1-Methyl-tryptophan can interfere with TLR signaling in dendritic cells independently of IDO activity.

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Running title: 1-MT can modify dendritic cell functions

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Abstract

1-methyl-tryptophan (1-MT) is a competitive inhibitor of indoleamine 2,3-dioxygenase that can break tolerance and induce fetus, graft and tumor rejection. Because of its broad effect on immune-related mechanisms, the direct action of 1-MT on human monocyte-derived dendritic cells (DC) was analyzed. It is shown here that 1-MT effect on DC is dependent on the maturation pathway. 1-MT had no effect on DC stimulated by the TLR3 ligand polyI:C but strongly enhanced the Th1 profile of DC stimulated with TLR2/1 or TLR2/6 ligands. 1-MT induced drastic changes in the function of DC stimulated by the TLR4 ligand LPS. These cells could still activate allogeneic and syngeneic T cells but stimulation yielded T cells secreting IL-5 and IL-13 rather than IFNγ. This action of 1-MT correlated with an increased phosphorylation of p38 and ERK MAP-kinases and sustained activation of the transcription factor c-Fos. Inhibiting p38 and ERK phosphorylation with synthetic inhibitors blocked the effect of 1-MT on LPS-stimulated DC. Thus, 1-MT can modulate DC function depending on the maturation signal and independently of its action on IDO. This is consistent with previous observations and will help further understanding the mechanisms of DC polarization.

Introduction

1-methyl-tryptophan[3] (1-MT) is a competitive inhibitor of indoleamine 2,3-dioxygenase (IDO) which is a rate limiting 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 psitacci, human cytomegalovirus 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).

1-MT has been initially used to block the immune privilege of placenta (11). 1-MT treatment of pregnant mice 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 local concentration of Trp in the placenta, allowing T cell activation. 1-MT can also break tolerance for tumor cells (9, 10). Mice receiving 1-MT before injection of tumor cells developed tumors later than control mice and mice receiving 1-MT during tumor growth developed tumors more slowly than control mice. 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 auto Ag (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, 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 $Gram^-$ bacteria are recognized by TLR4, components of $Gram^+$ bacteria and bacterial lipopeptides are recognized by TLR2/TLR1 or TLR2/TLR6, and polyI:C (pIC) mimicking double-stranded RNA is recognized by TLR3. TLR use various adaptors activating different signaling pathways. Briefly, all TLR except TLR3 can use the MyD88 adaptor leading to NFκB activation. TLR3 uses the TRIF adaptor that activates the transcription factor IRF-3 and NFκB. TLR4 can also activate a MyD88-independent pathway involving the adaptors TRAM and TRIF, triggering IRF3 activation and NFκB stimulation (25).

The polarization of T cells is dependent on various factors including the origin of DC, their degree of maturation and kinetic of activation (26-30). Myeloid DC, like monocyte derived-DC, prime mainly Th1 cells secreting large amounts of IFNγ 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 IFNγ, 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. Semi-mature DC with high expression of costimulatory molecules and low secretion of pro-inflammatory cytokines have also been described and may stimulate regulatory T cells (31). Lanzavecchia *et al.* 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 non polarized cells (28).

IDO can be induced in vitro or in vivo by various agents like cytokines (IFNγ, TNFα), 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 monocyte-derived DC expressing active IDO after IFNγ 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). Since 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 while DC stimulated with TLR2 ligands had an increased ability to stimulate IFNγ 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 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, Uppsala, Sweden). Monocytes were purified by immunomagnetic depletion (Dynal, Oslo, Norway) using a cocktail of monoclonal Ab anti-CD19 (4G7 hybridoma), anti-CD3 (OKT3, ATCC, Rockville, MD, USA) and anti-CD56 (NKH1, Beckman Coulter, Fullerton, CA, USA). 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, Paris, France) supplemented with 2 mM glutamine, 10 mM Hepes, 40 ng/ml gentamycin (Life Technologies, Rockville, MD, USA) and 10% FCS. Differentiating monocytes were treated at day 5 with 1 mM 1-methyl-DL-tryptophan or 2,5 mM of Trp or 60 µM of kynurenine (Sigma-Aldrich, St Quentin-Fallavier, France) and at day 6 with 1 µg/ml LPS (Escherichia coli, serotype 0127:B8, Sigma-Aldrich), 10 µg/ml polyI:C (pIC - Amersham Biosciences), 10 µg/ml peptidoglycan (PGN) of Staphylococcus aureus (Sigma-Aldrich) or 10 µg/ml of Pam3CSK4 (Pam - Axxora, San Diego, CA). 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, an inhibitor of MEK1/2 (Biomol, Plymouth Meeting, PA, USA), or 25 µM SB203580, an inhibitor of p38-MAPK (Biomol), were added 30 min before 1-MT treatment. All DC were more than 95% pure as assessed by CD14 and CD1a labeling.

Phenotype

Phenotype was analyzed on a FACScalibur (BD Biosciences, Le Pont de Claix, France) using

FITC-conjugated anti-CD14, -HLA-DR, -CD80, -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, TNFα and IL-13 levels were determined using cytokine-specific ELISA kits (Endogen, Woburn, MA, USA). IL-12 p40 and p70 were assayed using ELISA kits from Biosource (Camarillo, CA, USA). IL-2, IL-4, IL-5, IL-10 and IFNγ were determined using the human Th1-Th2 cytokine CBA kit I (BD Biosciences).

