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*J Immunol* 2000; 165:2374-2381; doi: 10.4049/jimmunol.165.5.2374
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Mycophenolate Mofetil Impairs the Maturation and Function of Murine Dendritic Cells

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The immunosuppressive drug, mycophenolate mofetil (MMF), has been successfully introduced in allogeneic transplantation medicine and, more recently, in the treatment of autoimmune skin disorders. MMF inhibits lymphocyte proliferation via a blockade of the enzyme inosine 5'-monophosphate dehydrogenase, an enzyme on which lymphocytes solely depend to generate the purines necessary for DNA/RNA synthesis. To investigate the effects of MMF on cutaneous immune responses, a murine model of contact hypersensitivity (CHS) was used, with oxazolone or trinitrochlorobenzene as a contact allergen. Compared with the respective vehicle, i.p. applied MMF significantly inhibited the elicitation and, surprisingly, the induction of CHS responses. This prompted further studies into the effects of MMF on Ag presentation. Bone marrow-derived dendritic cells (DC) were cultured with GM-CSF and IL-4 in the presence of MMF and were tested for their Ag-presenting capacity. Sensitization and elicitation of CHS and delayed-type hypersensitivity responses by s.c. injected haptenated DC were reduced upon preincubation of DC with MMF. CHS responses were not impaired upon resensitization, indicating that MMF does not induce hapten-specific immunotolerance. In addition, MMF decreased the ability of DC to stimulate allogeneic T cells in MLR assays. Accordingly, flow cytometric analyses revealed a dose-dependent reduction of the expression of CD40, CD80, CD86, I-A, and ICAM-1 on DC with a concurrent reduction of IL-12 production. These data suggest that MMF, in addition to affecting T lymphocytes, directly affects APC, resulting in an impairment of immune responses. They furthermore point to a possible role of inosine 5'-monophosphate dehydrogenase in the maturation of DC.

Received for publication August 4, 1999. Accepted for publication June 12, 2000.

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1 This work was supported by Grants BE 1580/2-2, Gr1022/3-3, SFB293/B1, and SFB 492/B2 from the German Research Association and a supportive grant from the Hoffmann-La Roche AG.

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3 Abbreviations used in this paper: MMF, mycophenolate mofetil; CHS, contact hypersensitivity; CM, complete medium; DC, dendritic cells; DNBS, dinitrobenzene sulfonic acid; DNFB, dinitrofluorobenzene; DTH, delayed-type hypersensitivity; EC, epidermal cells; IMPDH, inosine 5'-monophosphate dehydrogenase; LC, Langerhans cells; MELR, mixed epidermal cell-lymphocyte reaction; MnX, mean fluorescence intensity; MPA, mycophenolic acid; TNCB, trinitrochlorobenzene.

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to produce even higher quantities of IL-12, thus consigning T cells to Th1 responses (12). Recently, expression of Th1 cytokines, in particular IFN-γ, in a graft rejection model has been shown to be associated with acute graft rejection (13). In contrast, the predominant expression of Th2 cytokines, such as IL-4 and IL-10, was implicated in long term survival of the allograft.

In the present work we show that MMF inhibits allospecific and hapten-specific immune responses not only by repressing T cell proliferation but also by directly exerting its inhibitory effects on APC. A decrease in the ability of MMF-treated mice to induce or elicit contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) responses was shown to be dependent on the dose of administered MMF. MMF did not induce immunotolerance in this model, as mice not further treated with MMF after the initial challenge mounted CHS responses similar to vehicle-treated mice when re sensitized and rechallenged 2 wk later. The capacity of epidermal cells to stimulate allogeneic T cells was significantly decreased in mice chronically treated with MMF, although there was no difference in the number of I-A<sup>+</sup> cells found in epidermal ear sheets compared with that in control mice. Furthermore, flow cytometric analyses revealed a reduction of the expression of CD40, CD80, CD86, I-A, and ICAM-1 on bone marrow-derived DC treated with MMF. This was accompanied by a reduction of T cell stimulatory capacities in MLR, and a concurrent reduction of IL-12 production and a decrease in the capacity of DC to stimulate allogeneic T cells. These data suggest that MMF, in addition to affecting T lymphocytes, is able to suppress Ag presentation by attenuating functional maturation of DC, resulting in impaired immune responses. As IMPDH is the main target of the inhibitory effects of MMF, the results of this study also point to a possible role of IMPDH in DC development. Thus, this study elucidates another one of the underlying mechanisms resulting in the potent immunosuppressive effects of MMF in graft rejection and its beneficial effects on transplant maintenance.

