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Mycophenolate mofetil inhibits differentiation, maturation and allostimulatory function of human monocyte-derived dendritic cells

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SUMMARY

We have studied the effect of mycophenolate mofetil (MMF), a new drug used in prevention of transplant rejection, on differentiation, maturation and allostimulatory activity of human monocyte-derived dendritic cells (MDDC). MDDC were generated *in vitro* with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 in the presence or absence of MMF. MMF reduced the number of immature MDDC in culture, dose-dependently, by inducing apoptosis and inhibited their stimulatory activity on allogeneic lymphocytes. These changes correlated with down-regulation of co-stimulatory and adhesion molecules such as CD40, CD54, CD80 and CD86. No differences were observed in mannose receptor (MR)-mediated endocytosis, measured by the uptake of fluorescein isothiocyanate (FITC)-dextran. MDDC differentiated in the presence of MMF showed significantly reduced maturation upon stimulation with lipopolysaccharide, as judged by lower expresson of CD83 and co-stimulatory molecules, lower production of tumour necrosis factor (TNF)-α, IL-10, IL-12 and IL-18 as well as lower stimulation of alloreactive T cells including naive $CD4^+$ $CD45RA^+$ T cells. In contrast, MDDC matured in the presence of MMF showed a more marked decrease in the FITC-dextran uptake than mature MDDC cultivated without MMF and the phenomenon correlated with down-regulation of the MR expression. These results suggest that MMF impairs differentiation, maturation and function of human MDDC *in vitro*, which is an additional mechanism of its immunosuppressive effect.

Keywords alloreactivity cytokine production endocytosis human dendritic cells mycophenolate mofetil phenotype

INTRODUCTION

Mycophenolate mofetil (MMF), a new immunosuppressive drug [1–3], has been used in different models of allogeneic transplantation [4–8], autoimmune skin disorders [9,10] and rheumatoid arthritis [11]. In human medicine, MMF is licensed for the prevention of renal transplant rejection [1,2].

MMF is a morpholinoethylester of mycophenolic acid (MPA), its active metabolite [2]. The compound has been shown to inhibit inosine 5?-monophosphate dehydrogenase (IMPDH), a key enzyme in the purine synthesis pathway of lymphocytes [12,13]. Because T and B lymphocytes rely solely on this pathway for the production of guanosine nucleotides, the proliferation of these cells is specifically inhibited by MMF [14,15]. The drug not only blocks lymphocyte proliferation, but also prevents humoral responses to antigens and mitogens [16,17] and some other functions of lymphocytes [18,19].

The induction of immune response following allotransplantation involves a complex interaction between antigen-presenting cells (APC) and T lymphocytes [20]. Among APC, dendritic cells (DC) play a central role in antigen presentation and stimulation of naive T cells. Upon encountering an antigen at a peripheral site, such as transplanted tissue, immature DC migrate to the draining lymph node where they mature. The process includes up-regulation of co-stimulatory molecules, reduction of endocytosis, processing of antigenic peptides and their presentation to T lymphocytes. The contact between mature DC and specific T cells leads to proliferation and differentiation of T cells to Th1 effector cells [21,22].

Recent experiments in mice showed that MMF impairs maturation and function of DC [23]. In addition, MMF together with 1α, 25-dihydroxyvitamin D3 induces in mice differentiation of DC with a tolerogenic phenotype [24]. Similar experiments have not been performed in humans. In this work we showed that MMF, at therapeutic concentrations achieved *in vivo* (10 μM) [25],

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significantly inhibited differentiation, maturation, cytokine production, endocytic and allostimulatory activity of human monocyte-derived DC (MDDC) *in vitro*.

MATERIALS AND METHODS

Cytokines and reagents

Human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) (Leucomax, spec. activity $4{\cdot}44 \times 10^6$ IU) was obtained from Sandoz-Schering Plough, Basle, Switzerland. Human recombinant IL-4 was from Roche Diagnostics GmbH, Mannheim, Germany. MMF (Cellcept®) was from F. Hoffman-La Roche SA, Basle, Switzerland). Stock solutions were prepared by dissolving the drug in dimethylsulphoxide (DMSO) and then in RPMI-1640 serum free medium (ICN, Costa Mesa, CA, USA). Aliquots were kept frozen at –20°C. Lipopolysaccharide (LPS) and propidium iodide (PI) were from Sigma, Munich, Germany.

