Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis

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Mycophenolic acid and methotrexate inhibit lymphocyte cytokine production via different mechanisms.

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CHAPTER 3

Abstract

Mycophenolic acid (MPA) and methotrexate (MTX) are immunosuppressive drugs used for the treatment of various immunological disorders. MPA is an inhibitor of inosine monophosphate-dehydrogenase and MTX is a folate antagonist that inhibits tetrahydrofolate-reductase. Production of T cell cytokines in whole blood cultures as well as in PBMC cultures is inhibited by a low concentration of both drugs. Inhibition of cytokine production after monocyte stimulation was less evident. The mechanism by which inhibition is achieved is different for both drugs. Inhibition of T cell cytokine production by MPA was more profoundly and started earlier compared to the inhibition by MTX. MTX induced apoptosis in T cells that became activated, whereas MPA prevented activation of T cells by arresting the cells in the G0/G1 phase of the cell cycle. Addition of guanosine and adenosine can overcome this cell cycle arrest, even after several days. Furthermore MPA inhibited the expression of activation markers HLA-DR and CD71 on T cells.

The observation that MTX cannot prevent T cell activation but induces apoptosis in activated T cells and that MPA reversibly prevents activation of T cells could explain the immunosuppressive effects both drugs.
Introduction

Mycophenolate mofetil and methotrexate (MTX) are immunosuppressive drugs used for treatment of immunological disorders. MTX has shown its efficacy in rheumatoid arthritis (RA) in placebo controlled trials (1,2) whereas the use of mycophenolate mofetil or its active part mycophenolic acid (MPA) for treatment of RA is still experimental (3,4). MPA was reported to be an effective drug in preventing rejection in renal transplant patients (5,6). Recently it was suggested that MPA could be a useful drug to treat HIV infection since administration to HIV-infected subjects resulted in reduction of the number of activated T cells and of virus isolated from purified CD4+ T cells (7). By which mechanism MPA and MTX are immunosuppressive is not clear. MPA is a selective inhibitor of inosine monophosphate dehydrogenase (IMPDH), the rate limiting step in the de novo synthesis of (d)GTP (8).

MTX is a folate antagonist. Addition of MTX to cell cultures will lead to the inhibition of de novo synthesis of purines and pyrimidines (9). In view of the extreme sensitivity of lymphocytes to inhibition of de novo nucleotide synthesis, T and B cells are likely candidates to be affected by the drugs (10). It has been reported that MPA blocks proliferative responses in human T cells but that IL-2 production and CD25 expression is not changed (11). Nagy et al. have shown that MPA inhibits superantigen-induced cytokine production in human T cells whereas it has no effect on LPS- or mitogen-induced cytokine production (12). In the mouse, in vitro as well as in vivo, MPA inhibited TNF-α and IFN-γ production by T cells whereas it did not affect IL-6 production (13). In another mouse study it was reported that in vivo application of MPA two hours before an LPS challenge inhibited TNF-α production, whereas TNF-α production induced by anti-CD3 was not inhibited or even increased (14). In splenocytes from SLE prone and normal mice MPA inhibited lymphocyte proliferation, the production of immunoglobulins and autoantibodies (15).

Mizoribine, another IMPDH inhibitor, has been found to block T cell proliferation in the G0/1 phase (16). Early events in T cell activation such as mRNA expression of c-myc, IL-2, c-myb, CD25 and cdc2 kinase were unaffected. Subsequently it has been reported that MPA also blocked lymphocyte proliferation in the G0/1 phase (17) (18). Conversely, it has been described that MPA can induce apoptosis in several human cell lines and in activated T cells (7,19). Recently we have shown that MTX, at low concentrations, effectively inhibits cytokine production after polyclonal T cell stimulation in human whole blood cultures, whereas MTX hardly affects cytokine production after stimulation with LPS or SAC (Gerards et al., submitted). This effect of MTX is due to induction of apoptosis in activated T cells (20). We decided to study the effect of MPA on cytokine production in human whole blood cultures. We observed that similar to MTX, MPA is a potent inhibitor of cytokine production but by a completely different mechanism.

Materials and methods

Cells and cell cultures.