**Materials and Methods**

**Mice**

Five- to 8-wk-old specific pathogen-free female BALB/c and C57BL/6 mice were obtained from Harlan-Winkelmann (Borchten, Germany) and housed according to institutional regulations.

**CHS and DTH responses**

CHS experiments were performed as previously described (14, 15). Briefly, mice were sensitized by painting 100 μl of 0.15% trinitrochlorobenzene (TNCB) or 2% oxazolone (Sigma, St. Louis, MO) in acetone/corn oil (4/1) onto the shaved back. For elicitation of CHS responses, 10 μl of 0.8% TNCB or 0.5% oxazolone, respectively, were painted on both sides of each ear 5 days after immunization. CHS was determined by the degree of ear swelling of the hapten-exposed ear compared to the ear thickness before challenge and was measured with a spring-loaded micrometer (Mitutoyo, Tokyo, Japan) 24 h after challenge. Mice that were ear challenged without prior sensitization served as negative controls. Groups of BALB/c mice (n = 5) were treated with 30 μg/g MMF i.p. in DMSO/PBS/MMF i.p. in DMSO/PBS/MMF i.p. in DMSO/PBS (1/10) 24, 12, and 1 h before immunization as well as 12 and 24 h after immunization. Another group received MMF 24, 12, and 1 h before challenge with oxazolone and 12 and 24 h after challenge. Control groups were treated accordingly with DMSO/PBS (1/10). In another experiment, mice were sensitized by s.c. injection of dinitrobenzene sulfonic acid (DNBS)-coupled DC and challenged with 12 μl of 0.3% dinitrofluorobenzene (DNFB) in acetone/corn oil (4/1) at the ear. DC were pulsed with DNBS by incubating DC with 1 mM DNBS in complete medium (CM; RPMI 1640 containing 10% FCS, 50 μg/ml gentamycin, 2 mM glutamine, 0.1 mM nonessential amino acids, and 20 μg/ml gentamycin (all from PAA, Linz, Austria)) at 37°C for 90 min. DC were washed extensively with PBS to remove any unincorporated hapten, and 4 × 10<sup>6</sup> DC (suspending in 200 μl PBS) were then injected s.c. into the lower abdomen. Control groups received nonpulsed DC.

DTH experiments were performed by sensitizing mice by epicutaneous application of 100 μl of 0.3% DNFB onto the shaved lower back. One week after sensitization, DTH was elicited by challenging mice by injection of 4 × 10<sup>6</sup> haptenated DC or LC-depleted epidermal cells (EC; in 100 μl of PBS) into one hind footpad. Control groups were sensitized or challenged with nonpulsed DC or LC-depleted EC. The intensity of the DTH reaction was assessed as the degree of footpad swelling of the injected site compared with that of the untreated contralateral footpad and was measured with a micrometer 24 h after challenge.

**Immunofluorescent staining of epidermal ear sheets**

Epidermal sheets were stained essentially as previously described (16). Briefly, ears were mechanically split into dorsal and ventral sides and incubated in 20 mM EDTA (pH 7.3) for 3.5 h at 37°C. Epidermal sheets were collected, washed with PBS, and fixed in acetone at −20°C for 20 min. After washing, sheets were incubated in 1% BSA/PBS for 1 h. Staining with the Ab anti-I-A (clone M5/114; Roche Diagnostics, Mannheim, Germany) was performed overnight at 4°C. After washing, sheets were incubated with FITC-conjugated goat anti-rat IgG (PharMingen, Hamburg, Germany) for 1 h, washed extensively, mounted onto slides, and examined using a Zeiss Axiovert microscope.