Mixed Lymphocyte Reaction

T lymphocytes were purified after Ficoll-Hypaque and Percoll gradient centrifugation by immunomagnetic depletion using a cocktail of monoclonal Ab anti-CD19 (4G7), anti-CD56 (NKH1), anti-CD16 (3G8), anti-CD14 (RMO52) and anti-glycophorin A (11E4B7.6) (Beckman Coulter). T lymphocytes were more than 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 triplicates with 2.10^5 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 IFNγ measurement.

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 triplicates with 2.10^5 allogeneic T

cells in 200 µl at 1/20 DC/T cell ratio. 25 µg/ml purified tetanus neurotoxin (kindly provided by Dr Villiers, INSERM U548, CEA 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 IFNγ 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 rhIL-2 (Biosource), washed and restimulated with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (VWR International, Fontenay-sous-Bois, France) for 5 h. 10 ng/ml Brefeldin A (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-IFNγ monoclonal Ab and PE-labeled anti-IL-5 and IL-13 monoclonal Ab (BD Biosciences).

IDO expression and activity

Total RNA was extracted from cells collected at day 7 using RNeasy Mini kit (Qiagen, Courtaboeuf, France). 100 ng of total RNA was reverse transcribed using the thermoscript RT-PCR system (Life Technologies). Primers used for PCR amplification are: 5'-GCTTTCACACAGGCGTCATA-3' and 5'-GGTCATGGAGATGTCCGTAA-3' for IDO, and 5'-GGAGGTGTAATGGACGTTA-3' and 5'-CTGAGACTCCTTGCCATAG-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 catabolized 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. Optical density was measured at 490 nm. 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, 2.10^6 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 RIPA buffer containing 1 mM PMSF and 1% protease inhibitors. Phosphorylated and total ERK and p38 MAPK were quantified by specific ELISA (Assay Designs Inc, Ann Arbor, MI, USA).

For studies on c-Fos phosphorylation, 2.10^6 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 / 0,3% delipidated BSA. Cells were treated with the different TLR ligands for 1, 2, 4 or 6 hours. Cells were then washed twice with cold PBS and pellets were lyzed in RIPA buffer containing 1 mM PMSF and 1% protease inhibitors. Phosphorylated c-Fos was quantified by a chemoluminescent ELISA (Endogen) and normalized to the amount of protein determined with the microBCA assay kit (Pierce, Rockford, IL, USA).

Results

The 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 to 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 IFNγ secretion although DC treated with pIC or PGN were less efficient than DC treated with LPS or Pam (table I). 1-MT pre-treatment of pIC-activated DC had no effect on IFNγ, IL-5 and IL-13 secretion by T cells. IFNγ production in MLR was enhanced by 1-MT when DC were activated with either PGN or Pam while IL-5 was not modified and IL-13 was slightly increased (table I). In contrast, 1-MT pre-treatment of LPS-activated DC yielded cells with a reduced ability to stimulate IFNγ production by T cells (table I). DC treated with 1-MT and LPS had an increased ability to induce IL-5 and IL-13 production by T cells in MLR, compared to LPS-treated DC.

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

1-MT induces a Th2 polarization of DC matured with LPS

1-MT-treated DC activated by LPS had a reduced ability to induce IFNγ 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 kinetic of secretion of IFNγ, IL-5 and IL-13 was thus analyzed. In MLR with control LPS-treated DC, reasonable amount of IFNγ could be detected at day 2 of coculture while IL-5 and IL-13 began to be detectable at day 3. Cytokine concentration in the supernatants progressively increased until day 5 (Fig. 1A). The kinetic of IFNγ production was not modified by 1-MT pretreatment of DC although the quantity of cytokine released was drastically reduced. The kinetic 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 IFNγ, these secretions did not follow a first peak of IFNγ 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 IFNγ (58% of $IFN\gamma^+$ T cells) (Fig. 1B). 19% of these T cells also produced IL-13. Only 5% of T cells presented a Th2 profile with production of IL-13 without IFNγ. No IL-5 producing T cells were detected under these experimental conditions. DC pre-treated with 1-MT before LPS activated an increased number of T cells producing IL-13 without IFNγ (36% versus 5% in control MLR) while only 30% of T cells produced IFNγ (versus 58% in control MLR). 5% 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.

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

Since allogeneic MLR does not reflect an antigen-specific response, we examined the effect of 1-MT treatment of DC in an autologous response to tetanus neurotoxin. As shown in figure 2, the results found in allogeneic MLR can be observed in an antigen specific response. Without antigen, DC induced basal secretions of cytokines by T cells and 1-MT pre-treatment of LPS-activated DC yielded cells with a reduced ability to stimulate IFNγ 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 antigens to T cells. When antigen is added, all secretions are increased and the impact of 1-MT pre-treatment on LPS-stimulated DC is more striking, inducing a strong reduction of DC ability to stimulate IFNγ 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 mature DC 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. 1-MT pre-treatment had no effect on phenotypic maturation induced by pIC or Pam. Obvious effects of 1-MT were observed when DC maturation was induced by PGN and LPS. Indeed, for PGN- or LPS-matured DC, the expression of maturation markers was reduced by 1-MT pre-treatment although these markers were still expressed at high level (Fig. 3). An intermediate expression was also observed for CD80, CD83, CD54, 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 pre-treatment 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. 1-MT pre-treatment of LPS or PGN-stimulated DC was characterized by a strong reduction in secretion of IL-6, IL-10, IL-12p70 and TNFα (table II). In all conditions, cells remained negative for IFNγ and IL-1β secretion and DC viability was not modified (data not shown). 1-MT alone did not affect the basal level of cytokine secretion by iDC (table II).