DC cultures

Highly enriched monocytes (about 75–85% puritiy) were obtained and purified from buffy coats of healthy volunteers by lymphoprep, gradient (Nycomed, Oslo, Norway) and plastic adherence, as described previously [26]. Experiments were performed according to the guidelines approved by the Ethical Committee of MMA, Belgrade and with the informed consent of the subjects involved. In certain experiments monocytes were purified from peripheral blood mononuclear cells (PBMNC) using a monocyte separation kit by MACS technology (Myltenyi Biotec, Bergish, Gladbach, Germany), following the manufacturer's instructions. In brief, PBMNC were incubated with a cocktail of hapten-coupled monoclonal antibodies (MoAbs) to CD3, CD7, CD19, CD45RA and CD56, followed by antihapten MoAb coupled with super-paramagnetic MACS MicroBeads. Magnetically labelled lymphocytes and natural killer (NK) cells were removed and the negative fraction was collected. The purity of $CD14^+$ cells in the negative fraction was 92–96%. Monocytes were usually cultivated for 6–7 days in four-well tissue culture plates (Flow, Irvine, Scotland, UK) in 4 ml of complete RPMI-1640, HEPES/sodium bicarbonate buffered medium with the addition of 10% heat-inactivated fetal calf serum (FCS) (ICN), streptomycin, gentamycin, penicillin and 2-ME (Sigma). The medium was supplemented with 100 ng/ml of GM-CSF and 5 ng/ml of interleukin (IL)-4. After 3 days, cultures were fed with 3 ml of fresh medium with GM-CSF and IL-4. After 6 or 7 days non-adherent cells (predominantly immature MDDC), were transferred to new plates and half the medium was replaced with GM-CSF and IL-4 alone or GM-CSF and IL-4 with 1 μg/ml LPS. Cells were cultivated for an additional 2 days. In cultures with MMF, the compound was added at different concentrations at the beginning of monocyte cultivation and at the time of medium replacement.

Flow cytometry

Non-adherent MDDC, cultivated with or without MMF, were collected, washed in phosphate buffered saline (PBS) with 0·1% sodium azide and 2% FCS (PBS/FCS) and adjusted at concentrations of 2×10^5 cells/tube. Cells were stained in suspension using appropriate dilutions of the following MoAbs to: HLA-DR (Becton-Dickinson), CD14, CD40, CD54, CD80, CD83 and CD86 (Serotec) and mannose receptor (MR) (TNO, Rijswik, the Netherlands). CD14 and CD80 were conjugated with fluorescein isothiocyanate (FITC), CD86 was coupled with PE, whereas other MoAbs were unconjugated. After washing in PBS/FCS antimouse (Fab-2) Ig-FITC antibody (Serotec) with 5% normal human serum was added to cells, incubated previously with unconjugated MoAbs. Controls consisted of samples with irrelevant mouse MoAbs reactive with rat antigens. After washing, cells were analysed on a flow cytometer, EPICS XL-MCL (Coulter, Krefeld, Germany). At least 5000 events per sample were analysed.

Endocytosis assay

MR-mediated endocytosis was measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. Cells (2×10^5 per sample) were incubated in RPMI-1640 medium with the addition of FITC-dextran (1 mg/ml; mol. mass 40·000; Sigma) for 60 min at 37°C or at +4°C (for control binding). After incubation, MDDC were washed twice with PBS and fixed with 1% formaldehide. The uptake of FITC-dextran by MDDC, cultivated previously under different condtions, was determined by flow cytometry. At least 5000 cells per sample were analysed.

Cytokine assays

After 6 days MDDC, cultivated with or without MMF, were replated (6×10^5 cells/ml) in medium with GM-CSF/IL-4 and LPS or GM-CSF/IL-4, LPS and MMF. Cells were cultivated for an additional 2 days and supernatants were collected. TNF-α was determined using the L929 cytotoxic assay as described [27], whereas IL-10, IL-12 and IL-18 were determined using a sandwich ELISA assay, following the manufacturer's instructions. IL-10 and IL-12 ELISA kits were from R&D Systems (Minneapolis, USA) and IL-18 was from MBL (Nagoya, Japan).