**Generation and culture of bone marrow-derived DC**

DC were generated as described by Inaba et al. (17) with some modifications. In brief, bone marrow cells were collected from murine tibias and femurs, suspended by vigorous pipetting, passed through a nylon mesh to remove debris, resuspended in CM, and cultured in tissue dishes for 2 h. Nonadherent cells were collected, and aliquots of 1 × 10<sup>6</sup> cells were placed in 24-well plates (Becton Dickinson, Heidelberg, Germany) containing 1 ml of CM supplemented with 150 U/ml GM-CSF (R&D Systems, Wiesbaden, Germany) and 75 U/ml IL-4 (PharMingen). After 2 days of incubation (37°C, 5% CO2), 600 μl of medium was removed, and the same volume of fresh CM containing 150 U/ml GM-CSF and 75 U/ml IL-4 was added. On days 5 and 7 of culture nonadherent cells were transferred into six-well plates containing CM supplemented with cytokines (3 × 10<sup>5</sup> cells/well). After a total of 8 days of incubation, most of the nonadherent cells in culture had acquired typical dendritic morphology. These cells were harvested and used as a source of DC in subsequent experiments. At this time point LPS (0.1 μg/ml; Sigma) was added to the cell cultures in some experiments for overnight incubation. Subsequently, supernatants from these cultures were collected and stored at −20°C until they were used for cytokine quantifications. Unless otherwise noted, cells were cul tured in the presence of MMF or the vehicle from day 1 on. MMF was added at every change of the culture medium.

**Flow cytometry of DC**

Expression of cell surface molecules was quantitated by flow cytometry as follows. Aliquots of 2 × 10<sup>5</sup> DC were incubated with mAb against BMA (BMA, Augst, Switzerland), CD11b (clone M1/70; PharMingen), CD19 (ID3; PharMingen), CD40 (HM40-3; American Type Culture Collection, Manassas, VA), NLDC145 (BMA), or rat IgG as an isotype control (Dianova, Hamburg, Germany) for 60 min on ice (1 μg/ml diluted in PBS/1.0% FCS (v/v)). The cells were washed with PBS/1.0% FCS (v/v) and labeled with FITC-conjugated goat anti-rat IgG or FITC-conjugated goat antihamster IgG (PharMingen or Diavon, respectively), diluted 1/50 in PBS/1.0% FCS (v/v) for 30 min on ice. At the end of this incubation cells were washed, propidium iodide (100 μM; Sigma) was added, and the cells were subsequently analyzed in an EPICS-XL flow cytometer (Coulter, Krefeld, Germany). Propidium iodide fluorescence was used to detect dead cells, which were then gated out.

**EC preparations**

EC were prepared as previously described (16) from truncal skins of mice. Subcutaneous fat and panniculus carnosus were removed, after which the skins were floated dermal side down on 0.4% trypsin in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS for 60 min at 37°C. Epidermal sheets were collected and dissociated by gentle stirring for 20 min. The resulting EC were filtered through nylon gauze and washed. I-A<sup>+</sup> cells were depleted by incubation on plates coated with anti-I-A mAbs (clone M5/114; Roche). Dead cells were removed by treatment with 0.05% trypsin and 80 μg of DNase/ml in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS for 5 min at room temperature. Aliquots were analyzed by flow cytometry before and after depletion.

**Mixed epidermal cell-lymphocyte reactions (MELR)**

Mice were treated with 30 μg/g/day MMF i.p. in DMSO/PBS (1/10) or vehicle alone for 30 days. The MELR was performed as described by
Grabbe et al. (16). Briefly, 2 × 10^5 nylon-wool-enriched C3H splenic T cells were cocultured with varying numbers of freshly prepared EC from MMF-treated and untreated BALB/c mice. EC were enriched for I-A^+ cells by depletion of Thy-1-bearing cells. EC were incubated in anti-Thy 1.2 mAb (clone NEL-001, Sigma) for 30 min on ice, followed by washing and subsequent incubation in low toxicity rabbit complement (Cedarlane Laboratories, Hornby, Canada) for 30 min at 37°C. Dead cells were removed by treatment with 0.05% trypsin and 80 μg of DNase/ml in Ca^2+/-Mg^2+-free PBS for 5 min at room temperature. Cells were cultured in RPMI 1640 medium supplemented with 1.5% mouse serum/ml, 5 μg of indomethacin/ml, and 50 μM 2-ME. Serial dilutions of triplicate samples of epidermal cells enriched for I-A^+ cells were mixed with a constant number (2 × 10^5) of allogenic T cells. Cells were cultured for 6 days at 37°C in round-bottom 96-well plates and then pulse-labeled with 1 μCi of [3H]thymidine/ml for 24 h. Subsequently, cells were harvested, and the incorporation of [3H]thymidine was measured.

