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DIFFERENTIATION AND FUNCTION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS UNDER THE INFLUENCE OF LEFLUNOMIDE

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Abstract - Leflunomide is an immunosuppressive drug effective in experimental models of transplantation and autoimmune diseases and in the treatment of active rheumatoid arthritis (RA). Having in mind that it has been shown that some other immunosuppressive drugs (glucocorticoids, mycophenolate mofetil, sirolimus etc.) impair dendritic cell (DC) phenotype and function, we investigated the effect of A77 1726, an active metabolite of leflunomide, on the differentiation and function of human monocyte-derived dendritic cells (MDDC) *in vitro*. Immature MDDC were generated by cultivating monocytes in medium supplemented with GM-CSF and IL-4. To induce maturation, immature MDDC were cultured for 2 additional days with LPS. A77 1726 (100 μM) was added at the beginning of cultivation. Flow cytometric analysis showed that MDDC differentiated in the presence of A77 1726 exhibited an altered phenotype, with a down-regulated surface expression of CD80, CD86, CD54 and CD40 molecules. Furthermore, the continuous presence of A77 1726 during differentiation and maturation prevented successful maturation, judging by the decreased expression of maturation marker CD83, costimulatory and adhesive molecules on A77 1726-treated mature MDDC. In addition, A77 1726-pretreated MDDC exhibited a poor stimulatory capacity of the allogeneic T cells and a low production of IL-10 and IL-18. These data suggest that leflunomide impairs the differentiation, maturation and function of human MDDC *in vitro*, which is an additional mechanism of its immunosuppressive effect.

Key words: Alloreactivity, cytokine production, human dendritic cells, leflunomide, phenotype

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INTRODUCTION

Leflunomide is a well-known immunoregulatory and disease-modifying anti-rheumatic drug that is structurally different from other immunosuppressive agents (Bartlett and Schleyerbach, 1985; Bartlett, 1986; Bartlett et al., 1996; Brazelton and Morris, 1996). This isoxazole derivate has been shown as a potent inhibitor of T- and B-cell proliferation without evident cytotoxic activity (Siemasko et al., 1996; Elder et al., 1997). After oral administration, leflunomide is almost completely converted to its active metabolite, termed A77 1726, chemically (2Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl) phenyl] but-2-enamide. The metabolite is a potent inhibitor of the enzyme dihydroorotate dehydrogenase (DHODH), a key enzyme in the *de novo* synthesis of uridine monophosphate (Davis et al., 1996; Williamson et al., 1996). *De novo* synthesis of pyrimidines is not only essential for RNA and DNA synthesis and thus the clonal expansion of activated lymphocytes, but also for phospholipid synthesis and protein glycosylation (Allison et al., 1993; Cherwinski et al., 1995). In addition, at least five structurally different tyrosine kinases, predominantly expressed in T cells, have so far been described as targets of A77 1726 inhibition. Many of them are involved in antigen receptor and growth factor receptor signaling events

(Mattar et al., 1993; Xu et al., 1995; Xu et al., 1996; Elder et al., 1997). However, A77 1726 is a 150- to 900-fold more potent inhibitor of DHODH than tyrosine kinases (Williamson et al., 1996; Knecht and Loffler, 1998).

Although leflunomide has been approved for the treatment of rheumatoid arthritis (RA), experiments performed on different animal models showed that A77 1726 and two other leflunomide metabolites with much shorter plasma half-lives (FK 778 and FK 779) were effective immunosuppressants in experimental organ transplantation (Lirtzman et al., 1996; Lucien et al., 1996; Lin et al., 1998).

Alloreactivity is a complex immunological phenomenon associated with the recognition of alloantigens, activation of alloreactive T cells and their differentiation into effectors, which reject the transplanted graft. In these processes the cross-talk interplay between antigen-presenting cells (APC) and T-cells is of crucial importance. Among APC, dendritic cells (DC) are the only APC with the ability to stimulate naive T cells. DC are a heterogeneous population in terms of their phenotype, function, maturation stage and migration pattern. Myeloid DC that could be easily generated *in vivo* from monocytes are the best-characterized DC subset, usually assessed as stimulators in the allogeneic MLR. Based on previous observations that A77 1726 is able not only to inhibit T cell proliferation, but also to interfere with cell-cell contact and cell adhesion, we postulated that the metabolite of leflunomide also affects DC (Dimitrijevic and Bartlett, 1996).

In this work we showed that A77 1726 impairs the differentiation and maturation of human monocyte-derived DC (MDDC) *in vitro*, resulting in their lower allostimulatory activity.