Allogeneic mixed leukocyte reaction (MLR)

PBMNC were isolated from buffy coats using Lymphoprep gradient. T cells were purified from PBMNC using immunomagnetic sorting with pan-T cell or $CD4^+$ isolation kits (MACS, Myltenyi Biotec), following the manufacturer's instructions. $CD4^+$ $CD45RA^+$ T cells were isolated by addition of anti-CD45RO antibody (Serotec) to $CD4^+$ cells followed by anti-mouse Ig microbeads and subsequent depletion of positive cells. The purity of T cells or T cell subsets recovered in negative fractions was higher than 95% as checked by anti-CD3 FITC, anti-CD4 FITC and anti-CD45RA PE MoAbs (all from Serotec) and flow cytometry.

Purified T cells, $CD4^+$ T cells or $CD4^+$ $CD45RA^+$ T cells (2×10^5 cells/well) were cultivated with different numbers of allogeneic MDDC in complete RPMI medium + 10% FCS in 96-well, flat-bottomed cell culture plates for 5 days. Cells were pulsed with [^{3}H]-thymidine (1 μCI/well, Amersham, Bucks, UK) for the last 18 h. Labelled cells were harvested onto glass fibre filters. Radioactivity was counted in a Beckman scintilation counter and expressed as counts per minute (cpm) ± s.d. of triplicates.

Apoptosis assay

In MDDC cultures, using monocytes purifed by immunomagnetic sorting, non-adherent and loosely adherent cells were collected after 3 and 5 days of cultivation and their numbers were counted. Apoptosis was determined as described previously [28]. Cells were centrifuged and the pellets were gently resuspended in 1·5 ml hypotonic PI solution (50 μg/ml PI in 0·1% sodium citrate plus 0·1% Triton X-100, Sigma). Cells were left in the dark at

room temperature for 30 min and then analysed. The PI fluorescence of individual nuclei was measured by flow cytometry. The percentage of apoptotic cells (subdiploid DNA peak in the DNA fluorescence histogram) was determined.

RESULTS

MMF impairs differentiation of MDDC

We studied first the effect of MMF on differentiation of MDDC. Human monocytes, purifed from PBMNC by immunomagnetic sorting, were cultivated with different concentrations of MMF. At day 3 most cells became non-adherent, displayed short cytoplasmic protrusions and down-regulated CD14, whereas 10–20% cells were loosely adherent. The appearance of cultures was similar at day 5, but most cells were $CD14^-$ (Table 1).

MMF decreased survival of MDDC dose-dependently, and the finding correlated with increased apoptosis. At day 3, MMF slightly prevented down-regulation of CD14, whereas after 5 days the differences in CD14 expression between MMF-treated and control cells were not statistically significant. (Table 1).

Phenotypic characteristics of MMF-treated MDDC

Based on previous findings, we chose the 10 μM dose of MMF for further experiments and for phenotypic characterization of MDDC. As shown in Fig. 1, MDDC generated in control cultures with GM-CSF and IL-4 for 8 days expressed the phenotype of immature DC, such as high levels of MHC class II, moderate levels of CD40, CD54 and CD86 and a low level of CD80. The cells were mainly $CD83^-$. MDDC developed in the presence of MMF showed reduced expression of all the molecules examined, except MHC class II.

MDDC have been induced to mature by LPS. As presented in Fig. 1, maturation of these cells was followed by up-regulation of all molecules examined and by the expression of CD83. MMF significantly inhibited maturation of MDDC as manifested by lower expression of CD54, CD80, CD83 and CD86. No significant effect was seen regarding the expression of MHC class II molecules and CD40.

MMF inhibited allostimulatory function of MDDC

We examined further whether the observed effect in down-regulation of co-stimulatory and adhesion/signalling molecules by MDDC treated with MMF correlated with their allostimulatory function. Figure 2 shows that MMF-treated immature MDDC caused less stimulation of both allogeneic T lymphocytes as well as naive $CD4^+$ $CD45RA^+$ T cells, compared to MDDC generated without MMF.

As expected, mature MDDC were more potent stimulators of allogeneic T cells than immature MDDC. MDDC matured in the presence of MMF and LPS had lower allostimulatory activity for $CD3^+$ T cells and $CD4^+$ $CD45RA^+$ T cells than MDDC matured in the presence of LPS alone (Fig. 2). Similar results were achieved using purified total allogeneic $CD4^+$ T cells as responders (not shown).