MLR

DC were cultured in the continuous presence of various concentrations of MMF in CM, washed extensively with PBS, resuspended in MLR medium (RPMI containing 10% FCS, 100 μM HEPES, 1 mM pyruvate, 50 μM 2-ME, 2 mM glutamine, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin (all from PAA)), and applied in graded doses to 2 × 10^5 allogenic T cells in 96-well round-bottom plates. T cells were obtained from spleens of C3H/BLA mice by nylon-wool adherence and subsequent elimination of residual contaminating cells with Ab-coated T cell isolation columns (Cellect mouse T cell immunocolumns, Biotech, Alberta, Canada). The resulting cell preparation contained <0.1% I-A^+ cells. After 4 days, T cell proliferation was measured by adding 1 μCi of [3H]thymidine followed by an overnight incubation period and quantification of incorporated [3H]thymidine by subsequent liquid scintillation counting.

Detection of IL-12

IL-12 production by DC was determined by solid phase sandwich ELISA assays (Laboserv, Giessen, Germany) according to the manufacturer’s specifications. This kit allows the detection of natural IL-12 (p70) in addition to the free p40 subunit of IL-12 with a minimum detectable dose of <2 pg/ml. Supernatants from 10^5 DC/ml propagated in the presence of 0.1, 0.01, and 0.001 μM MMF or in DMSO were assayed after 8 days of culture and overnight stimulation with LPS (0.1 μg/ml; Sigma). Reombinant cytokines were used to generate standard curves, with concentrations of IL-12 determined using the linear portion of the curve. The background values were determined by replacing the recombinant cytokines with DMSO or tissue culture medium. All experiments were performed in triplicate.

Statistical evaluation

The significance of differences between the mean values obtained for CHS, DTH, and MLR experiments was assessed by two-tailed Student’s t test for unpaired data; p < 0.05 was considered significant.

Results

MMF impairs murine CHS and DTH responses, but does not cause induction of hapten-specific immunotolerance

As MMF has a direct influence on T cells, we were interested in investigating its effects on murine CHS responses. The effects of MMF on CHS responses to the hapten oxazolone were evaluated. Groups of BALB/c mice were treated with MMF at different time points before immunization with oxazolone as well as after immunization. The dosage of MMF used was ascertained by preliminary experiments based on the dosage in various animal models described previously (7, 18, 19). Multiple injections were performed, as MMF is rapidly converted to MPA and MPA has an apparent half-life of 17.9 h (7). Another group received MMF before and after challenge with oxazolone. Control groups were treated accordingly with the vehicle only. As a measure of CHS, ear swelling was assessed 24 h after challenging the ear skin. The results of a representative experiment are presented in Fig. 1A. In comparison to vehicle-treated control animals (immunized and challenged), the groups of mice treated with MMF during the elicitation of CHS exhibited reduced ear swelling, indicating that the afferent phase of CHS is impaired by MMF. Surprisingly, MMF also reduced CHS responses when administered upon initial hapten application, indicating that the sensitization phase of CHS was impaired. Compared with their respective vehicle-treated control groups, MMF significantly inhibited the induction (32%) and elicitation (38%) of CHS. Similar results were obtained when TNBC was used as a contact allergen (data not shown).