MATERIALS AND METHODS

Cytokines and reagents

Human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) (Leucomax, spec. activity 4.44×10^6 IU) was obtained from Sandoz-Schering Plough, Basle, Switzerland. Human recombinant interleukin (IL)-4 was from Roche Diagnostics GmbH, Mannheim, Germany. A77 1726, a leflunomide metabolite, was obtained from Hoecht Marion Roussel, Germany. Stock solutions were prepared by dissolving the drug in RPMI-1640 serum free medium (ICN, Costa Mesa, CA). Aliquots were kept frozen at –20°C. Lypopolysaccharide (LPS) was from Sigma, Munich, Germany.

DC cultures

Highly enriched monocytes (about 75-85 % purity) were purified from the buffy coats of healthy volunteers by a Lymphoprep gradient (Nycomed, Oslo, Norway) and plastic adherence as described (Čolić et al., 2003; Čolić et al., 2004). Monocytes were cultivated for 6-7 days in 4-well tissue culture plates (Flow, Irvine, Scotland) in 4 ml of complete RPMI-1640, HEPES/sodium bicarbonate buffered medium with the addition of 10% heat-inactivated fetal calf serum (FCS) (ICN, Costa Mesa, CA), streptomycin, gentamicin, penicillin and 2-ME (Sigma, Munich, Germany). The medium was supplemented with 100 ng/ml of GM-CSF and 5 ng/ml of IL-4. After day 3, the cultures were fed with 3 ml of fresh medium with cytokines. On day 6 (or in some experiments on day 7), non-adherent cells (predominantly immature DC) were transferred to new plates and half the medium was replaced with cytokines or cytokines with 1 μg/ml LPS. The cells were cultivated for an additional 2 days. In the cultures with A77 1726, the compound was added at different concentrations at the beginning of monocyte cultivation and at the time of medium replacement.

Flow cytometry

Immature or mature non-adherent MDDC cultivated with or without A77 1726 were collected, washed in PBS with 0.1% sodium azide and 2% FCS (PBS/FCS) and adjusted at concentrations of $1\text{-}2 \times 10^5$ cells/tube. The cells were stained in suspension using appropriate dilutions of the following monoclonal antibodies (mAbs) to: HLA-DR (Becton-Dickinson), CD1a,

CD40, CD54, CD80, CD83 and CD86 (Serotec), CD14 (ICN). CD1a and CD80 mAbs were conjugated with FITC, CD86 with PE, whereas other mAbs were unconjugated. After washing in PBS/FCS, an anti-mouse (Fab-2) Ig-FITC antibody (Serotec) with 5% normal human serum was added to the cells previously incubated with unconjugated mAbs. The control consisted of samples with irrelevant mouse mAbs reactive with rat antigens. After washing, the cells were analyzed on an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany). At least 5000 events were analyzed.

Cytokine assays

After 6 days of cultivation, control immature MDDC and A77 1726-treated MDDC were replated (6 x 10^5 cells/ml) in complete medium with cytokines and LPS or LPS and A77 1726. The cells were cultivated for 2 days and supernatants were collected. TNF-α was determined using the L929 cytotoxic assay as described (Hay and Cohen, 1989). IL-10, IL-12 and IL-18 were determined using a sandwich ELISA assay following the instructions of the manufacturers. The IL-10 and IL-12 ELISA kits were from R&D (Minneapolis, USA) and IL-18 was from MBL (Nagoya, Japan). Detection limits were 0.5 pg/ml for IL-12, 3.9 pg/ml for IL-10, 12.5 pg/ml for IL-18, and 23.5 pg/ml for TNF-α.

Effect of A77 1726 on allogeneic lymphocytes proliferation (two-way MLR and allogeneic MLR)

Peripheral blood mononuclear cells (PBMNC) were isolated from the buffy coats using a Lymphoprep gradient. In some experiments, the T cells were purified from PBMNC using immunomagnetic sorting with a pan-T cell isolation kit by MACS technology (Myltenyi Biotec, Bergish Gladbach, Germany), following the instructions of the manufacturer. The purity of the T cells recovered in the negative fraction was higher than 95%, as checked by anti-CD3 FITC mAb (Serotec) and flow cytometry.

To test the effects of the increasing doses of A77 1726 on allogeneic T cell proliferation, a total of 2 x 10^5 purified T cells were stimulated in a 96-well plate either by allogeneic lymphocytes (2 x 10^5) or 2.5 x 10^3 immature MDDC or mature MDDC in the absence or presence of A77 1726 (10-200 μM). After this, we assessed the allostimulatory capacity of the A77 1726-treated MDDC, immature or mature, on allogeneic T cell stimulation. MDDC as stimulators were cocultured with 2 x 10^5 responders in flat-bottom 96-well microtiter plates. The different DC/T cells ratios were used. After 5 days of culture, and in order to assess cell proliferation, the cells were pulsed with [3H]-thymidine for the last 18 h (1 μCi/well, Amersham, Books, UK). Labeled cells were harvested onto glass fiber filters. Radioactivity was counted in a Beckman scintillation counter and expressed as cpm ± SD of triplicates.