Heparinised blood collected from healthy volunteers was used for whole blood (WB) cultures and for isolation of peripheral blood mononuclear cells (PBMC). All cell cultures were performed in culture medium (Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100µg/ml) and 50 µM 2-mercaptoethanol). WB was diluted 1:10 in culture medium supplemented
with 0.1% fetal bovine serum (FCS, Bodinco, Alkmaar, the Netherlands) and 15 U/ml heparin, all endotoxin free. PBMC were isolated from freshly drawn blood and separated over a Percoll gradient (d=1.078, Pharmacia Fine Chemicals AB, Uppsala, Sweden). PBMC were cultured (2x10^5 cells/ml) in culture medium supplemented with 5% FCS and 20 μg/ml human transferrin (Sigma-Aldrich, Steinheim, Germany). All cells were cultured in 200 μl at 37°C in the presence of 5% CO₂ in flat bottom plates (Nunc, Roskilde, Denmark). Stimuli and drugs were directly added to the cell culture, unless otherwise indicated. The duration of the cultures is indicated for each experiment. 

**Antibodies, stimuli and drugs.**

Anti-CD3 (CLB.T3/4.E, CLB, Amsterdam, the Netherlands) and anti-CD28 (CLB.CD28/1) were used for stimulation of the cells at 1 μg/ml. Other stimuli were LPS (100 pg/ml, a gift from Dr. J. Poolman, RIVM, Bilthoven, the Netherlands) and Staphylococcus aureus cells (SAC, Pansorbin, 1:4000, Calbiochem, La Jolla, CA). Human recombinant IL-2 (Chiron corp., Emeryville, CA) was used at 100 units/ml. The drugs MPA (GibcoBRL, Grand Island, NY) and MTX (Lederle, Hoofddorp, the Netherlands) were freshly diluted from a 2.5 mg/ml stock in each experiment. For FACS analysis we used anti-TNF-α (CLB-TNF/5) anti-CD25, anti-HLA-DR, Ki-67 (all FITC-labeled), anti-CD69, anti-CD71 (both PE-labeled) anti-CD33 (PE-Cy5 labeled) and anti-CD2 (biotin-labeled) mAbs. These mAbs were purchased at the CLB, except for Ki-67 (Immunootech, Marseille, France). Guanosine and adenosine (cell culture tested) were obtained from Sigma-Aldrich.

**Cytokine measurements.**

The production of cytokines was measured in the supernatant of the cell cultures. Supernatant was harvested at indicated times and stored at −20°C until use. IL-8, IL-6, IL-4, IL-13, TNF-α and IFN-γ were measured with ELISA kits (PeliKine-compact, CLB) according to the protocol. In short, mAbs were coated on flat bottom microtiter plates (Nunc, Maxisorb) overnight in 100 μl 0.1M Na-bicarbonate at pH 9.6. All incubations were performed in 100 μl at room temperature. Plates were washed 5 times with PBS 0.02% Tween-20 (Mallinckrodt Baker, Deventer, the Netherlands). Samples were incubated together with a biotinylated mAb for 2 hours in high performance ELISA (HPE) buffer (CLB). Plates were washed 5 times (PBS, 0.02% Tween-20) and incubated for 30 minutes with streptavidin-labeled poly-horseradish peroxidase (CLB) 1:1000 diluted in PBS/2% skimmed milk. Plates were washed 5 times and developed with 0.003% H₂O₂, 100 μg/ml 3,5,3',5'-tetramethylbenzidine (Merck, Darmstadt, Germany) in 0.11 M Na-acetate, pH 5.5. The reaction was stopped with an equal volume (100 μl) of 2M H₂SO₄. Only for IL-13 an additional blocking step of 30 minutes with PBS/2% milk was required after the O/N coating step. GM-CSF was measured via a similar protocol as the other cytokines. The GM-CSF Abs were a kind gift from Dr. G. Trinchieri (the Wistar Institute, Philadelphia, PA). In this assay the coating Ab was anti-GM-CSF 9.1 (used at 2 μg/ml), the biotinylated Ab was anti-GM-CSF 16.3 (0.1 μg/ml). rGM-CSF (Sandoz, Basel, Switzerland) was used for the preparation of a standard curve. For intracellular TNF-α staining 5x10^5 cells/ml were cultured, to prevent secretion of cytokines 1μl/ml GolgiPlug (Pharmingen, San Diego, CA) was added 18 hours before harvesting the cells. 