The overall data indicate that 1-MT pre-treatment 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 while 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 in regards of the lack of inhibition by 1-MT following PGN stimulation. No IFNγ secretion was detected in DC culture supernatants, confirming that IDO induction can be IFNγ-independent.

Kynurenine is the main catabolite of Trp and is involved in the inhibition of T cell proliferation (18, 22). Since its production is inhibited by 1-MT, we asked whether addition of kynurenine during the pre-treatment of DC with 1-MT could restore a normal phenotypic and functional maturation induced by LPS. Figure 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). 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 these results strongly suggest 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 pre-treated 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. SB203580 (SB) is a specific inhibitor of p38-MAPK and PD98059 (PD) inhibits MEK activation thus preventing ERK phosphorylation. Addition of SB before LPS maturation yielded DC that could not induce IFNγ 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 pre-treated with PD and LPS secreted

similar amounts of IFNγ, IL-5 and IL-13 compared to 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 pre-treated with 1-MT before stimulation with LPS. As expected, in presence of SB, DC pre-treated or not by 1-MT were not able to induce IFNγ 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. 5C,D). Treating DC with both PD and 1-MT before stimulation with LPS restored their ability to induce IFNγ 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. 6A,B). All the TLR ligands increased the ratio of phosphorylated p38 to total p38 although with different strength and kinetics. The pre-treatment of DC with 1-MT did not modify the outcome of p38 activation induced by TLR stimulation with pIC while 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 pre-treated with 1-MT. The most striking differences were observed with LPS stimulation. p38 phosphorylation still occurred at 5 minutes but was followed by a second and more important peak at 15 minutes (Fig. 6A).

Like for p38, 1-MT pre-treatment had no effect on ERK phosphorylation following pIC stimulation. ERK phosphorylation was reduced following PGN signaling or delayed

following Pam signaling. The profile of pre-treatment with 1-MT were the same as those observed for p38 except for the TLR4 ligand LPS. When DC had been pre-treated with 1-MT, ERK phosphorylation was more intense and was maintained more than 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 pre-treatment 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). Like for ERK phosphorylation, pre-treating 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 pre-treated with 1-MT. The most striking difference was again observed for LPS. Pre-treatment 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.* that a sustained activation of ERK results in a phosphorylation and stabilization of c-Fos correlating with a Th2 response (49). All 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 of LPS seems to play a central role in the generation of DC with defective Th1 function and improved Th2 function.

Discussion

1-MT is a competitive inhibitor of Trp for the enzyme IDO (50). 1-MT was successfully used in vivo to break the immune privilege of placenta and tolerance against grafts, auto Ag 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 only recently investigated (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 mature DC. 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 IFNγ$^+$ T cells. This resulted in the secretion of high amounts of IL-5 and IL-13 and low amounts of IFNγ during MLR. These results were reproduced with an antigen-specific response, indicating that 1-MT could affect immune responses in vivo. 1-MT had minor or no effect 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 pre-treatment appeared to favor IFNγ 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.* 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 (51).

Actually, 1-MT effects on DC maturation is not correlated to 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 mimick or counteract 1-MT effect on DC function (Fig. 4C). So all these data suggest that the effects of 1-MT on DC function are independent of IDO catabolic 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 T cell-derived signals, and serotonin has been shown to act on mature DC by decreasing their secretion of IL-12 and TNFα after LPS maturation (52, 53). These possibilities are currently under investigation.

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 previously reported (45-48). p38-MAPK is mainly involved in CD83, CD80 and CD86 upregulation and in TNFα 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 to 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) while the IFNγ locus becomes less accessible (55-58). Since 1-MT pre-treatment of LPS-stimulated DC results in the stimulation of an increased number of $IL13^+$ T cells and a decreased number of $IFN\gamma^+$ T cells, and since 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 pre-treatment of DC stimulated with LPS strengthened and maintained the activation of c-Fos, while 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.* showing that a sustained ERK phosphorylation in DC results in a phosphorylation and stabilization of c-Fos and in a Th2-polarization of DC (49).

$CCR6^+$ $CD123^+$ 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 an 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 both on MyD88 dependent and independent pathways. The impact of 1-MT on phenotype and cytokines induced by LPS, PGN or Pam suggests an interference of 1-MT on the MyD88-dependent pathway. However, the differential effect of 1-MT on the ability of DC to induce IL-13, IL-5 and IFNγ secretion by T cells most likely reflects a complex action of 1-MT at various levels of the signaling pathways. Interestingly, 1-MT pre-treatment does not modify the Th1 response induced by DC activation with an anti-CD40 antibody (data not shown), reinforcing the notion that the effect of 1-MT is dependent on the signaling pathway. Perturbation of these signaling pathways through various adaptors remains to be analyzed in more details.

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. 1-MT constitutes a pharmacologic agent useful to manipulate the immune response in vivo. In regards of its action on IDO and involvement in the rupture of tolerance, 1-MT has been proposed to be used in cancer therapy. According to our results which suggest that 1-MT 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 1-MT in clinical protocols should be examined carefully.

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Footnotes

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3 - Abbreviations: 1-MT, 1-methyl-tryptophan; Trp, tryptophan; DC, dendritic cell; iDC, immature DC; IDO, indoleamine 2,3-dioxygenase; mDC, mature DC; pIC, polyI:C; PGN, peptidoglycan; Pam, Pam3CSK4; RLU, relative luminescence unit.

Figure Legends

Figure 1. 1-MT induces a Th2 function of DC stimulated with LPS. (A) Kinetic of secretion of IFNγ, IL-5 and IL-13 in MLR supernatants. MLR were conducted with control iDC (□), LPS-stimulated DC (○) and LPS-stimulated DC pre-treated with 1-MT (▲). Cytokines were measured in MLR supernatants at the indicated times. Mean ± SD of triplicates of one representative experiment out 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 IFNγ 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 out of three.