Statistical analysis

Differences in parameters between the control and the A77 1726-treated MDDC were evaluated using the Mann-Whitney U-test. A p value less than 0.05 were considered significant.

RESULTS

A77 1726 impairs differentiation of MDDC

The first part of this work was designed to study the effect of A77 1726 on the *in vitro* differentiation of MDDC. Blood monocytes (mostly CD14+ CD1a-) (Fig. 1A) were cultivated for 7 days in a DC culture medium (RPMI+10% FCS supplemented with GM-CSF and IL-4) alone (Fig. 1B), or with the addition of different concentrations of A77 1726 (25 – 200 μM) (Fig. 1C-F). Our preliminary experiments showed that after 7 days 70-85% of the monocytes developed into non-adherent veiled cells, whereas 15-30% cells remained adherent, macrophage-like cells. To check cell yields in the A77 1726-treated cultures, we collected non-adherent and adherent cells. The cell yield was only marginally reduced in the cultures using 25 μM, 50 μM and 100 μM concentrations of A77 1726, whereas the concentration of 200 μM was cytotoxic and resulted in about 40% cell recovery (data not shown).

In the control cultures, about 70% of the cells expressed CD1a, a DC marker, whereas 30% expressed CD14, a monocyte/macrophage marker (Fig.1B). The absence of CD83 expression (data not shown) suggests that most cells were immature DC. A77 1726 reduced the expression of CD1a in a dose-dependent manner. In contrast, with the lowest concentration of the metabolite (25 μM) an increase in the percentage of the CD14+ cells was observed, followed by a gradual decrease in expression with increasing concentrations of A77 1726 (Fig. 1C-F).

Based on the FS/SS profile (Fig. 1), cells cultivated in the presence of A77 1726 became smaller, had less branching and fewer veiled structures and a lower FS profile compared to the control MDDC. Cumulatively, these results suggest that the leflunomide metabolite at concentrations between 25 μM and 100 μM impairs the differentiation of MDDC without significant cytotoxic effect. Therefore, in the next experiments, we mostly used the 100 μM concentration of A77 1726.

Phenotypic analysis of MDDC cultured for 9 days in control DC medium or DC medium with addition of A77 1726 is presented in Fig. 2A. Control, immature MDDC were strongly positive for HLA-DR (97.4 ± 2.2%; MIF 70.8 ± 7.6), CD40, CD54 and CD86, and expressed a moderate level of CD80. Only a small subset of these cells were CD83+ (10.4 ± 4.2%). A77 1726 significantly inhibited the percentage of cells expressing CD80 (47% of inhibition) or MIF for CD40, CD54 and CD86. Expression of CD83 was almost undetectable while HLA-DR was not significantly changed compared to control.

A77 1726 inhibits maturation of MDDC

Immature MDDC generated in the presence of GM-CSF and IL-4 were induced to mature at day 7 by the addition of LPS. At day 9 the phenotype of the cells was analyzed. The results presented in Fig. 2B show that the maturation of MDDC was followed by upregulation of CD83, CD80, CD86, HLA-DR (98.4 ± 1.3%; MIF 154.2 ± 13) and CD54 on the MDDC, whereas the expression of the CD40 marker was not significantly changed compared to immature MDDC (Fig. 2A i B).

The addition of LPS to the A77 1726-treated immature MDDC cultures resulted in the inhibition of MDDC maturation. The phenomenon was confirmed by a significant down-regulation of the maturation marker CD83 and decreased MIF for CD80 and CD54 molecules. The reduced percentage of cells expressing CD80 and decreased density of CD86 and CD40 were measured in the A77 1726 samples after stimulation with LPS, but these differences did not reach statistical significance. A77 1726 treatment had no influence on HLA-DR expression or MIF (Fig. 2B).

A77 1726 blocks proliferation of allogeneic lymphocytes and significantly decreases allostimulatory capacity of MDDC

Leflunomide has been shown to be a potent inhibitor of lymphocyte proliferation. This is confirmed in our experiments using classical two-way MLR. As can be seen in Fig. 3A, the inhibition of proliferation of allogeneic lymphocytes was about 60% using a 10 μM concentration of A77 1726, whereas concentrations between 100 μM and 200 μM almost completely blocked cell proliferation. A similar, dose-response effect of the leflunomide metabolite on the proliferation of allogeneic lymphocytes was seen using immature and mature MDDC as stimulators (Fig. 3B). MDDC cultivated with A77 1726 (100 μM) showed a decreased allostimulatory capacity using purified T cells (Fig. 3C, D) as responders. The effect was observed with both the immature MDDC treated with A77 1726, and in A77 1726-treated MDDC induced to mature with LPS; it was stronger at higher DC/lymphocyte ratios.