**FACS analysis.**

Induction of apoptosis was measured by staining the cells with annexin-V (FITC-labeled, Bender Medsystems, Vienna, Austria). All incubations were performed at 4°C. Cells were washed 3 times with buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1
mM MgCl$_2$ and 0.5% (w/v) bovine serum albumin, pH 7.4) Cells were incubated for 20 minutes with annexin-V (1:400) and biotinylated anti-CD2 (1:10), washed 3 times, incubated with streptavidin-allophycocyanin (APC) (1:750, PharMingen) and measured on the FACScalibur (BectonDickinson, San Jose, CA). For the analysis of activation markers, stimulated or unstimulated cells, cultured with or without MPA, were analyzed after 24 hours for CD25 and CD69 expression and after 72 hours for Ki-67, HLA-DR and CD71 expression. After the cells were washed 3 times with PBS, 0.5% BSA and 0.02% Sodium azide (PBA), the cells were incubated with mAb’s (1:10) for 20 minutes in the presence of 3 mg/ml human gammaglobulins (CLB), to block non-specific binding. Before staining with Ki-67, TNF-α and anti-CD3 mAb, cells were permeabilised by incubation for 10 minutes with FACS lysing solution and FACS permeabilising solution (Becton Dickinson) respectively, according to the manufacturers protocol, and washed 2 times. Thereafter cells were washed 3 times and measured on the FACScan or FACScalibur (Becton Dickinson). Appropriate isotype control mAbs (CLB) were used in each experiment.

Results

Effect of MPA and MTX on T cell cytokine production in WB and PBMC.

Whole blood of 8 different healthy donors was stimulated with a T cell stimulus (anti-CD3 plus anti-CD28 mAbs) in the presence of MPA and MTX for 4 days resulting in the production of T cell cytokines. As an example we show that MPA dose-dependently inhibited the GM-CSF production (Fig. 1A). The inhibition of GM-CSF production by MTX started at a lower concentration than that by MPA (Fig. 1B). In addition to the inhibition of GM-CSF production, MPA also inhibited the production of IL-4, IL-13, IL-8, IFN-γ and TNF-α (Fig. 2). Dose response curves of cytokine inhibition by MPA were similar for each cytokine. MTX inhibited the production of these cytokines as well, except that of IL-8, but the inhibition was not as strong as that observed with MPA (Fig. 2). In this experiment the amount of supernatant restricted the number of cytokines we could test. A prominent cytokine missing in this experiment is IL-2. In separate experiments we have amply tested the effect of MPA and MTX on IL-2 production. Indeed IL-2 is inhibited by both drugs at relatively early time points. However that IL-2 is a difficult cytokine to measure in supernatants because it is consumed by proliferating T cells. For that reason, at later time points IL-2 levels strongly decrease, also in cultures without drugs.

For the production of cytokines by T cells IL-2 is essential (15). Consequently, inhibition of cytokine production could be secondary to early depletion of IL-2. However addition of excess (100U/ml) of IL-2 at the start of the cultures did not alter the inhibition of either drug (not shown). The observed effects are therefore not caused by IL-2 starvation of the cells.

The kinetics of inhibition by MPA and MTX are different. At day one inhibition by MPA in 8 donors varied between 50 and 80% whereas MTX had no effect (fig. 3). At day two inhibition by MPA was more than 90% and inhibition by MTX between 40 and 75%. At day 3 both drugs efficiently inhibited cytokine production (fig. 3).
Figure 1: The effect of MPA and MTX on the production of GM-CSF. WB (1:10 diluted) of healthy donors was stimulated with anti-CD3/anti-CD28 and incubated with a concentration range of MPA and MTX for 4 days. Without addition of a drug, the GM-CSF production ranges from 37330-136050 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug. 1A, the dose dependent inhibition of GM-CSF production by MPA of 4 of the donors is shown. 1B, inhibition of GM-CSF production by MPA (○) is compared to the inhibition by MTX (●) (mean of 8 donors, +/- SEM).

Figure 2: The effect of MPA and MTX on cytokine production after T cell stimulation. WB of 8 healthy donors was stimulated with anti-CD3/anti-CD28 and incubated with 2 μg/ml MPA (○) or 2 μg/ml MTX (●). Cytokine production was measured by ELISA after 4 days. Without addition of a drug, values of the different donors ranged from: 165-2500 pg/ml IL-4, 75250-394600 pg/ml IL-8, 3315-12075 pg/ml IL-13, 37330-136050 pg/ml GM-CSF, 47775-393050 pg/ml IFNγ and 470-10984 pg/ml TNF. The values on the y-axis are percentages of the production in the absence of the drug.
Figure 3: The kinetics of GM-CSF inhibition by MPA and MTX. WB of 8 healthy donors was stimulated by anti-CD3/anti-CD28 mAb without a drug or in the presence 2 μg/ml MPA (○) or 2 μg/ml MTX (●). At the indicated times the GM-CSF production was measured by ELISA. The values on the y-axis are percentages of the GM-CSF production in the absence of the drug. Without addition of a drug values of the different donors ranged from 370-1635 pg/ml at day 1, 2925-15940 pg/ml to day 2 and 20840-95470 pg/ml at day 3.