Effect of MMF on cytokine production by mature MDDC

We also studied production of TNF-α, IL-12, IL-10 and IL-18 by MDDC induced to mature by LPS. Culture supernatants of six different donors were analysed. All cytokines were detected, but considerable variations in their levels were observed. In cultures with MMF and LPS, production of IL-18 was almost completely inhibited, whereas the levels of IL-10, TNF-α and IL-12 were reduced by about 87%, 80% and 71%, respectively (Table 2).

Effect of MMF on endocytic activity of MDDC

It is known that immature DC posses stronger endocytic activity than mature DC. This is confirmed in our experiments using the FITC-dextran uptake, an MR-mediated endocytosis assay. MMF did not significantly modulate the FITC-dextran uptake by immature MDDC, as presented in Fig. 3a, or slightly decreased endoc-

Table 1. Effect of MMF on survival, apoptosis and CD14 expression by human MDDC

Cultures (days)	MMF (μM)	Survival (%)	Apoptosis (%)	CD14 expression (%)
0	None	100%	2·3 ± 0·6	93·2 ± 2·7
3	None	93·7 ± 4·3	3·7 ± 1·3	34·2 ± 7·9
	2·5	89·3 ± 6·9	5·0 ± 1·8	46·2 ± 5·1*
	5	89·6 ± 4·8	5·7 ± 1·5	50·0 ± 7·3*
	10	80·2 ± 5·5**	8·5 ± 3·1*	51·7 ± 9·5*
	25	65·7 ± 5·6**	13·5 ± 3·3**	52·5 ± 9·9*
5	None	88·2 ± 6·4	5·0 ± 1·4	9·7 ± 2·9
	2·5	82·2 ± 8·5	6·7 ± 1·2	9·7 ± 4·6
	5	77·7 ± 5·3*	6·5 ± 1·7	7·7 ± 1·8
	10	75·3 ± 5·0*	12·0 ± 1·4**	11·5 ± 3·4
	25	53·1 ± 6·4***	19·5 ± 4·0**	11·2 ± 3·3

Monocytes, isolated from PBMNC by immunomagnetic sorting (contaminating lymphocytes were less than 2%) were cultivated in 24-well plates (1×10^6 cells/well in 1 ml of medium) in control GM-CSF/IL-4 medium or GM-CSF/IL-4 medium with different concentrations of MMF. After 3 and 5 days, non-adherent and loosely adherent cells, easily detached by pipetting, were collected and counted. Cells were stained with propidium-iodide as described [28] or with anti-CD14 MoAb followed by a secondary FITC-antibody and analysed by flow cytometry. Apoptotic cells were regarded those with hypodiploid nuclei. Survival of cells (%) was determined on the basis of starting number of monocytes used as 100%. *$P < 0·05$; **$P < 0·01$; ***$P < 0·005$ ($n = 4$) compared to corresponding cultures without MMF.