A77 1726 modulates cytokines production by mature MDDC

During maturation MDDC secrete various cytokine, but some of them, such as IL-12 and IL-10, are very important because they induce the Th1 and Th2

immune response, respectively. We found that A77 1726 reduced IL-10 and IL-18 secretion and had no influence on measured IL-12 and TNF-α levels, compared to the control, mature MDDC (Table 1).

Table 1. Effect of A77 1726 on cytokine production by mature MDDC.

pg/ml	n	mature MDDC			A77 1726-treated MDDC		
TNF-α	7	11639	±	9498	12340	±	10398
IL-12	6	35	±	30	26	±	27
IL-10	6	810	±	227	285	±	148*
IL-18	6	29	±	27	ND		

Immature MDDC were obtained by cultivating monocytes with GM-CSF and IL-4 for 7 days. A77 1726 (100 μM) was added at the beginning of monocyte cultivation. At day 7, non-adherent cells were collected, transferred to 6-well plates (5×10^5 cells/ml, 3 ml/well) and induced to mature by adding LPS (1 μg/ml) to DC culture medium with cytokines. A77 1726 was added together with LPS in previously A77 1726-treated cultures. After 48 h supernatants are collected and the levels of cytokines were determined by ELISA (IL-12, IL-10, and IL-18) or bioassay (TNF-β). Results are presented as the mean ± SD.
*$p < 0.05$ compared to mature MDDC. n-number of donors; ND-non-detectable.

DISCUSSION

Immunosuppressive agents are used to control allograft rejection and to inhibit lymphocyte activation and proliferation in the treatment of autoimmune diseases. In recent years, evidence has accumulated that these diversely acting agents also affect the development and functional immunobiology of dendritic cells, *in vitro* and *in vivo*, by inducing DC with a tolerogenic phenotype and function. It has been shown that immunosuppressive agents, such as glucocorticoids, mycophenolate mofetil (MMF), sirolimus and others, impair DC differentiation, maturation, allostimulatory capacity and cytokine production (Piemonti et al., 1999; Woltman et al., 2000; Woltman et al., 2001; Čolić et al., 2003).

It is well known that A77 1726, an active metabolite of a relatively new immunosuppressive drug leflunomide, shows antiproliferative and antiadhesive effects. To the best of our knowledge, although Dimitrijević et al. showed that the migration of DC might be influenced by *in vivo* administration of leflunomide (Dimitrijević et al., 1998), this study presents the inhibitory effect of leflunomide on the differentiation, maturation and function of human MDDC generated *in vitro*.

Our work was initiated by preliminary experiments designed to study the effects of different concentrations of A77 1726 on *in vitro* differentiation of MDDC. We showed that A77 1726 at concentrations between 25 μM and 100 μM impairs differentiation, based on the up-regulation of CD1a and down-regulation of CD14 expression, without a significant cytotoxic effect. On the basis of these data and the therapeutic plasma concentrations of the active metabolite in patients with RA (100-150 μM), in the next experiments we used a 100 μM concentration of A77 1726. Phenotypic analysis showed that 80-90% of immature MDDC expressed HLA-DR, CD40, CD54 and CD86, 47% were CD80 positive, while only a small subset was CD83+. The obtained results are in agreement with the data showing that DC *de novo* express CD80 and upregulate the expression of CD40, HLA-DR, CD4, CD11b, CD11c (Pickl et al., 1996; Palucka et al., 1998). The continuous presence of leflunomide during the differentiation of MDDC was followed by a significantly inhibited density of co-stimulatory and adhesive molecules on the cell surface (CD40, CD54 and C86), and a reduced percentage of cell expression of CD80. CD83 was almost undetectable, while the other markers were not significantly changed. This finding is in accordance with the markedly suppressed surface expression of CD40 and CD80, and the prevented neo-expression of CD1a in leflunomide-treated DC (Kirsch et al. 2005). The obtained phenotype of A77 1726-treated MDDC points to a decreased co-stimulatory and adhesive function of these cells and an unchanged antigen-presenting function, judging by the unaltered HLA-DR expression.