Effect of MPA and MTX on monocyte cytokine production.
Stimulation of WB with bacterial products LPS or SAC induces production of IL-6, IL-8 and TNF-α in monocytes. The production of these cytokines was hardly affected by MTX (Gerards et al., submitted). The LPS- or SAC-induced IL-6, IL-8 and TNF-α production was slightly inhibited by MPA (Fig. 4). The effects of MPA and MTX on cytokine production by PBMC were comparable to the effects of the drugs on the cytokine production by WB. In experiments with PBMC, the production of IL-8 was not measured, since isolated PBMC produce high levels of IL-8 in the absence of stimuli (not shown).

Figure 4: The effect of MPA and MTX on LPS induced cytokine production. WB of 8 healthy donors was incubated with LPS (100 pg/ml) in combination with 2 μg/ml MPA (○) or 2 μg/ml MTX (●). After 24 hours, the production of cytokines was measured by ELISA. The production of IL-6 ranges from 1853-3780 pg/ml, IL-8 from 5257-26030 pg/ml and TNF from 270-1053 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug.
Apoptosis induction.
It was reported before that both MTX and MPA induce apoptosis in T cells. We performed flow cytometric studies with PBMC to assess the effects of MPA and MTX on cell viability in low-density cultures. The morphology of the cells was analyzed after a 4-day culture period. Unstimulated cells remained small while anti-CD3/anti-CD28 stimulated cells formed blasts. Cells stimulated in the presence of MPA remained small, cells stimulated in the presence of MTX first formed blasts and then appeared to become apoptotic; they became more granular and shrunk (Fig. 5A). This notion was supported by annexin-V binding studies. In unstimulated cell cultures neither of the drugs induced annexin-V binding (Fig. 5B). Stimulated cells bound slightly more annexin-V, than resting cells did. Stimulation of the cells in the presence of MPA had no effect on annexin-V binding whereas stimulation in the presence of MTX led to extensive annexin-V binding (Fig. 5B). The induction of apoptosis starts after two days and the number of annexin-V positive cells increases in time.
The induction of apoptosis by MPA described in literature was found both on T cell lines (19) and on activated peripheral blood T cells (7). The PBMC used in our study were resting at the time MPA and the stimulus were added. Indeed, when MPA was added to PBMC stimulated earlier, MPA did induce annexin-V binding (Fig. 5C). MPA addition induced annexin-V binding to PBMC as soon as 24 hours after stimulation (not shown).
The effects of MPA are reversible.
Our data indicated that, rather than killing the cells, MPA prevents proliferation of freshly isolated cells. We next studied whether the effects of MPA might be reversible. Addition of 100 μM guanosine and 200 μM adenosine, together with the stimulus and MPA, was found to prevent the inhibitory effects of the drug (not shown). To see whether it is not only possible to prevent the inhibitory effects of MPA but also to reverse the inhibition, we stimulated PBMC in the presence of MPA and cultured them for various periods before addition of guanosine and adenosine. Both proliferation and cytokine production were restored in the cells cultured with anti-CD3/anti-CD28 and MPA after addition of guanosine and adenosine at day 4 (Fig. 6). Untreated PBMC were cultured in the same plate. These were stimulated at the day of guanosine and adenosine addition and served as a control for the capacity of the cells to proliferate and produce cytokines. We established that the proliferation and cytokine production in response to anti-CD3/anti-CD28 of PBMC that have been cultured in medium for 4 days prior to stimulation is comparable to when the stimulus is given directly. It was possible to reverse the effects of MPA by guanosine and adenosine addition for up to 14 days (not shown).
Figure 5: A differential effect of MPA and MTX on the induction of apoptosis. PBMC were not stimulated or stimulated with anti-CD3/anti-CD28 in the presence of MPA or MTX. After 4 days apoptosis was measured by staining the cells with annexin-V. 5A: FACS scatter plots of: non-stimulated PBMC (upper left panel), PBMC stimulated with anti-CD3/anti-CD28 (upper right panel) and stimulated in the presence of MTX (lower left panel) or MPA (lower right panel). 5B. Annexin-V binding to PBMC. The percentage annexin-V binding cells within the CD2 population is depicted on the Y-axis. Unstimulated PBMC (left three bars) and PBMC stimulated with anti-CD3/antiCD28 (right three bars) in the presence of no drug (white), MPA (gray) and MTX (black). Similar results were obtained in 3 separate experiments. 5C: MPA induces apoptosis in activated PBMC. PBMC are stimulated with anti-CD3/anti-CD28. At day 3 cells were washed and cultured with 100 U/ml IL-2, without MPA (white) or with 2 µg/ml MPA (gray). At day 6 cells were stained with annexin-V and were analyzed on the FACS. The percentage annexin-V binding cells within the CD2 population is depicted on the y-axis. Similar results were obtained in 2 separate experiments.
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**Figure 6:** Adenosine and Guanosine reverse the effects of MPA. PBMC are cultured with the additions indicated. At day 8 3H-thymidine incorporation and cytokine production are assessed. Similar results are obtained in 4 separate experiments. Error bars indicate the SEM of triplicate cultures.