Figure 2. 1-MT pre-treatment of LPS-stimulated DC induces a Th2 response of tetanus neurotoxin specific T cells. Control iDC, 1-MT-treated DC, LPS-stimulated DC and LPS-stimulated DC pre-treated with 1-MT were cocultured with autologous T cells in presence (opened bars) or not (filled bars) of 25 µg/ml of tetanus neurotoxin (Ag). IL-2 (A), IFNγ (B), IL-5 (C) and IL-13 (D) were measured in supernatants recovered after 5 days of cocultures. Mean ± SD of triplicates of one representative experiment out of two.

Figure 3. Effect of 1-MT on phenotypic maturation. Control iDC were obtained at day 7 without addition of a maturation agent. Control mDC were obtained at day 7 after addition at day 6 of LPS, pIC, PGN or Pam. When indicated, 1-MT was added at day 5 before TLR stimulation at day 6. Phenotype was analyzed at day 7. CD86 and CD40 expression of control iDC (dotted line), TLR-stimulated DC (filled profile) and TLR-stimulated DC pre-treated with 1-MT (thick line). (A) TLR4 stimulation by LPS. (B) TLR3 stimulation by pIC. (C)

TLR2/6 stimulation by PGN. (D) TLR2/1 stimulation by Pam. Data from one representative experiment out of five. (E) CD14/CD1a surface expression was analysed on control immature DC (iDC), DC treated with the different TLR ligands at day 6 of differentiation, and DC treated with 1-MT at day 5 and with different TLR ligands at day 6. All cells were recovered and examined at day 7. The percentage of $CD1a^+/CD14^-$ cells is indicated in the quadrant.

Figure 4. The effect of IDO activity and kynurenine on DC function. (A) Expression of IDO mRNA. At day 7, total RNA from iDC, DC treated with LPS, pIC, PGN or Pam was amplified by RT-PCR for IDO and S12. (B) IDO activity was measured by a kynurenine assay at day 7 in supernatants of control iDC, TLR-stimulated DC, TLR-stimulated DC pre-treated with 1-MT. Kynurenine concentration was normalized to 100% for control iDC (absolute values between 35 and 185 μM). Mean ± SD from five independent experiments. (C) LPS-stimulated DC (black bars), LPS-stimulated DC pre-treated with kynurenine (grey bars), LPS-stimulated DC pre-treated with 1-MT (opened bars), LPS-stimulated DC pre-treated with 1-MT and kynurenine (hatched bars), LPS-stimulated DC pre-treated with Trp (dotted bars) and LPS-stimulated DC pre-treated with 1-MT and Trp (vertical bars) were harvested at day 7 and cocultured with T lymphocytes. Data are shown for 1/20 DC/T cell ratio and were similar for the other ratios. IFNγ, IL-5 and IL-13 were measured in MLR supernatants at day 5. Cytokine secretion was normalized to 100% for control mDC. Mean ± SD of triplicates of one representative experiment out of three.

Figure 5. p38-MAPK and ERK pathways in DC polarization. (A, B) Control LPS-mDC (black bars) or DC pre-treated with 25 μM SB203580 (A) or with 40μM PD98059 (B) before addition of LPS at day 6 (grey bars) were cocultured with T cells. Data are shown for 1/20 DC/T cell ratio and were similar for other ratios. Secretions of IFNγ, IL-5 and IL-13 were

measured in coculture supernatants at day 5. Cytokine secretion was normalized to 100% for control mDC. Mean ± SD of three independent experiments. (C, D) p38-MAPK inhibitor prevents Th2 polarization of LPS-stimulated DC. Control LPS-stimulated DC (black bars), LPS-stimulated DC pre-treated with 1-MT (opened bars) or LPS-stimulated DC pre-treated with SB203580 and 1-MT (hatched bars) were cultured with T cells. Secretions of IFNγ (C), IL-5 and IL-13 (D) were measured in coculture supernatants at day 5. Cytokine secretion was normalized to 100% for control mDC. Mean ± SD of three independent experiments. (E, F) MEK/ERK pathway inhibitor restores the Th1 polarization of DC treated by 1-MT and LPS. Control LPS-stimulated DC (black bars), LPS-stimulated DC pre-treated with 1-MT (opened bars) or LPS-stimulated DC pre-treated with PD98059 and 1-MT (grey bars) were cultured with T cells. Secretions of IFNγ (E), IL-5 and IL-13 (F) were measured in the supernatants at day 5 of coculture. Cytokine secretion was normalized to 100% for control LPS-stimulated DC. Mean ± SD of three independent experiments.

Figure 6. Differential effect of 1-MT on p38-MAPK, ERK and c-Fos activation induced by different TLR ligands. (A, B) Time course of p38 and ERK phosphorylation induced by 1-MT pre-treatment and TLR stimulation. DC were treated at day 5 with 1-MT (△) and stimulated at day 6 with LPS, pIC, 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 out of three. (C) Time course of c-Fos phosphorylation induced by 1-MT pre-treatment and TLR stimulation. DC were treated at day 5 with 1-MT (△) and stimulated at day 6 with LPS, pIC, PGN or Pam for the indicated periods of time. Control TLR-stimulated DC (■) were not treated with 1-MT. Phosphorylated c-Fos was quantified in cell lysates and normalized

compared to the total protein content. Results are shown as phosphorylated c-Fos (RLU)/µg protein ratio. Data from one representative experiment out of three.