The exposure of immature MDDC to bacterial cell products, such as LPS, induced the maturation

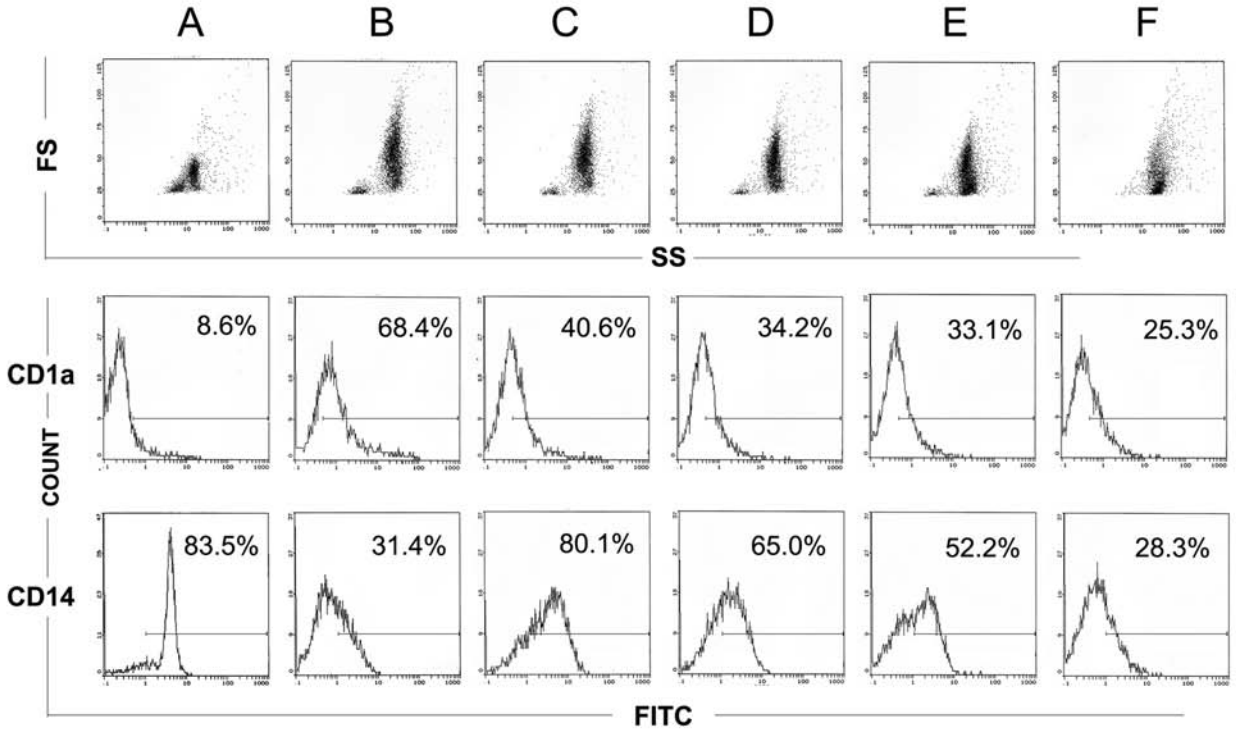

Fig. 1. A77 1726 impairs *in vitro* differentiation of human MDDC.
Monocytes isolated from PBMNC were cultivated in (A) RPMI + 10 % FCS medium, (B) DC medium (RPMI + 10 % FCS medium supplemented with GM-CSF and IL-4) or DC medium with different concentrations of A77 1726: (C) 25 μM, (D) 50 μM, (E) 100 μM and (F) 200 μM. After 7 days, non-adherent and loosely adherent cells that were easily detached by pipetting were collected. Forward scatter/side scatter profile and CD1a and CD14 expression were analyzed by flow cytometry.

of DC that was associated with a significant up-regulation of the maturation marker, CD83, as well as other molecules, CD80, CD86, CD54, HLA-DR, whereas the expression of CD40 was not significantly changed compared to the immature MDDC. Maturation was inhibited in the presence of A77 1726; this was confirmed by the lower expression of CD83, and 32% of inhibition and down-regulation of CD40, CD54, CD80 and CD86. A similar phenotype of DC differentiated and activated in the presence of 150 μM A77 1726 was obtained in the study of Kirsch and collaborators (Kirsch et al., 2005). The described effects of leflunomide on MDDC differentiation and maturation were in correlation with the effect of several other immunosuppressive agents published so far. Indeed, drugs such as corticosteroids, sirolimus, MMF and cyclosporin A inhibited the differentiation, maturation and/or function of human DC (Piemonti et al., 1999; Woltman et al., 2000; Woltman et al., 2001; Čolić et al., 2003).