**Figure 7:** The effect of MPA on activation markers. PBMC are not stimulated (white bars) stimulated with anti-CD3/anti-CD28 in the absence (gray bars) and presence (black bars) of MPA. After 24 hours the expression of CD25 and CD69 was measured, after 72 hours the expression of HLA-DR, CD71 and Ki-67 was measured on the FACS. Similar results are obtained in 3 separate experiments.

**The effect of MPA on activation markers.**

In T cells, MPA induces an arrest in the G0/1 phase of the cell cycle (not shown) (18) (17). To more precisely define the point in the cell cycle, at which the cell is arrested by MPA, the expression of T cell activation markers was measured. CD69 and CD25 appear on the cell surface a few hours after stimulation whereas HLA-DR and CD71 appear after 2 days. It has been shown that the nuclear antigen Ki-67 is expressed in the late G1 phase just before cells enter the S phase (21). In our cultures Ki-67 positive cells appear 2 days after stimulation.
For optimal detection the expression of the early activation markers CD69 and CD25 was determined after 24 hours. Expression of late activation markers HLA-DR and CD71 and the nuclear antigen detected by Ki-67 was determined after 72 hours. The expression of CD69 and CD25 was not influenced by MPA, whereas the expression of HLA-DR and CD71 was decreased in the presence of MPA (Fig. 7). Ki-67 staining was completely inhibited in the presence of MPA (Fig. 7). Similar results were obtained when the cells were pre-incubated with MPA for 48 hours prior to stimulation with anti-CD3/anti-CD28. As expected MTX had no effect on the expression of either activation marker (not shown).

The effect of MPA and MTX on intracellular TNF-α staining.

From the results described above we concluded that MPA inhibits cytokine production independent of proliferation and MTX does not. To further investigate this hypothesis we stained PBMC intracellularly for TNF-α production at an early time point when the cells were not proliferating. After stimulation for two days, 46% of the T cells produce TNF-α; these are all CD69 positive cells. MTX did not influence intracellular TNF-α staining. In the presence of MPA only 10% of the T cells produced TNF-α (Fig. 8).

Figure 8. Intracellular TNF-α staining. PBMC were not stimulated or stimulated with anti-CD3/anti-CD28 in the presence of MPA or MTX. GolgiPlug was added after 28 hours, 16 hours later the cells were harvested and stained for CD3 and TNF-α after permeabilisation. Non-stimulated PBMC (upper left panel), PBMC stimulated with anti-CD3/anti-CD28 (upper right panel) and stimulated in the presence of MTX (lower left panel) or MPA (lower right panel). CD3 positive cells are shown in dotplots, with CD69 staining on the y-axis and TNF-α staining on the x-axis. The percentage of cells in each quadrant is indicated in the upper right corner of every plot. Similar results are obtained in 3 separate experiments.
Discussion.

MPA and MTX are used as immunosuppressive drugs but the mechanism by which the immunosuppression is achieved is disputed. Here we show that MPA, like MTX, strongly inhibited T cell cytokine production in whole blood and PBMC cultures. Furthermore, production of IL-6, IL-8 and TNF-α induced by monocyte activation is slightly decreased by MPA, and even less affected by MTX. The mechanism by which these drugs affect cytokine production turns out to be different. Addition of MPA to PBMC prevents blastogenesis but does not lead to apoptosis. In contrast, addition of MTX induces apoptosis in activated T cells as shown before (20).