Table I: Secretion of cytokines in MLR supernatants

	IL-2 [a]	IFNγ [b]	IL-5 [b]	IL-13 [b]
iDC	44 ± 14	441 ± 141	10 ± 0,2	47 ± 10
1-MT	75 ± 9	236 ± 134	1 ± 0,4	31 ± 6
LPS	168 ± 56	36126 ± 376	31 ± 5	345 ± 94
LPS+1-MT	306 ± 19	7547 ± 1720	112 ± 48	982 ± 102
pIC	106 ± 36	2019 ± 947	58 ± 23	542 ± 32
pIC+1-MT	246 ± 0,2	2088 ± 1067	23 ± 4	585 ± 36
PGN	187 ± 74	2657 ± 626	121 ± 21	1024 ± 72
PGN+1-MT	470 ± 109	10011 ± 2939	114 ± 61	1553 ± 35
Pam	82 ± 23	21001 ± 4204	23 ± 5	698 ± 87
Pam+1-MT	179 ± 44	43186 ± 12048	46 ± 5	1314 ± 57

[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 CBA and are expressed in pg/ml. Means ± SD from five independent experiment.

Table II: Cytokine secretion ns by DC treated with the different TLR ligands

	IL-6 [a]	**IL-10** [a]	**IL-12p70** [a]	**TNFα** [a]
iDC	119 ± 32	132 ± 101	2 ± 2	111 ± 53
1-MT	87 ± 33	91 ± 7	0	78 ± 34
LPS	24200 ± 10767	2333 ± 1168	1667 ± 566	9222 ± 1863
LPS+1-MT	5283 ± 1402	1194 ± 362	162 ± 97	3855 ± 1634
pIC	313 ± 116	78 ± 23	0,0	377 ± 143
pIC+1-MT	346 ± 103	82 ± 15	0,0	837 ± 352
PGN	4975 ± 834	6269 ± 1504	0,0	9673 ± 2433
PGN+1-MT	953 ± 455	1688 ± 66	0,0	3143 ± 1383
Pam	379 ± 135	204 ± 129	0,0	891 ± 324
Pam+1-MT	320 ± 217	163 ± 93	0,0	549 ± 372

[a] maximum values of cytokine secretion by DC treated with the different TLR ligands ± 1-MT. Cytokine secretions were quantified by ELISA and are expressed in pg/ml. Means ± SD from 5 independent experiments.