Hapten-immunized mice that had been treated with MMF or vehicle alone were reimmunized 2 wk after the initial challenge. Five days later, mice were rechallenged. After rechallenge, MMF-treated mice were able to mount CHS responses comparable to

FIGURE 1. MMF suppresses the induction and elicitation of CHS responses. CHS responses are expressed as the average ear swelling, with error bars representing SDs for each group of five mice. BALB/c mice were immunized with 2% oxazolone and challenged with 0.5% oxazolone. A, Mice were immunized and challenged (a); challenged only (b); treated with 30 μg/g MMF (c) or vehicle (d) alone i.p. 24, 12, and 1 h before as well as 12 and 24 h after immunization; or treated with MMF (e) or vehicle (f) alone i.p. 24, 12, and 1 h before challenge and 12 and 24 h after challenge. Ear swelling was assessed 24 h after challenge. **, p < 0.0001 for vehicle vs MMF-treated mice before immunization and challenge; *, p < 0.016 for immunized and vehicle-treated mice before challenge vs immunized and MMF-treated mice before challenge. B, Mice were treated with MMF or vehicle, immunized, and challenged. Two weeks later mice were reimmunized and 5 days later rechallenged. Ear swelling was assessed 24 h after rechallenge. *, p < 0.001 for vehicle vs MMF-treated mice.
FIGURE 2. MMF inhibits the capacity of DC to induce hapten-specific primary immune responses. CHS responses are expressed as the average ear swelling, with error bars representing SDs for each group of five mice. DC were cultured in 0.1 or 0.01 μM MMF, coupled with DNBS, and s.c. injected. Mice were challenged 1 wk later with DNFB, and ear swelling was measured 24 h later. **, p < 0.0001 for DC cultured in 0.1 μM MMF vs untreated DC; *, p < 0.0001 for DC cultured in 0.01 μM MMF vs untreated DC.

FIGURE 3. MMF inhibits the capacity of DC to induce hapten-specific secondary immune responses. DTH responses are expressed as the average footpad swelling, with error bars representing SDs for each group of five mice. BALB/c mice were immunized by painting 0.5% DNFB onto the lower shaved back. DC were cultured in 0.1 or 0.01 μM MMF or vehicle, coupled with DNBS, and injected into the hind footpad. Footpad swelling was assessed 24 h later. **, p < 0.0001 for DC cultured in 0.1 μM MMF vs untreated DC; *, p < 0.0006 for DC cultured in 0.01 μM MMF vs untreated DC.

FIGURE 4. Chronic treatment with MMF leads to a reduced stimulatory capacity of epidermal cells to stimulate allogeneic T cells. Mice were treated with 30 μg/g/day MMF. Thirty days later EC were prepared and enriched for I-A$^+$ cells from skin. Graded doses of EC were used to stimulate allogeneic T cells in MELR. EC from vehicle-treated EC were used as the control. *, p < 0.001 for EC prepared from mice treated with 0.1 μM MMF vs EC isolated from vehicle-treated mice.

flow cytometric analysis) or I-A-depleted EC that had been exposed to hapten were injected. There were no significant differences in DTH responses when uncoupled or hapten-coupled I-A-depleted EC instead of DC were used for sensitization, indicating that DC were not just an unspecific vector of the hapten (data not shown). In both experimental approaches there was a significant reduction of the hapten-specific immune response, indicating that MMF had had a direct and dose-dependent effect on the Ag-presenting function of the DC.

Effects of chronic MMF treatment on number and function of epidermal LC

As the impaired immune responses could also be attributed to a reduction in the numbers of APC as a result of MMF treatment, mice were chronically treated with 30 μg/g for 30 days. Subsequently, the numbers of LC, the resident DC of the skin, were evaluated after immunofluorescent staining of earsheets with I-A. There were no significant differences in terms of numbers of LC between the MMF-treated mice (718 ± 32/mm$^2$) and the control mice (704 ± 23/mm$^2$).

To test the functional ability of epidermal cells derived from MMF-treated mice to stimulate T cells, epidermal cells were prepared from mice chronically treated with MMF or vehicle and enriched for LC content. These EC were used to stimulate allogeneic T cells in MELR. As demonstrated in Fig. 4, EC of MMF-treated mice were significantly impaired in their ability to stimulate T cells, pointing to a functional impairment of Ag-presenting capacities of their LC.

