MT is a competitive inhibitor of Trp for IDO; however, 1-MT action on LPS-stimulated DC was not suppressed by an excess of Trp that could displace 1-MT from the enzyme (Fig. 4C). Thus, all of these results strongly support the idea that 1-MT could inhibit the Th2 shift induced by 1-MT.
suggested that the effect of 1-MT on TLR signaling in DC is independent of IDO activity.

**p38-MAPK, ERK, and c-Fos in DC polarization**

p38-MAPK and ERK have been shown to play a role in DC maturation and in the type of T cell response they can elicit (45–48). We thus asked whether these pathways could be differentially engaged when DC were pretreated with 1-MT before activation with the different TLR ligands. The functional consequences of specific inhibitors of these two kinases on DC maturation was therefore examined. SB is a specific inhibitor of p38-MAPK and PD inhibits MEK activation, thus preventing ERK phosphorylation. Addition of SB before LPS maturation yielded DC that could not induce IFN-γ secretion by allogeneic T cells, confirming the involvement of p38-MAPK in LPS-induced maturation of DC (Fig. 5A). Secretion of IL-5 and IL-13 by T cells was not affected and remained at its low basal level. Allogeneic T cells cocultured with DC pretreated with PD and LPS secreted similar amounts of IFN-γ, IL-5, and IL-13 compared with T cells cocultured with control mDC, suggesting that the MEK/ERK pathway is not essential in LPS-induced maturation (Fig. 5B). The effect of both inhibitors was then investigated on DC pretreated with 1-MT before stimulation with LPS. As expected, in presence of SB, DC pretreated or not by 1-MT were not able to induce IFN-γ secretion by T cells. However, in the presence of SB, 1-MT lost its ability to generate DC that could induce IL-5 and IL-13 secretion by T cells (Fig. 5, C and D). Treating DC with both PD and 1-MT before stimulation with LPS restored their ability to induce IFN-γ secretion by T cells, suggesting that the MEK/ERK pathway is involved in the inhibition of Th1-type responses by 1-MT (Fig. 5E). In contrast, the MEK/ERK pathway did not seem to regulate the ability of DC to induce IL-13 and IL-5 secretion by T cells (Fig. 5F). The data suggest that ERK and p38-MAPK can interfere with DC polarization and may be involved in the shift of DC function induced by 1-MT in LPS-activated DC.

Therefore, we looked directly at the activation of these two kinases triggered by the different TLR ligands. Activation of p38-MAPK and ERK pathways was determined by the ratio of phosphorylated enzyme to total enzyme by ELISA. In iDC, the treatment with 1-MT alone does not activate ERK and p38 (Fig. 6, A and B). All of the TLR ligands increased the ratio of phosphorylated p38 to total p38, although with different strength and kinetics. The pretreatment of DC with 1-MT did not modify the outcome of p38 activation induced by LTR stimulation with pIC, whereas it slightly reduced the level of activation induced by TLR2/6 stimulation without affecting the kinetics (Fig. 6A). The activation induced by Pam was delayed when cells were pretreated with 1-MT. The most striking differences were observed with LPS stimulation. p38 phosphorylation still occurred at 5 min but was followed by a second and more important peak at 15 min (Fig. 6A).

As for p38, 1-MT pretreatment had no effect on ERK phosphorylation following pIC stimulation. ERK phosphorylation was reduced following PGN signaling or delayed following Pam signaling. The profile of pretreatment with 1-MT was the same as those observed for p38 except for the TLR4 ligand LPS. When DC had been pretreated with 1-MT, ERK phosphorylation was more intense and was maintained >45 min (Fig. 6B).

It has been suggested that sustained ERK signaling in DC results in the phosphorylation and stabilization of the immediate early gene product c-Fos, therefore leading to a Th2 polarization of DC (49). We therefore looked at the state of phosphorylation of c-Fos following pretreatment of DC with 1-MT before TLR stimulation. Correlating with ERK phosphorylation, we found that all TLR ligands activated c-Fos. pIC was the weakest inducer, whereas the other TLR ligands were quite similar activators (Fig. 6C). As for ERK phosphorylation, pretreating DC with 1-MT did not affect c-Fos activation induced by pIC and decreased the level of activation induced by the TLR2/6 ligand without affecting the kinetics. The activation of c-Fos induced by Pam was delayed when DC were pretreated with 1-MT. The most striking difference was again observed for LPS. Pretreatment of DC with 1-MT increases and lengthens the activation of c-Fos induced by the TLR4 ligand, therefore corroborating the finding of Agrawal et al. (49) that a sustained activation of ERK results in a phosphorylation and stabilization of c-Fos correlating with a Th2 response. All of these results were confirmed by intracellular staining of the phosphorylated forms of ERK, p38 and c-Fos (data not shown).

The data strongly suggest that the determination of DC polarization implies p38, ERK, and c-Fos, and that 1-MT modifies the polarization of LPS-matured DC by regulating the level and the kinetic of activation of these three pathways. Inappropriate activation of the MEK/ERK pathway by 1-MT in the presence...