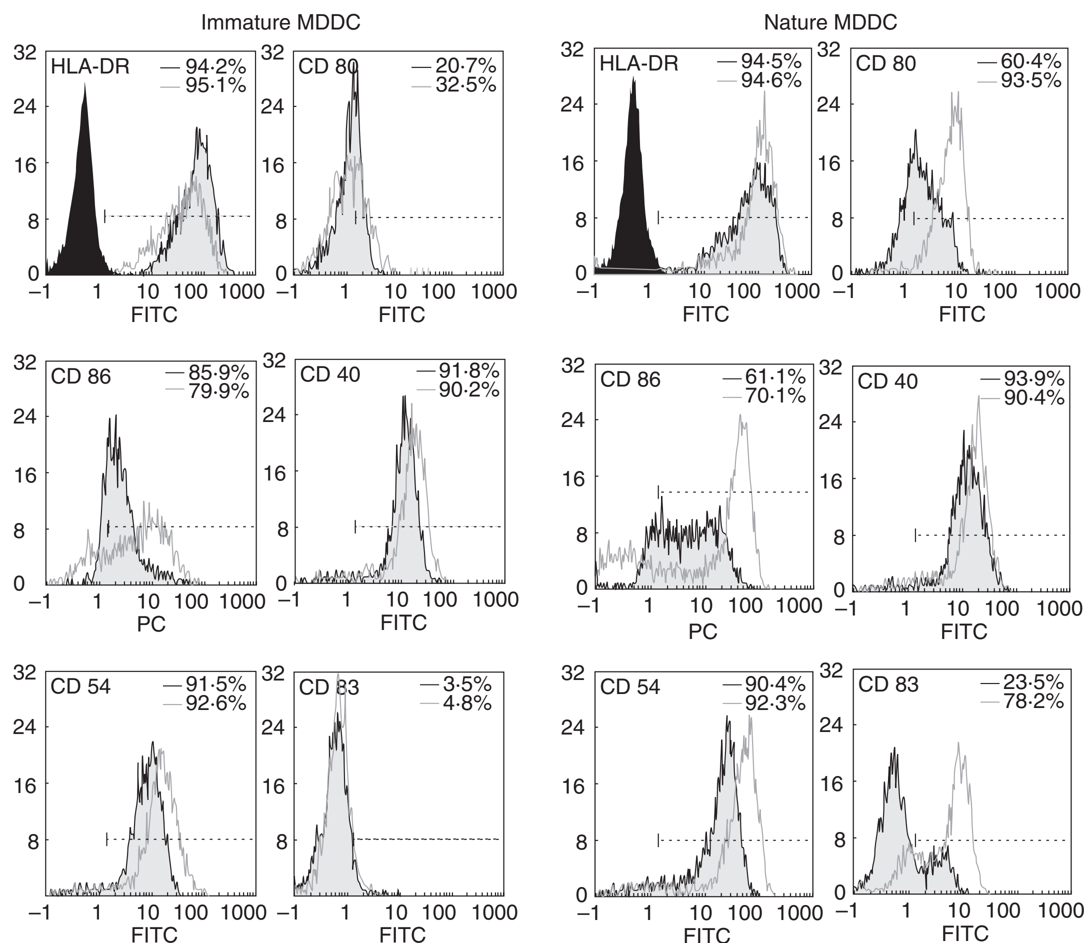

Fig. 1. Effect of MMF on phenotypic characteristc of MDDC. Immature MDDC were generated by cultivating monocytes (isolated by adherence to plastic) with GM-CSF and IL-4 for 8 days, whereas mature DC were prepared by subsequent cultivation of immature DC from day 6 to day 8 with LPS. MMF (10 μM) was present from the beginning of monocyte cultivation. MDDC were peleted and stained in suspension using different MoAbs (CD80-FITC, CD86-PE, HLA-DR, CD40, CD54 and CD83 unconjugated, followed by antimouse, Ig-FITC) and analysed by flow cytometry. Results are presented as histograms of fluorescence and percentages of positive cells. Grey histograms represent fluorescence of MDDC cultivated with MMF, whereas white histograms show fluorescence of control MDDC cultivated without MMF. Horizontal bars were positioned on the basis of non-specific fluorescence (cells stained with irrelevant mouse MoAbs). Negative controls (black histograms) are presented on HLA-DR histograms. Similar profiles were obtained using other controls (not shown). The results shown here are representative of three independent experiments.

Table 2. Effect of MMF on cytokine production by MDDC matured in the presence of LPS

	Levels (pg/ml)	
Cytokine	Control	MMF
TNF-α	8885·3 ± 4759·0	1808·7 ± 1815·4**
IL-10	1216·8 ± 598·6	152·9 ± 177·5***
IL-12	63·5 ± 54·3	18·3 ± 26·2*
IL-18	129·3 ± 109·9	0·4 ± 1·1**

MDDC generated in the presence of 10 μM MMF or without MMF (control) were induced to mature at day 6 by LPS (1 μg/ml) for an additional 2 days. In cultures with MMF the compound (10 μM) was added together with LPS. The levels of cytokines were determined as described in Materials and methods. Results are presented as mean ± SD ($n = 6$). *$P < 0·05$; **$P < 0·01$; ***$P < 0·005$ compared to values in the control.

ytosis in some cultures (data not shown). However, MMF additionally lowered endocytic activity of mature MDDC and the finding correlated with down-regulation of the MR expression (Fig. 3b).