MMF impairs the expression of cell surface T cell-stimulating molecules

Although it is well known that MMF affects lymphocyte proliferation, the inhibition of induction of CHS was unexpected. These data prompted us to study the effects of MMF on APC in vitro. As only mature, not immature, DC are capable of activating naive T cells, it was conceivable that the impairment of the Ag-presenting capacities of DCs propagated in the presence of MMF may be due to alterations in the expression of costimulatory and MHC molecules. To investigate cell surface expression of immunomodulatory molecules, DC were prepared and cultured in the continuous presence of various concentrations of MMF (0.001, 0.01, and 0.1 μM).
The expression of the cell surface molecules CD40, CD80, CD86, ICAM-1, NLDC-145 (DEC205), and I-A (MHC class II), all of which are up-regulated upon DC maturation (9, 10), was then assessed by flow cytometry. In the presence of MMF, DC expressed lower amounts of these molecules, reflecting that MMF inhibits DC differentiation from bone marrow precursors (Fig. 5). Although expression of these markers was somewhat reduced at a concentration of 0.001 μM MMF, it did not differ markedly from that of the vehicle-treated controls. These effects were not owing to an increase in the percentage of dead cells, as determined by trypan blue exclusion or flow cytometry with propidium iodide. The number of cells was only slightly reduced in cultures containing 0.1 μM MMF compared with vehicle-treated or untreated cultures. In a typical experiment cultures from untreated or vehicle-treated DC contained $8.1 \times 10^6$ DC/ml, whereas DC from 0.1 μM MMF-treated cultures contained $7.65 \times 10^6$ DC/ml.

DC and macrophages originate from the same precursors and can be distinguished by various cell surface markers depending on their stage of differentiation. Furthermore, DC are predominantly separated from macrophages during culture by their differences in adherence to plastic (9, 20). To ascertain whether the reduction in surface molecule expression was due to altered differentiation of precursor cells by the culture conditions with MMF, flow cytometry using the macrophage-related surface molecules CD11b, ER-TR9, ER-HR3, F4/80, and BM8 as well as the B-cell lineage marker CD19 was performed on day 7 with cells cultured in 0.01

**FIGURE 5.** MMF reduces the expression of immunomodulatory cell surface molecules in DC. DC were prepared from BALB/c mice and propagated in the presence of 0.1 or 0.01 μM MMF or vehicle. Expression of cell surface molecules was assessed using flow cytometry with fluorescently labeled Abs. The results of a representative experiment are given as the percent positive DC as well as the MnX of the total population. Vehicle-treated DC were used as the control.
μM MMF, 0.1 μM MMF, or vehicle or with untreated cells. Expression of CD11b (59.8%; mean fluorescence intensity (MnX), 4.90) and BM8 (29.6%; MnX, 2.60) was considerably increased when using 0.1 μM MMF as compared with vehicle (CD11b: 12.9%; MnX, 1.87; BM8: 16.6%, MnX, 1.88). These molecules have been reported to be widely expressed on immature DC and macrophages depending on their stage of maturation (9, 10). For all other markers no major differences were observed (data not shown). Taken together, these results suggest a direct effect of MMF on DC by retarding or impairing DC maturation.

To assess whether a normal functional maturation would occur after termination of MMF exposure, DC were cultivated in the presence of 0.01 and 0.1 μM MMF or vehicle alone or the cells were left untreated to day 7. At this point, the cultures were split and half of the respective cultures was cultivated without MMF, whereas the other half was further cultivated in the presence of MMF or vehicle alone. DC were subjected to flow cytometric analysis of the cell surface Ags CD40, CD80, CD86, and I-A 2 days later. There was a distinct increase in costimulatory molecule expression in DCs that had been propagated as of day 7 without MMF compared with the respective cells that had been further propagated in the presence of MMF (data not shown). To confirm the maturation of DC after discontinuation of the immunomodulatory effects of MMF on the functional level of T cell stimulation, the DC cultivated to days 7 and 9 were used in MLR. As shown in Fig. 6, the results of the flow cytometric analyses were corroborated by the results of the MLR, in that DC cultivated with MMF to day 7 had no inhibition of T cell-stimulating capacities, whereas DC cultivated in MMF until day 9 were significantly impaired. This time course dependency further supports the conclusion that MMF impairs DC maturation.