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**Table II. Cytokine secretions by DC treated with different TLR ligands**

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<tr>
<th></th>
<th>IL-6*</th>
<th>IL-10*</th>
<th>IL-12p70*</th>
<th>TNFα*</th>
</tr>
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<td>111 ± 53</td>
</tr>
<tr>
<td>1-MT</td>
<td>87 ± 33</td>
<td>91 ± 7</td>
<td>0</td>
<td>78 ± 34</td>
</tr>
<tr>
<td>LPS</td>
<td>24,200 ± 10,767</td>
<td>2,333 ± 1,168</td>
<td>1,667 ± 566</td>
<td>9,222 ± 1,863</td>
</tr>
<tr>
<td>LPS + 1-MT</td>
<td>5,283 ± 1,402</td>
<td>1,194 ± 362</td>
<td>162 ± 97</td>
<td>3,855 ± 1,634</td>
</tr>
<tr>
<td>pIC</td>
<td>313 ± 116</td>
<td>78 ± 23</td>
<td>0</td>
<td>377 ± 143</td>
</tr>
<tr>
<td>pIC + 1-MT</td>
<td>346 ± 103</td>
<td>82 ± 15</td>
<td>0</td>
<td>837 ± 352</td>
</tr>
<tr>
<td>PGN</td>
<td>4,975 ± 834</td>
<td>6,260 ± 1,504</td>
<td>0</td>
<td>9,673 ± 2,433</td>
</tr>
<tr>
<td>PGN + 1-MT</td>
<td>953 ± 455</td>
<td>1,685 ± 66</td>
<td>0</td>
<td>3,143 ± 1,383</td>
</tr>
<tr>
<td>Pam</td>
<td>379 ± 135</td>
<td>204 ± 129</td>
<td>0</td>
<td>891 ± 324</td>
</tr>
<tr>
<td>Pam + 1-MT</td>
<td>320 ± 217</td>
<td>163 ± 93</td>
<td>0</td>
<td>549 ± 372</td>
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</table>

* Maximum values of cytokine secretion by DC treated with the different TLR ligands with or without 1-MT. Cytokine secretions were quantified by ELISA and are expressed in picograms per milliliter. Means ± SD from five independent experiments are shown.
of LPS seems to play a central role in the generation of DC with defective Th1 function and improved Th2 function.

Discussion

The compound 1-MT is a competitive inhibitor of Trp for the enzyme IDO (50). It (1-MT) was successfully used in vivo to break the immune privilege of placenta and tolerance against grafts, autoantigens, and tumors (9–12, 16). In vitro, the effect of 1-MT was analyzed in various coculture systems, but the direct effect of 1-MT on DC has been investigated only recently (18–24, 43). It is shown here that 1-MT has a profound effect on DC function depending on the type of maturation signal provided. Treatment of iDC with 1-MT before LPS stimulation induced a Th1 to Th2 functional shift of mDC. DC treated with 1-MT before LPS maturation activated an increased number of IL-5+ and IL-13+ T cells but a reduced number of IFN-γ+ T cells. This resulted in the secretion of high amounts of IL-5 and IL-13 and low amounts of IFN-γ during MLR. These results were reproduced with an Ag-specific response, indicating that 1-MT could affect immune responses in vivo. There was minor or no effect by 1-MT on DC stimulated with the TLR3 ligand pIC that remained Th1-oriented. In contrast, when DC were stimulated with TLR2/1 or TLR2/6 ligands, 1-MT pretreatment appeared to favor IFN-γ production by T cells in MLR. The direct effect of 1-MT on the functional orientation of DC is thus dependent on the maturation signal detected by the DC. These results are in line with those of Hayashi et al. (51) showing that 1-MT can interfere with TLR9 stimulation in vivo in experimental asthma, inhibiting the Th1-protection induced by TLR9 ligand and restoring a Th2 profile of cytokine secretions.

Actually, the effects of 1-MT on DC maturation are not correlated with the inhibition of IDO activity (Fig. 4C). First, 1-MT has not the same effect on maturation triggered by pIC and LPS, although these two stimuli induce the same IDO activity. Moreover, 1-MT modifies the functional properties of DC treated with Pam or PGN without affecting IDO activity in these cells. IDO activity results in Trp depletion and accumulation of kynurenine, both processes inhibiting T cell activation in cocultures of DC with T cells. We found that addition of kynurenine on DC did not affect DC maturation and an excess of Trp did not mimic or counteract 1-MT effect on DC function (Fig. 4C). So, all of these data suggest that the effects of 1-MT on DC function are independent of IDO catalytic activity on Trp and that 1-MT may be acting on one or several other targets that are involved in DC polarization. One possible explanation is that 1-MT could interfere with Trp transporters under certain circumstances, therefore limiting the uptake of Trp by DC and interfering with protein synthesis (like cytokines). This dysregulation of cytokine secretions by DC would result in a Th2 bias of DC function. Another possibility is that 1-MT could influence more generally Trp metabolism in DC; it could inhibit IDO activity while increasing transport and activities of enzymes involved in serotonin formation. Actually, IDO and SERT (serotonin transporter) are reciprocally regulated in DC by cytokines. This dysregulation of cytokine secretions by DC would result in a Th2 bias of DC function. Another possibility is that 1-MT could influence more generally Trp metabolism in DC; it could inhibit IDO activity while increasing transport and activities of enzymes involved in serotonin formation. Actually, IDO and SERT (serotonin transporter) are reciprocally regulated in DC by cytokines. This dysregulation of cytokine secretions by DC would result in a Th2 bias of DC function.