The present study also showed that leflunomide efficiently inhibited lymphocyte proliferation regardless of which stimulators were used, allogeneic lymphocytes, immature or mature MDDC. The registered inhibitory effect of A77 1726 in classical two-way MLR and allogeneic MLR was strong and dose-dependent with almost completely blocked cell proliferation by higher concentrations of metabolite (100-200 μM). Leflunomide exerted an inhibitory effect on lymphocyte proliferation through a direct effect on the lymphocytes themselves or by an indirect effect on the MDDC as stimulators. As we mentioned before, MDDC

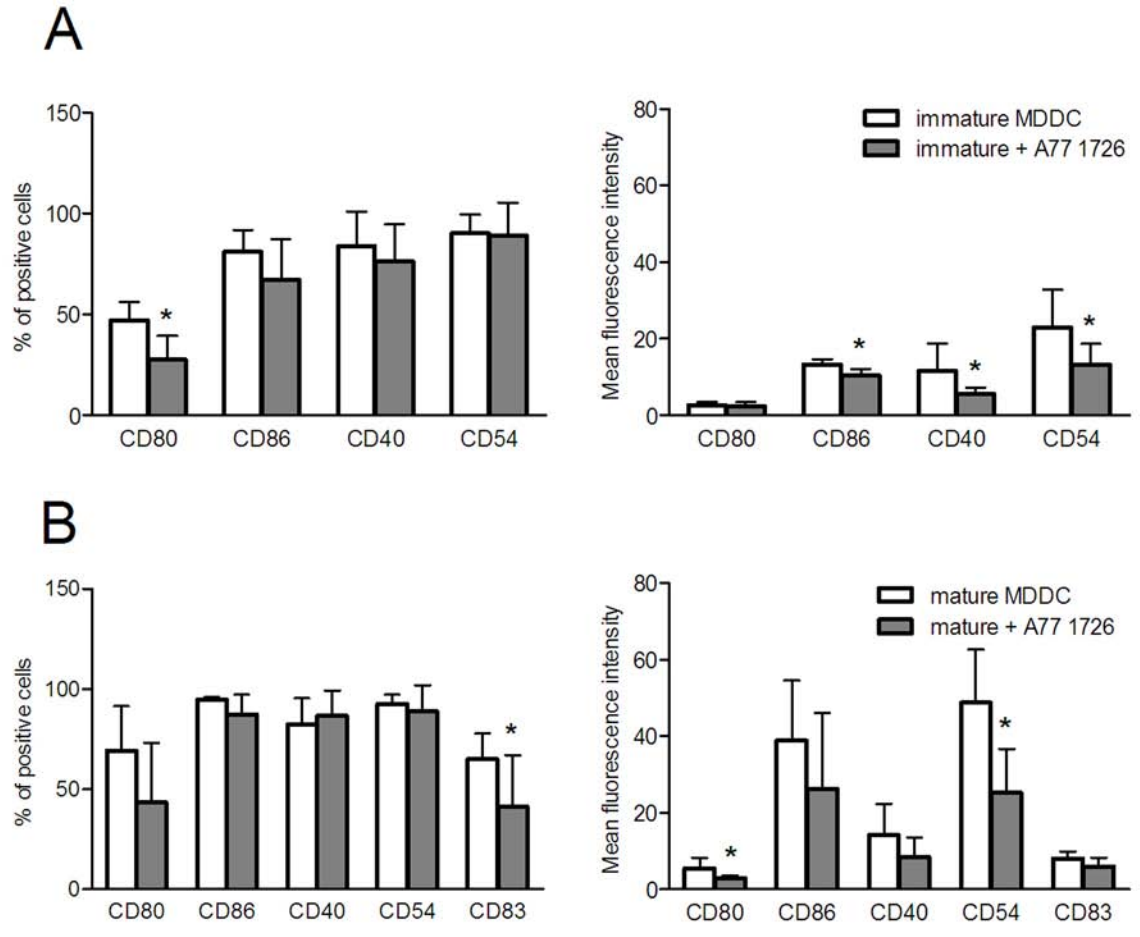

Fig. 2. Effect of A77 1726 on phenotypic characteristics of immature and mature MDDC.
(A) Immature MDDC were obtained by cultivating monocytes with GMCSF and IL-4 for 9 days. A77 1726 (100 μM) was added at the beginning of monocyte cultivation. (B) To obtain mature MDDC, at day 7, immature MDDC were induced to mature by adding LPS (1 μg/ml) to DCs culture medium with cytokines. A77 1726 was added together with LPS in previously A77 1726-treated cultures. At day 9 of culture, non-adherent control and A77 1726-treated MDDC were collected, stained in suspension using different MoAbs and analyzed by flow cytometry. Values are given as mean percentages of positive cells ± SD and mean fluorescence intensity ± SD calculated from four to seven independent experiments. *$p < 0.05$ compared to control, immature or mature MDDC.

cultivated with A77 1726 showed a decreased expression of costimulatory and adhesive molecules, and as consequence of these changes, a lower allostimulatory activity of both immature and mature A77 1726-treated MDDC in allogeneic MLR was observed. It has been published that leflunomide's antiproliferative effect on lymphocytes is a consequence of dihydroorotate dehydrogenase inhibition, the enzyme that is necessary for pyrimidine biosynthesis. In our pilot study (unpublished results), the addition of uridine (100 μM) could completely reconstitute the proliferation of lymphocytes in two-way MLR and allogeneic ML, when leflunomide is used in concentrations less that 100 μM. Interestingly, supplementation of uridine in the MDDC cultures in the presence of