**FIGURE 6.** Normal functional maturation of DC after discontinuation of MMF exposure. DC were prepared from BALB/c mice and propagated in the presence of 0.1 μM MMF or vehicle. On day 7 half the DC were cultured without MMF, and the other half of each culture was further cultivated with MMF. The T cell stimulatory effects of DC cultivated to day 7 and to day 9 with MMF or vehicle to day 9 were evaluated by MLR. Approximately twice the number of DC treated with 0.1 or 0.01 μM MMF were needed to attain a similar amount of thymidine incorporation as that in untreated control groups. DC treated with 0.001 μM MMF did not differ from vehicle-treated DC in their T cell stimulatory abilities.

**Discussion**

Immunosuppressive agents are routinely used to modulate allograft rejection and to prolong graft survival and maintenance in organ transplantation. Among the many drugs currently in use, the new immunosuppressive drug MMF is proving to be very effective in reducing the likelihood of allograft rejection. Studies of the mechanisms of the immunosuppressive effects of MMF generally focus on its effects on T or B cells (21, 22). This study was aimed at defining a new possible mode of action of the immunosuppressive drug MMF by examining whether MMF may exert its effects on APC.

**FIGURE 7.** Pretreatment of DC with MMF suppresses their capacity to produce IL-12. Supernatants of LPS-triggered DC from BALB/c mice cultured with 0.1 or 0.01 μM MMF or vehicle alone were assessed for their capacity to produce IL-12 as measured by ELISA. Values are expressed as picograms per milliliter. Results from one representative experiment of three from 20,000 DC are shown. The error bars depict SDs calculated from triplicate samples. *, p < 0.001 for vehicle-treated control vs DC treated with 0.1 μM MMF; not significant for vehicle-treated control vs DC treated with 0.01 or 0.001 μM MMF.

**MMF reduces the production of IL-12 by DC triggered by LPS stimulation**

IL-12 is produced in large amounts by mature, but not by immature, DC and therefore is a suitable surrogate marker for DC differentiation. To determine whether IL-12 production is altered in DC treated with MMF, the production of IL-12 was measured by ELISA. Supernatants were collected from LPS-triggered DC propagated in the presence of MMF. The results of a representative experiment are shown in Fig. 7. Pre-exposure of developing DC to 0.1 μM MMF considerably reduced their subsequent ability to secrete IL-12 upon stimulation by LPS. Doses of 0.1 μM MMF reduced IL-12 production by almost 40% compared with vehicle-treated DC, whereas concentrations of 0.01 and 0.001 μM MMF had little effect on IL-12 production.
The effects MMF exerts on Ag presentation were studied 1) in vivo using CHS and DTH assays as a measure of functional immune response, and 2) in vitro using bone marrow-derived DC as an example for APC to assess changes in their functional properties. Consistent with other studies, in which impairment of T cell functionality has been reported (23, 24), CHS responses after applying MMF in the T cell-dependent elicitation phase were reduced. Application of MMF before sensitization also resulted in a reduction of CHS responses, although Ag-specific immunotolerance was not induced in this model. As the interaction of T cells with APC is essential at this stage, these data are suggestive of a regulatory effect of MMF on APC function. These results were further corroborated by 1) the decrease in the allostimulatory capacity of EC in MELR, and 2) the reduction of hypersensitivity responses evoked by hapten-coupled DC following ex vivo treatment with MMF. Furthermore, this approach excluded any systemic effects MMF may have had on other cell types in vivo following i.p. injection of MMF.

Immune responses leading to allograft rejection are initiated by T cell-dependent recognition of foreign Ags displayed on allogeneic histocompatibility complexes on APC originating from the allograft (1). The complex interactions resulting in the modulation of T cell-mediated immune responses are directly dependent on Ag presentation, cognate interactions between T cells and APC, and the concomitant production of the necessary costimulatory molecules by alloreactive APC and T cells (reviewed in Refs. 9 and 25). This is also the fundamental mechanism underlying MLR, namely clonal expansion of alloreactive T cells following priming by APC. MLR assays can be used to make a prognosis on graft survival, with intense proliferation of T cells indicating an incompatibility donor (26–28). DC cultivated in the presence of MMF exhibited a dose-dependent reduction in their capacity to stimulate T cell proliferation in MLR assays, indicating a direct effect of MMF on DC. This conclusion was further substantiated using flow cytometric analyses, which revealed a dose-dependent reduction in the expression of immunomodulatory molecules crucial for T cell activation, namely CD40, CD80, CD86, ICAM-1, and I-A.