The interference of 1-MT with LPS signaling resulted in Th2-oriented DC. ERK and p38-MAPK appeared to be involved in this functional shift. The activation of ERK and p38-MAPK pathways during DC maturation has been reported previously (45–48). p38-MAPK is mainly involved in CD83, CD80, and CD86 up-regulation and in TNF-α and IL-12 secretion following LPS or anti-CD40 stimulation. Although ERK phosphorylation was detected in DC after TLR stimulation, its role in maturation is still controversial depending on the culture system used. T cell-polarizing activity of DC may depend on the balance between ERK and p38-MAPK activation triggered by maturation stimuli and environmental signals. In LPS-stimulated DC, pretreatment with 1-MT increased ERK phosphorylation and induced two peaks of phosphorylation of p38-MAPK. Inhibition of the MEK/ERK pathway partially prevented the effect of 1-MT and restored the Th1-oriented function of generated DC. Blocking the p38-MAPK pathway also partially prevented the effect of 1-MT on DC. DC treated with both a specific inhibitor of p38-MAPK pathway and 1-MT could not induce the secretion of IL-5 and IL-13 by T cells and thus DC did not...
acquire a Th2-oriented function. Further work is also needed to understand how Th1 and Th2 effector cells can be differentially activated as well as the relative predominance of the transcription factors T-bet and GATA3 in this process (54). It has already been described that the polarization of the Th response is associated with the accessibility of the chromatin. For a Th2 response, IL-4, IL-5, and IL-13 loci become more accessible to be transcribed (after demethylation or hyperacetylation of histones H3 and H4 and chromatin remodeling for example), whereas the IFN-γ locus becomes less accessible (55–58).

Because 1-MT pretreatment of LPS-stimulated DC results in the stimulation of an increased number of IL13+ T cells and a decreased number of IFN-γ+ T cells, and because these DC also induce Th2 cells in a recall Ag presentation test, chromatin remodeling at the loci of cytokine genes could be involved in the Th2 bias we observe.

TLR ligands also stimulated c-Fos phosphorylation. As observed for ERK phosphorylation, 1-MT pretreatment of DC stimulated with LPS strengthened and maintained the activation of c-Fos, whereas it had no effect on the phosphorylation of c-Fos triggered by TLR3 ligand. Accordingly, c-Fos phosphorylation was either reduced or delayed by 1-MT following TLR2 stimulation. This is in agreement with the results obtained by Agrawal et al. (49) showing that a sustained ERK phosphorylation in DC.
results in a phosphorylation and stabilization of c-Fos and in a Th2 polarization of DC.

CCR6⁺CD123⁺ DC expressing IDO represent a subset of monocyte-derived DC that are deficient in allostimulation and could play an important role in tolerance induction (21). We did not find this subset of DC in our culture system that generates a homogeneous population of CCR6⁺CD123⁺ DC expressing active IDO upon maturation. Understanding the correlation between IDO and DC differentiation and maturation is an active field of research with sometimes conflicting observations (37, 42, 43). Molecular mechanisms involved in the differential effect of 1-MT on DC according to the maturation stimulus need further investigation. It would be especially interesting to understand how 1-MT can interfere with TLR4 signaling mediated by LPS to generate Th2 DC. TLR3 signaling is MyD88 independent, TLR2 signaling is MyD88 dependent, whereas TLR4 signaling relies on both MyD88-dependent and -independent pathways. The impact of 1-MT on phenotype and cytokines induced by LPS, PGN, or Pam for the indicated periods of time. Control TLR-stimulated DC were not treated with 1-MT. Phosphorylated and total p38 (A) and phosphorylated and total ERK (B) were quantified in cell lysates by ELISA. Results are shown as phosphorylated/total protein ratio. Data from one representative experiment of three.

In conclusion, 1-MT offers a promising tool to study more precisely molecular mechanisms involved in the polarization of DC especially in response to TLR ligands and thus to pathogen components. The compound 1-MT constitutes a pharmacologic agent useful to manipulate the immune response in vivo. With respect to its action on IDO and involvement in the rupture of tolerance,
I-1MT has been proposed to be used in cancer therapy. According to our results, which suggest that I-1MT can act on other targets than IDO and that it can modulate DC function depending on the maturation status of DC, the conditions of use of I-1MT in clinical protocols should be examined carefully.

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Disclosures
The authors have no financial conflict of interest.

References