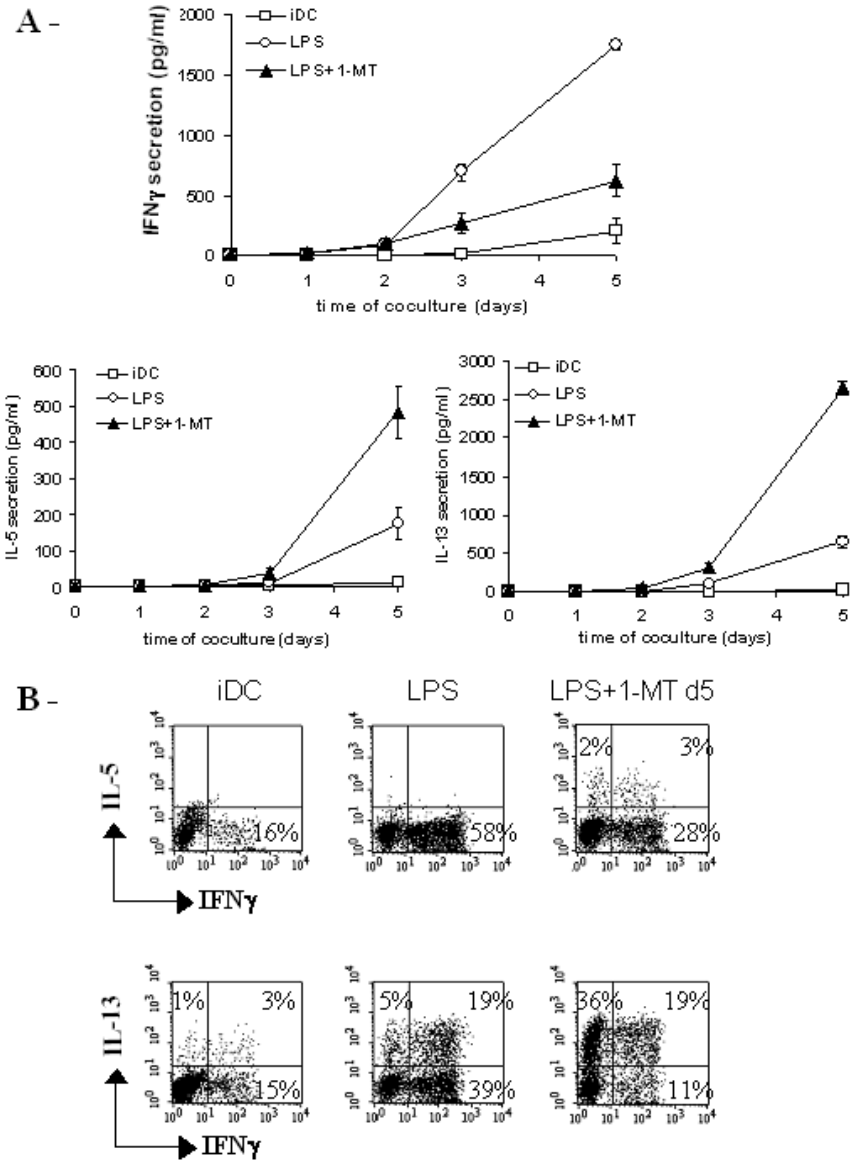

Figure 1

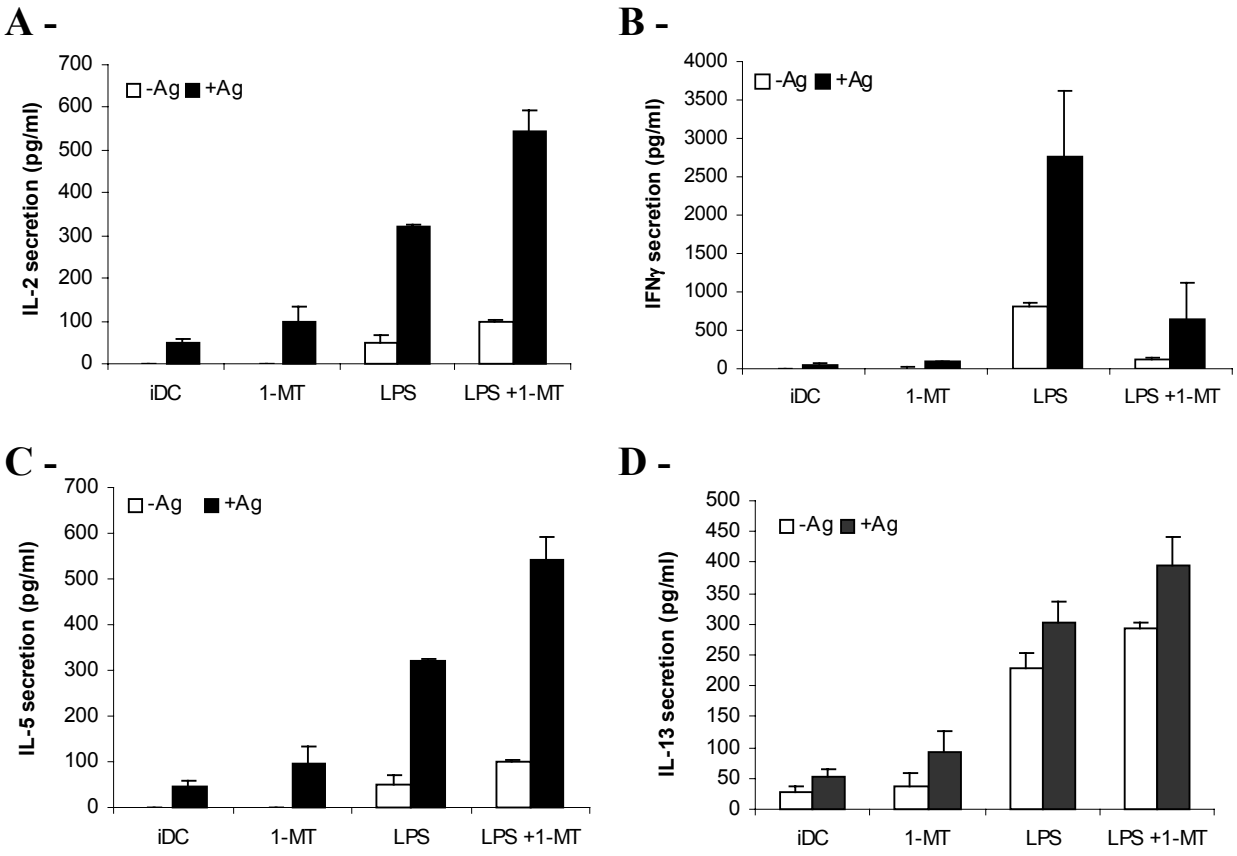

Figure 2