Th1-type cells are particularly involved in acute allograft rejection (13). Studies on patients receiving MMF immediately after transplantation or after the onset of rejection reactions revealed diminished rejection of the transplant (7). This would coincide with the similar effects evidenced by the inhibition of the elicitation of CHS in the animal model used in this study and the decrease in the expression of IL-12, a key mediator of T cell differentiation into Th1-type effector cells, by DC propagated in the presence of MMF. A number of groups have reported the absence of Th1-type cytokines, such as IFN-γ, in allograft recipients receiving tolerizing therapies (29–31). Recently, Lui et al. (22) reported a dose-dependent reduction of IFN-γ production, as assessed by mRNA levels, after treatment of mice with MMF. Furthermore, MPA, the bioactive metabolite of MMF, has been shown to have little effect on the expression of the Th1-immunosuppressive cytokine IL-10 in mouse spleen cells (18). Insufficient amounts of IL-12 and/or CD80 production by APC during Ag presentation have also been implicated in anergy and tolerance, both of which render T cells functionally unresponsive (32). Although the expression of these molecules was reduced when cells were treated with MMF, administration of MMF did not induce Ag-specific immunotolerance in vivo in the model used in this study.

The direct stimulation of recipient T cells by donor APC originating from the graft has long been considered to be critical in graft rejection. In the past several years evidence has accumulated that presentation of donor Ags originating from the transplant and being presented by MHC on the recipients APC, termed indirect recognition, is a mechanism involved in chronic rejection of allograft transplants (1, 33, 34). Wecker et al. (35) demonstrated that MHC class II-deficient skin grafts were able to elicit chronic rejection in SCID mice reconstituted with CD4+ T cells via MHC class II-restricted Ag recognition. Another study also confirmed that allograft rejection is linked to the indirect pathway of allorecognition (36). MMF has been shown to attenuate chronic rejection of transplants in rats and humans (37–39). In addition, graft rejection by DTH-like reactions by Th1 cells has been inferred as playing a role in chronic rejection (1, 40). Interestingly, MMF was also shown to decrease DTH-like responses using hapten-coupled MMF-treated DC in the mouse model employed in this study. Taking not only the inhibitory effects of MMF on T cells into account but also the role of APC in chronic rejection, this study sheds new light on additional beneficial effects of MMF in transplantation therapy.

Taken together, these results highlight a novel facet of MMF action, namely not only does MMF exert its immunosuppressive effects by suppressing T cell proliferation but also by impairing APC function, as assessed using bone marrow-derived DC as an exemplary APC cell type. Our studies indicate that this effect may be based on the retardation of DC maturation, because the expression of costimulatory cell surface markers is directly correlated to the duration and the concentration of MMF treatment. Although the molecular events leading to the immunosuppressive effects of MMF on DC remain to be resolved, one possible mechanism mediating these effects may well be the inhibition of IMPDH. If this were the case, as has been reported for T and B cells, DC and other cells of the immune system may also depend on the de novo synthesis of guanosine. This would allow new strategies and alternative implementations of immunosuppressive regimens, thus expanding therapeutic flexibility in treating immune disorders and transplant rejection.

References


FIGURE 8. Pretreatment of DC with MMF inhibits their allostimulatory capacity. DC were prepared from BALB/c mice, propagated in the presence of 0.1 or 0.01 μM MMF or vehicle, and used to stimulate allogeneic T cells from C57BL6 mice. Results from one representative experiment of three are shown. The ability of DC to stimulate allogeneic T lymphocytes was assessed by uptake of [3H]thymidine. *, p < 0.05 for vehicle-treated control vs DC treated with 0.001 μM MMF; **, p < 0.002 for vehicle-treated control vs DC treated with 0.1 μM MMF.