DISCUSSION

MMF has been shown to be a very potent immunosuppressive drug [3]. It reduces the likelihood of allogeneic rejection, acting as a selective inhibitor of lymphocyte proliferation [18]. In this study we demonstrated that MMF affects human MDDC *in vitro*. To our knowledge, this is the first report showing the direct inhibitory effect of MMF on human DC.

Our work has been initiated by the recent data in mice which showed that MMF impairs maturation and function of bone marrow-derived DC *in vitro* [23]. In addition, MMF in combination with 1α, 25-dihydroxyvitamin D3, by acting on DC, induces tolerance to fully mismatched islet allografts *in vivo* [24]. In most of

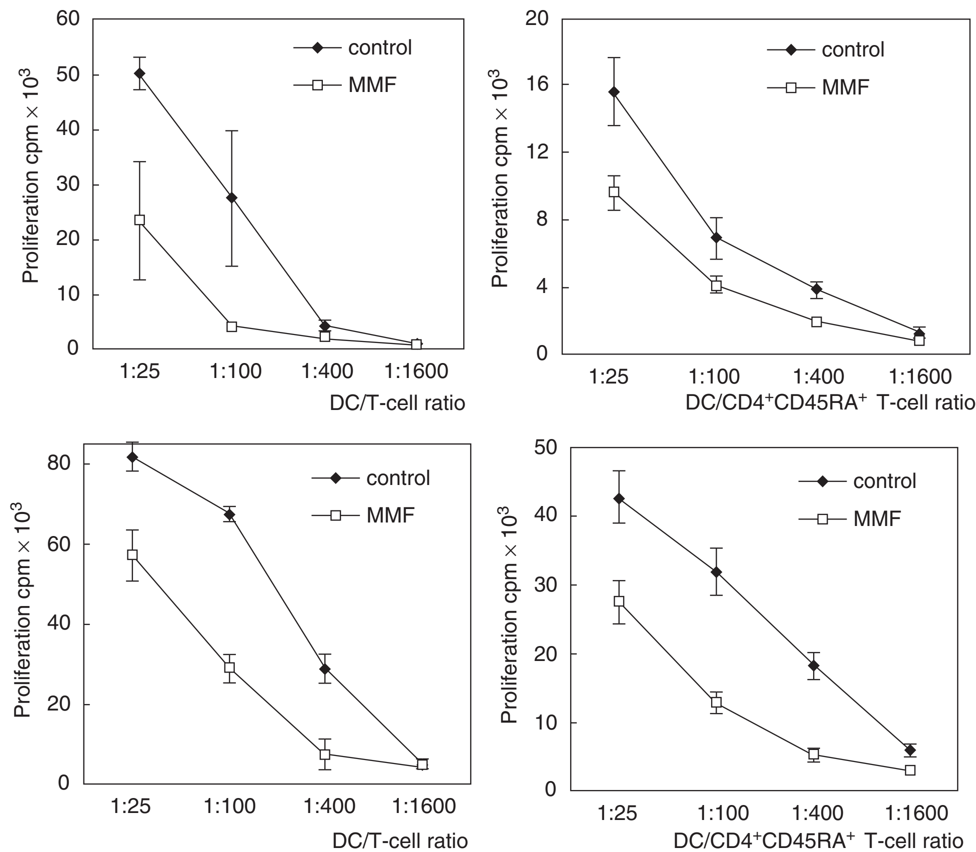

Fig. 2. Effect of MMF on the allostimulatory capacity of MDDC. Immature (a, c) and mature (b, d) MDDC were generated as described in Fig. 1. MMF (10 μM) was added at the beginning of monocyte cultivation and was present during the whole period. MDDC were washed three times and co-cultivated with allogeneic ($CD3^+$) T cells (a, b) or $CD4^+$ $CD45RA^+$ T cells (c, d) (prepared by negative immunomagnetic sorting, as described in Materials and methods) in 96-well plates, both at concentrations of 2×10^5 cells/well at different DC/T cell ratio. Cell proliferation was measured after 5 days using the [^{3}H]-thymidine uptake assay, as described previously. Results are presented as mean cpm ± s.d. (triplicates of one representative experiment). Similar results were obtained in additional four (T cells) and two experiments ($CD4^+$ $CD45RA^+$ T cells), respectively.

our experiments we used MMF at concentrations of 10 μM. The dose slightly reduced the number of DC in culture by inducing apoptosis, but caused significant phenotypic and functional changes of MDDC. It has been shown that in patients on standard MMF therapy plasmatic concentrations of the active metabolite of the drug reached levels of about 10 μM [25].