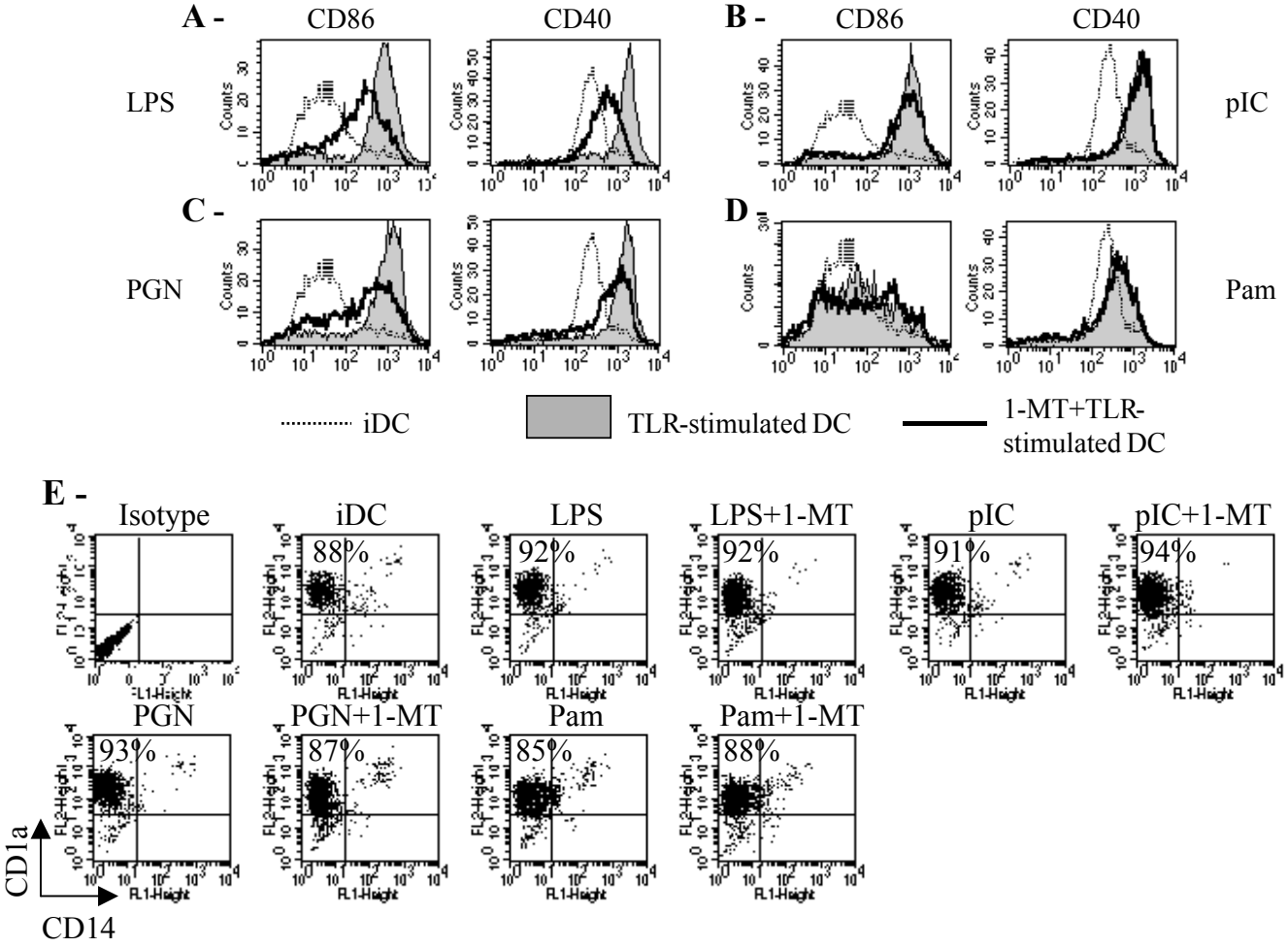

Figure 3

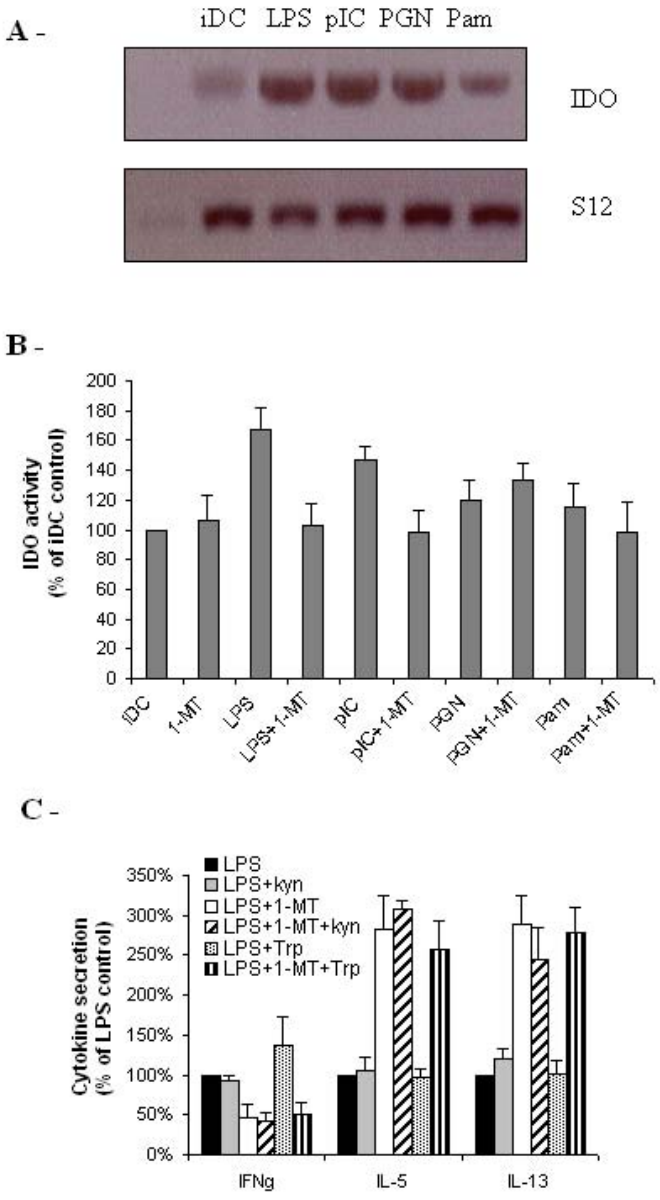

Figure 4