It has previously been reported that also MPA does induce apoptosis (7,10,19). The reason for this discrepancy is that we studied resting cells rather than proliferating cells or (continuously cycling) cell lines. Indeed, when MPA was given after stimulation, MPA did induce apoptosis in PBMC. This demonstrates that the effect of MPA on initially resting cells is different from the effect on proliferating cells.

MPA is a better inhibitor of cytokine production than MTX. This is probably due to the differences in kinetics. MTX targets blasts only and its kinetics of inhibition coincides with the kinetics of blastogenesis. MPA prevents blastogenesis; consequently, it works at an earlier stage than MTX. This is clearly demonstrated by the inhibition of intracellular TNF-α expression, at a time point where blasts are not yet present. Another remarkable difference in the inhibition induced by MPA and MTX is the effect on IL-8 production after T cell activation. MPA inhibited the IL-8 production but MTX did not. We found that IL-8 production depended on the endogenous production of TNF-α and GM-CSF by T cells and that IL-8 is not produced by T cells. MPA is a better inhibitor of both TNF-α and GM-CSF production than MTX. The residual (10%) TNF-α and GM-CSF production in the presence of MTX is sufficient to support full IL-8 production by a yet unidentified cell.

When activation marker expression is analyzed after anti-CD3/anti-CD28 stimulation, the inhibition of the expression by MPA follows the same kinetics as the inhibition of cytokine production. The expression of the activation markers appearing within hours was not affected by MPA, whereas the expression of markers that appear later (CD71, HLA-DR and Ki-67) was decreased. These results are in agreement with the observation that MPA blocks rat lymphocytes in the G0/1 phase, but has no effect on CD25 expression and with the finding that MPA blocks human PHA-activated T cells in G1 (17,18).

There are many reports showing that the inhibitory effects of MPA are prevented by addition of different guanosine nucleotides to the MPA-treated cell cultures (8,11,17,19,22,23). We used a combination of guanosine and adenosine to restore proliferation in PBMC cultured with MPA and anti-CD3/anti-CD28, as measured by cytokine production and [3H]-thymidine incorporation. The use of adenosine was necessary, since guanosine alone did not completely restore proliferation of the cells. The recovery of proliferation and cytokine production in cells even after 14 days of culture with MPA and anti-CD3/anti-CD28 proves that under these conditions apoptosis was not induced. The cells are stopped in their
activation process, before entering the S-phase and the process is completely reversible. This observation correlated well with the observation that peripheral blood mononuclear cells, isolated from patients treated with MPA respond normally to mitogenic stimulation in vitro (10). It has been shown before that depletion of (deoxy) guanosine nucleotides by inhibition of IMPDH is the reason for impaired DNA synthesis (10,11). Lymphocytes contain high levels of IMPDH and upon activation they express the IMPDH type II isoform, which is more sensitive to MPA than type I. Therefore, lymphocytes are a primary target for MPA (10). Although we show that MPA effectively inhibits T cell cytokine production, the exact mechanism remains to be elucidated.

Inhibition of proliferation will of course decrease the number of cytokine producing cells. However on day 2 T cell proliferation is hardly initiated whereas cytokine inhibition is already considerable. This means that inhibition of proliferation cannot be the sole explanation for the observed inhibition. In addition, the reduced production of monokines supports the notion that additional inhibitory mechanisms might be operational. Possibly, changes in metabolic pathways requiring guanosine nucleotides, such as RNA synthesis or G-protein-linked events and diminished expression of adhesion molecules (24) are important in the decreased production of cytokines.

In conclusion we show that MPA, effectively inhibits cytokine production by T cells. It does so by preventing T cells to become fully activated. In activated T cells MPA induces apoptosis; thus treatment with MPA may result in depletion of the in vivo activated T cells. Whether the reversible prevention of activation of resting T cells by MPA might have any consequences for the immune response remains to be investigated.

In the light of the successes of anti-TNF treatment in RA and Crohn's disease, our observation that TNF-\( \alpha \) production by both T cells and monocytes is inhibited by MPA renders MPA an attractive drug for RA treatment.

Our findings lead to the conclusion that MPA prevents T cell activation without depleting them. Its efficacy in graft-versus-host-disease or transplantation could be due to this mechanism. Early in the disease MPA will work by inhibiting T cell activation. At a later stage of treatment, when the acute inflammation is over the treatment with MPA can be discontinued because in the absence of the pro-inflammatory signals alloantigen presentation will probably results in a (tolerogenic) Th2 type response in the remaining alloresponsive T cells.

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