We observed the following phenotypic changes of MDDC in the presence of MMF: down-regulation of CD86, CD40 and CD54 on immature MDDC and CD80, CD86, ICAM-1 and CD83 on mature MDDC. As a consequence of these changes, lower allostimulatory activity of both mature and immature DC in MLR was observed. The significance of co-stimulatory molecules, CD40 and CD54, in stimulation and proliferation of naive T cells is well documented [22]. Mehling *et al*. [23] showed similar phenotypic and functional changes of murine DC, including lower production of IL-12, cultivated in the presence of 10 times lower concentrations of MMF than we used. These differences probably reflect species differences in reactivity of mouse and human DC to the drug.

It is known that LPS induces production of TNF-α, IL-10, IL-12 and IL-18 by MDDC [29]. All these cytokines were detected in our culture supernatants in the presence of LPS. TNF-α was detected in culture supernatants of immature MDDC, whereas levels of other cytokines were undetectable or very low (data not shown). TNF-α stimulated maturation of DC [30] induced production of IL-10 by activated monocytes [31] and together with prostaglandin E_2 stimulated production of IL-12 by human DC [30]. IL-12 is produced most abundantly by DC that are beginning to respond to maturation stimuli [32] and IL-12-driven Th1 immune response is enhanced significantly by IL-18 through the induction of INF-γ [33]. In contrast, IL-10 inhibits mainly the production of IL-12 and therefore promotes either Th2 response or tolerance [32,34].

We showed that MMF almost completely (IL-18) or significantly (TNF-α, IL-10 and IL-12) inhibited production of cytokines by MDDC stimulated with LPS. Except for decreased production of IL-12 [23,24], secretion of other cytokines by DC have not been examined directly. Durez *et al*. [35] showed that MMF inhibited production of TNF-α in mice treated with LPS, whereas the level of IL-10 was increased. Maksimivic-Ivanic *et al*. [36] also demonstrated an increase in the IL-10 level after MMF treatment, using a model of diabetes in rats. In other mouse models the IL-10 gene expression in spleen cells was not modulated significantly by MMF [37].

Decreased production of IL-18 and IL-12 by MDDC treated with MMF in our culture system suggests an additional mechanism for attenuation of transplant rejection by suppressing the