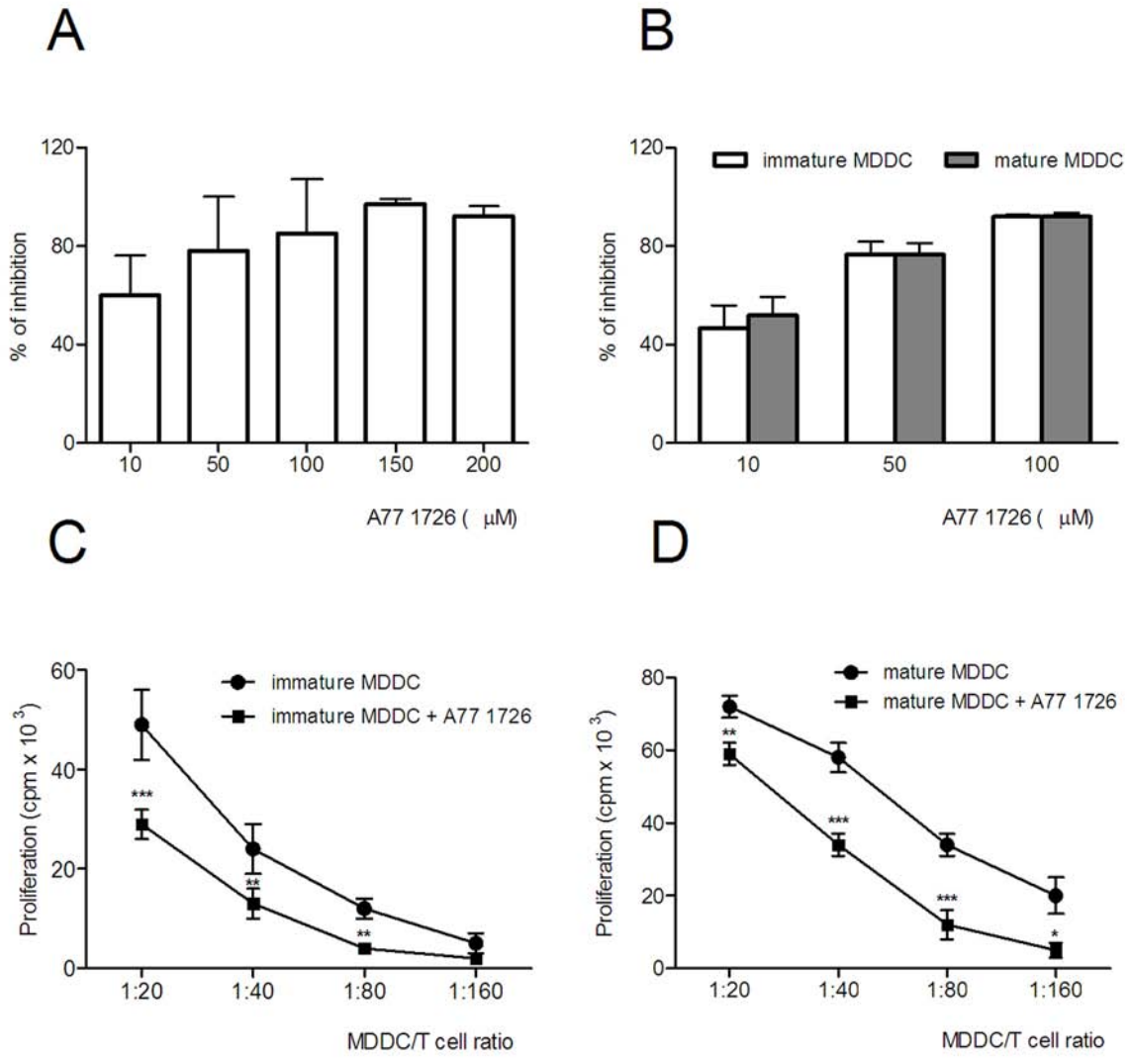

Fig. 3. A77 1726 inhibits lymphocytes proliferation induced by different stimulators.
(A, B) Purified T cells (2×10^5/well) were cultured in a 96-well plate with allogeneic lymphocytes (2×10^5/well), immature MDDC (2, 5×10^3/well) or mature MDDC (2,5 $\times 10^3$/well) in the absence or presence of A77 1726 (10-200 μM). After 5 days of culture, to assess cell proliferation, cells were pulsed with [3H]-thymidine for last 18 h and the incorporation of the radionuclide into DNA was further measured by β-scintillation counting. Data are expressed as the percentage of proliferation inhibition in the presence of A77 1726 compared to corresponding control cultures without drug. The data are from one representative experiment out of three performed. (C, D) Immature and mature MDDC were obtained as described in Materials and methods, in the presence or absence of A77 1726 (100 μM). MDDC were extensively washed and co-cultivated with 2×10^5 allogeneic T cells at different MDDC/T cell ratio. Cell proliferation was measured after 5 days using [3H]-thymidine uptake assay. Results are presented as the mean cpm ± SD of triplicate cultures. One representative of four experiments is shown.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to control, immature or mature MDDC.