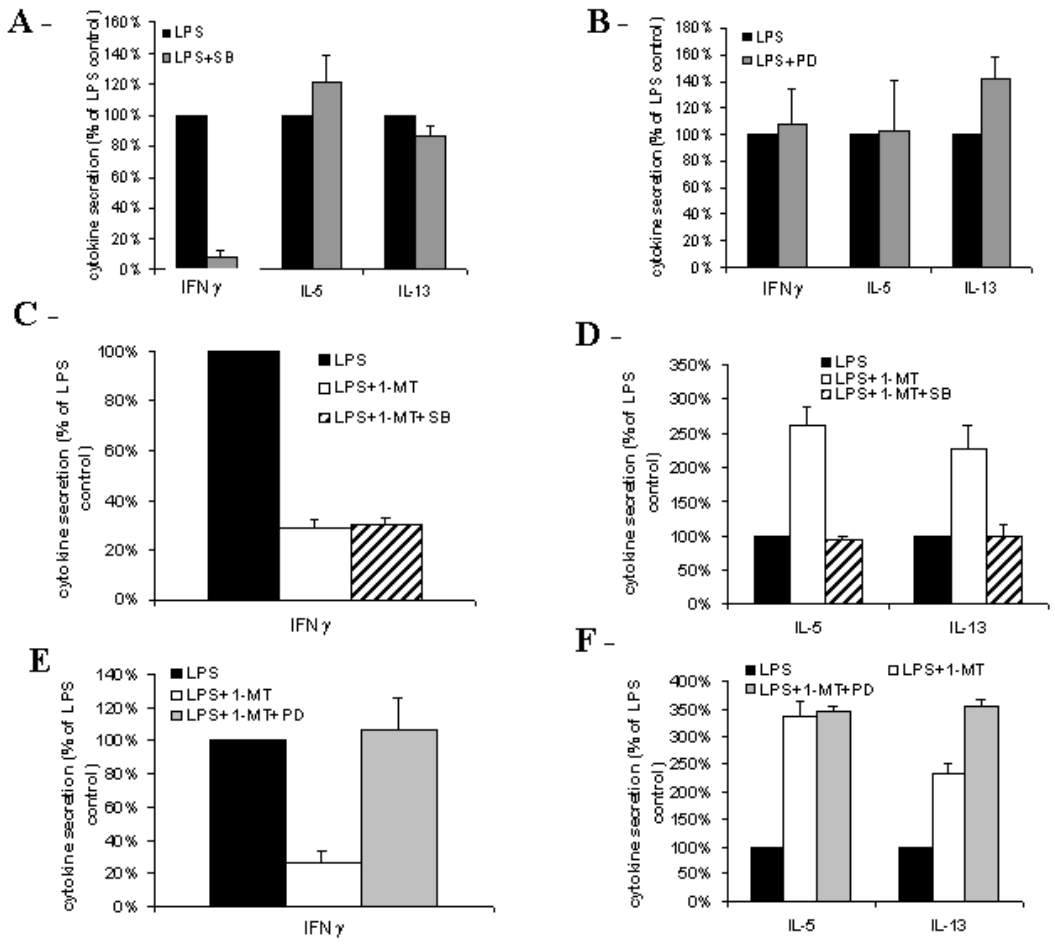

Figure 5

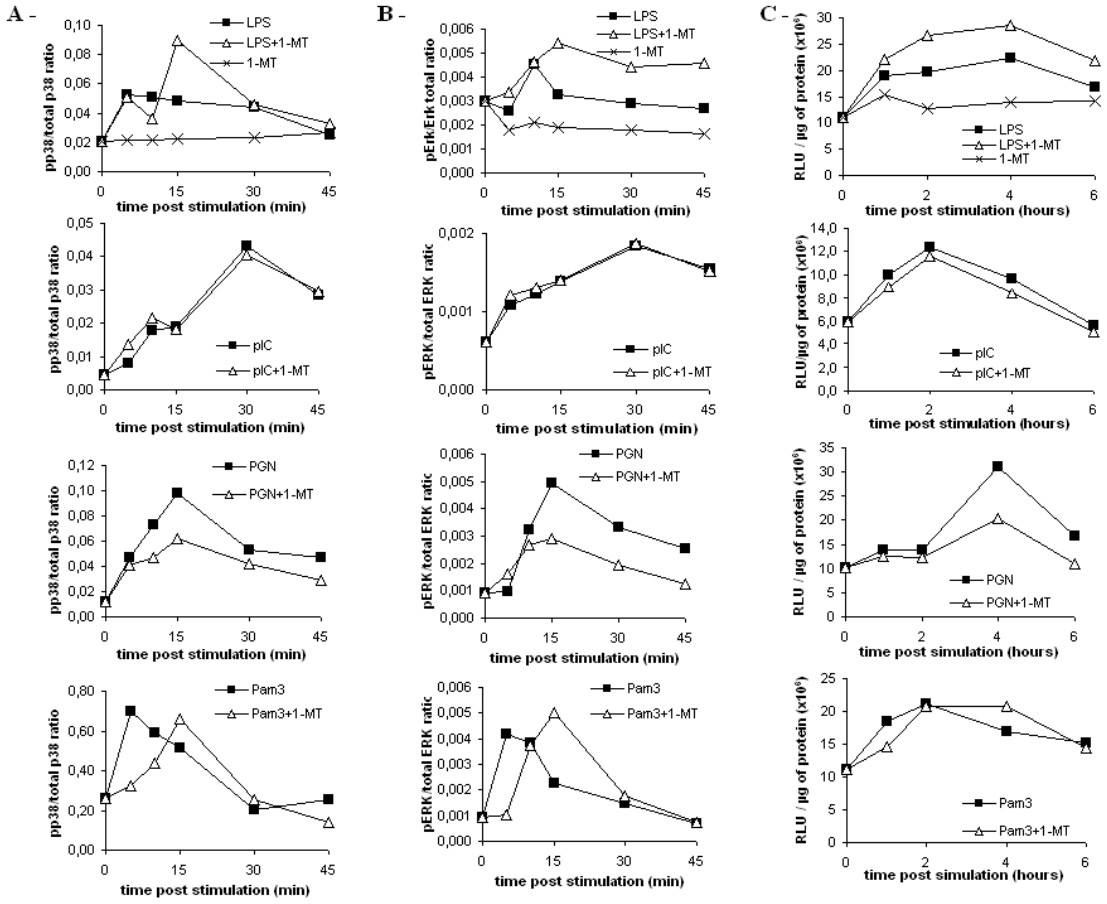

Figure 6