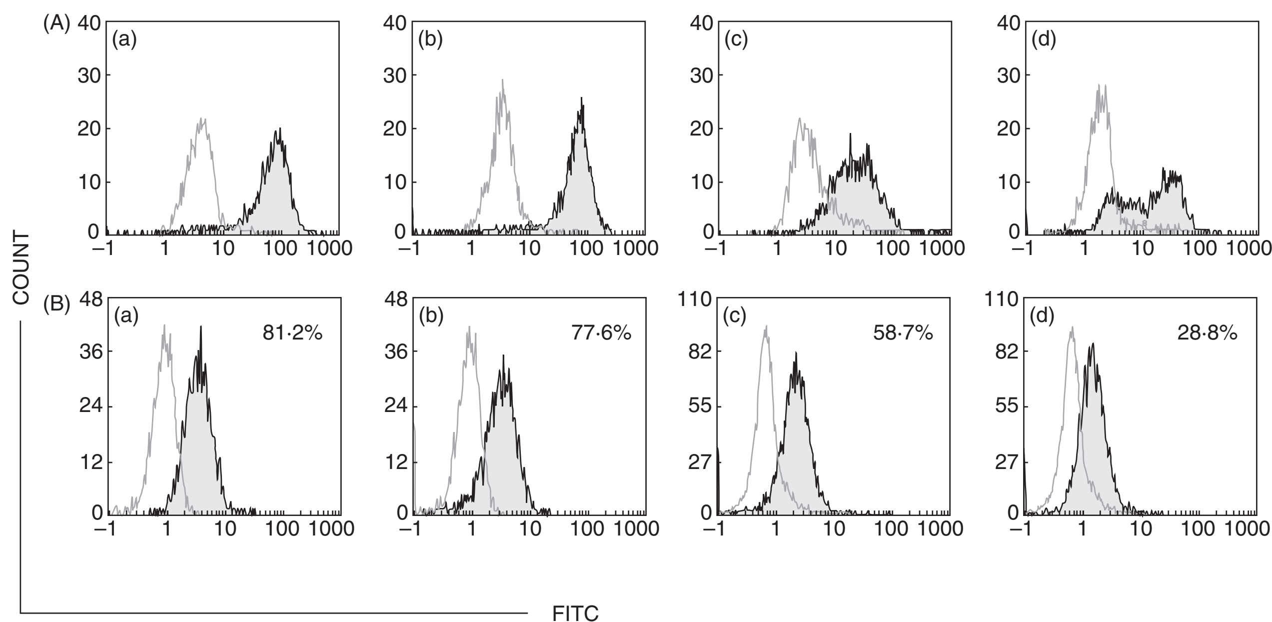

Fig. 3. Effect of MMF on endocytic activity (A) and MR receptor expression by MDDC (B). Immature MDDC (a, b) were generated by cultivating monocytes (isolated by adherence to plastic) with GM-CSF and IL-4 for 6 days. Mature MDDC (c, d) were obtained by subsequent cultivation of immature MDDC for an additional 2 days. MMF (10 μM) was present during the whole cultivation period of 8 days. (A) Cells were incubated with dextran-FITC (1 mg/ml) at + 4°C (white histograms) or 37°C (grey histograms) for 1 h. After washing cells were analysed by flow cytometry. Note lower endocytic activity of mature MDDC (c) compared to immature MDDC (a) and reduction of the dextran-FITC uptake by MDDC cultivated in the presence of LPS and MMF (d) compared to the fluorescence of MDDC, matured without MMF (c). (B) Cells were stained with anti-MR MoAb followed by antimouse Ig-FITC (grey histograms) or with a control MoAb (white histograms), as described. Results are presented as histograms of fluorescence and the percentages of positive cells from one representative experiment. Note lower expression of MR by MDDC matured in the presence of LPS (c) compared to immature MDDC (a) and a significant down-regulation of the MR expression by MDDC cultivated in the presence of LPS and MMF (d).

Th1 immune response [38]. It is known that insufficient amounts of IL-12 and CD80 expression by APC during antigen presentation have been implicated in anergy and tolerance, both of which render T cells functionally unresponsive [39].

We showed that MMF did not modulate significantly the MR-mediated endocytosis (FITC-dextran uptake) by immature MDDC. However, a significant reduction in endocytosis was observed by MDDC cultivated in the presence of MMF + LPS compared to MDDC cultivated with LPS alone, and the finding correlated with down-regulation of the MR expression. Similar experiments have not been performed until now. It is known that mature DC reduced endocytic activity [20,21]. Based on the phenotype profile of MMF-treated MDDC, induced to mature by LPS, an opposite effect could be expected. Piemonti *et al*. [40] showed that glucocorticoids affect the differentiation of MDDC by freezing the cells at an immature stage, and the effect was followed by an increase in MR-mediated endocytosis. Decreased endocytosis of MMF-treated DC could be associated with the direct action of MMF on MR. The hypothesis is based on previous observations that some anti-inflammatory effects of MMF, such as reduced recruitment of leucocytes to sites of inflammation, are connected with the inhibition of mannosylation of human monocyte surface glycoproteins, including adhesion molecules [41].

An intriguing question resulting from our work and the previous study [23] is whether the observed action of MMF on differentiation and maturation of DC is mediated through blocking of IMPDH isoforms and subsequent synthesis of guanosine. Our recent experiments in rats [42] and human (manuscript in preparation) showed that certain guanosine analogues stimulate maturation of DC and significantly abrogated the inhibitory effect of MMF on DC differentiation. These data suggest that not only lymphocytes, but also DC, may depend on the *de novo* synthesis of guanosine.

In conclusion, our results support the concept that MMF impairs not only the function of T and B cells, but also DC. Both T cells and DC are of key importance for alloreactivity and transplant tolerance. The results that MMF inhibits production of IL-10, IL-12 and IL-18 by MDDC open further studies regarding the influence of MMF on polarization of the immune response. Some of these experiments are currently in progress in our laboratory.

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