A77 1726 (100 μM) could not restore the MDDC allostimulatory capacity to the control level. Furthermore, the addition of uridine (50 μM) did not rescue DC differentiation and maturation, judging by obtained phenotype, from the effects of A77 1726, indicating that the inhibition of DHOD did not underlie the observed effects (Kirschet et al., 2005). Earlier studies performed on lymphocytes and some cell lines have established that leflunomide, in concentrations of 100 μM and higher, could also act as a tyrosine kinase inhibitor (Mattar et al., 1993; Xu et al., 1995; Xu et al., 1996; Elder et al., 1997). The epidermal growth factor receptor tyrosine kinase, lck, fyn and Janus kinase (JAK) 1 and JAK3 were first identified as the targets of A77 1726 inhibition. Siemasko et al. showed that leflunomide diminished the tyrosine phosphorylation of JAK3 and STAT6 in the absence or presence of uridine, and blocked IgG1 production (Siemasko et al., 1998). Since, it is difficult from our work to identify the mechanisms by which leflunomide inhibits the differentiation and maturation of MDDC, based on the used concentration of A77 1726 and the effect of added uridine, we could assume that its inhibitory action on tyrosine kinase is involved. Indeed, the altered differentiation of the MDDC could be the result of the inhibitory effect of leflunomide on JAK, a tyrosine kinase in the first step of the signal transduction pathway activated by numerous cytokines, especially IL-4 and GM-CSF (Lutz et al., 2002). Furthermore, leflunomide could inhibit the NF-kB activation (Kirsch et al., 2005) caused by GM-CSF (Ammon et al., 2000; Ebner et al., 2003), LPS (Rescigno et al., 1998) and TNF-α (Manna et al., 2000) and prevent, in the first place, MDDC maturation and to less degree differentiation.

Final functional maturation of MDDC is followed by the production of numerous cytokines. Depending of origin and type, as well as maturation stimulus, DC can produce TNF-α, IL-6, IL10, IL-12, IL-15, IL-18 and IFN-γ (Verhasselt et al., 1997). In this work, LPS-stimulated MDDC produced significantly higher levels of TNF-α, IL-12, IL-10 and IL-18 than the immature one. TNF-α induces the differentiation and maturation of DC, the production of IL-10 by activated monocytes and together with PGE2, the secretion of IL-12 by human DC (Reiser et al., 1997; Foey et al., 1998). IL-12 is a potent pro-inflammatory cytokine that plays a central role in the initiation and control of cell-mediated immunity as an inducer of Th1 response. IL-12-driven Th1 immune response is enhanced by IL-18 through the induction of IFN-γ (Nakanishi, 2002). In contrast, IL-10 inhibits Th1 development, promotes Th2 response or tolerance, and exerts negative regulatory roles for a wide variety of immune cells (Seewaldt et al., 2002).

On the one hand, we showed that exposure of MDDC to A77 1726 during maturation had no influence on the production of TNF-α and IL-12. These *in vitro* data did not correlate with the published results of other authors suggesting the inhibitory effect of leflunomide on TNF-α production by different cell type such as T cells (Deage et al., 1998), macrophages (Elkayam et al., 2003), MDDC (Kirsch et al., 2005) or IL-12 production by MDDC (Kirsch et al., 2005). The observed differences could, at least, be a consequence of different cultivation conditions, concentration of leflunomide metabolite and maturation stimulus or, finally, of the assays used for cytokine detection.

On the other hand, the reduced production of IL-10 and IL-18 that has been measured is in accordance with findings showing a decreased IL-10 levels *in vivo* and *in vitro* after leflunomide treatment (Kirsch et al., 2005; Litinsky et al., 2006). Other immunosuppressive agents, like cyclosporine A, tacrolimus and sirolimus to less degree suppressed or had no major effect on IL-12 and IL-10 production, with the exception of sirolimus and MMF, which inhibited IL-10 secretion like leflunomide (Woltman et al., 2000; Woltman et al., 2001; Čolić et al., 2003).

Taken together, our data show that leflunomide, in addition to its direct inhibitory action on T cells, inhibits the differentiation, maturation and function of human MDDC. These findings provide new information on the immunosuppressive/immunomodulatory function of leflunomide and could in future

contribute to the generation of new strategies for the therapeutic control of immunopathological reactions with an excessive immune response.

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