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Mycophenolic acid and methotrexate inhibit lymphocyte cytokine production via different mechanisms



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 profound 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 cell cycle in the G0/G1 phase. 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 of both these drugs.

Full text

INTRODUCTION

Mycophenolate mofetyl and methotrexate (MTX) are immunosuppressive drugs used for the treatment of immunological disorders. MTX has shown efficacy in rheumatoid arthritis (RA) versus placebo controlled trials [1, 2] whereas the use of mycophenolate mofetyl or its active molecule mycophenolic acid (MPA) for the treatment of RA is still experimental [3, 4]. MPA has been 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 in the amount 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 leads to the inhibition of *de novo* synthesis of purines and pyrimidines [9]. In view of the extreme sensitivity of lymphocytes to the 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 are 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-alpha and IFN-gamma 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-alpha production, whereas TNF-alpha production induced by anti-

CD3 was unchanged [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-mycb, 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 reported 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 effects 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 (100 U/ml), streptomycin (100 mug/ml) and 50 muM 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 (2×10^5 cells/ml) in culture medium supplemented with 5% FCS and 20 mug/ml human transferrin (Sigma-Aldrich, Steinheim, Germany). All cells were cultured in 200 mul 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, 1:4,000, Calbiochem, La Jolla, CA, USA). Human recombinant IL-2 (Chiron corp., Emeryville, CA, USA) was used at 100 units/ml. The drugs MPA (Gibco BRL, 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-CD3 (PE-Cy5 labeled) and anti-CD2 (biotin-labeled) mAbs. These mAbs were purchased from the CLB, except for Ki-67 (Immunotech, 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 the 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. Briefly, mAbs were coated onto flat-bottom microtiter plates (Nunc, Maxisorb) overnight in 100 µl 0.1 M sodium 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-horse-radish peroxidase (CLB) 1:1,000 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 sodium acetate, pH 5.5. The reaction was stopped with an equal volume (100 µl) of 2 M H₂SO₄. 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 using a protocol similar to that used for the other cytokines. The GM-CSF Abs were a kind gift from Dr. G. Trinchieri (the Wistar Institute, Philadelphia, PA, USA). 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, 5 × 10⁶ cells/ml were

cultured; to prevent secretion of cytokines 1µl/ml GolgiPlug (PharMingen, San Diego, CA, USA) 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 MgCl₂ 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 (Becton Dickinson, San Jose, CA, USA). 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 mAbs (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-alpha 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 manufacturer's protocol, and washed 2 times with PBA. After staining, they cells were washed 3 times with PBA 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 from 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 (Figure 1A). The inhibition of GM-CSF production by MTX started at a lower concentration than the inhibition induced by MPA (Figure 1B). In addition to the inhibition of GM-CSF production, MPA also inhibited the production of IL-4, IL-13, IL-8, IFN-gamma and TNF-alpha (Figure 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 (Figure 2). In this experiment, the amount of supernatant restricted the number of cytokines that could be tested. A prominent cytokine missing in this experiment is IL-2. In separate experiments, we 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, IL-2 is a difficult cytokine to measure in supernatants because it is taken up by proliferating T cells. For that reason, at later time points IL-2 levels strongly decrease, even in cultures without drugs.

IL-2 is essential for the production of cytokines by T cells. Consequently, inhibition of cytokine production could be secondary to early depletion of IL-2. However, addition of excess (100 U/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 (Figure 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 (Figure 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-alpha 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-alpha production was slightly inhibited by MPA (Figure 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).

Apoptosis induction

It was reported earlier 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 shrunken (Figure 5A). This notion was supported by annexin-V binding studies. In unstimulated cell cultures, neither of the drugs induced annexin-V binding (Figure 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 (Figure 5B). The induction of apoptosis started after two days and the number of annexin-V positive cells increased over time.

The induction of apoptosis by MPA described in literature was found both in T cell lines [19] and in activated peripheral blood T cells [7]. The PBMC used in our study were resting at the time that MPA and the stimulus were added. Indeed, when MPA was added to previously stimulated PBMC, MPA did induce annexin-V binding (Figure 5C). MPA addition induced annexin-V binding to PBMC as early as 24 hours after stimulation (not shown).

The effects of MPA are reversible

Our data indicated that, rather than killing the cells, MPA prevented proliferation of freshly isolated cells. We next studied whether the effects of MPA might be reversible. Addition of 100 μ M guanosine and 200 μ M adenosine, together with the stimulus and MPA, was found to prevent the inhibitory effects of the drug (not shown). To see whether it is possible not only 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 (Figure 6). Untreated PBMC were cultured in the same plate. These were stimulated the same day as guanosine and adenosine were added and served as a control for the capacity of the cells to proliferate and produce cytokines. We established that the proliferation and cytokine production of PBMC that had been cultured in medium for 4 days prior to stimulation, in response to anti-CD3/anti-CD28 were comparable to when the stimulus was given directly. It was possible to reverse the effects of MPA by addition of guanosine and adenosine, for up to 14 days (not shown).

The effect of MPA on activation markers

In T cells, MPA induces an arrest of the cell cycle at the G0/1 phase (not shown) [17, 18]. 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 appeared 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 (Figure 7). Ki-67 staining was completely inhibited in the presence of MPA (Figure 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-alpha 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-alpha production at an early time point when the cells were not proliferating. After stimulation for two days, 46% of the T cells produced TNF-alpha; these were all CD69 positive cells. MTX did not influence intracellular TNF-alpha staining. In the presence of MPA, only 10% of the T cells produced TNF-alpha (Figure 8).

Discussion

MPA and MTX are used as immunosuppressive drugs, but the mechanism by which the immunosuppression is achieved is discussed. 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-alpha induced by

monocyte activation was 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 elsewhere [20].

It has also been reported previously that MPA induces 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 its 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 the kinetics of inhibition coincide 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-alpha 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 IL-8 production but MTX did not. We found that IL-8 production depended on the endogenous production of TNF-alpha and GM-CSF by T cells, and that IL-8 is not produced by T cells. MPA is a better inhibitor of both TNF-alpha and GM-CSF production than MTX. The residual (10%) TNF-alpha 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 this 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 previously 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 barely 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.

CONCLUSION

In conclusion, we show that MPA, effectively inhibits cytokine production by T cells. It does so by preventing T cells from becoming 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 works by inhibiting T cell activation. At a later stage of treatment, when the acute inflammation is over, 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.

Acknowledgments. We would like to thank Dr. D. Hamann for helpful discussion and critical reading of the manuscript. This work is supported by a grant from the Dutch League Against Rheumatism (NR 98-1-402).

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Figures

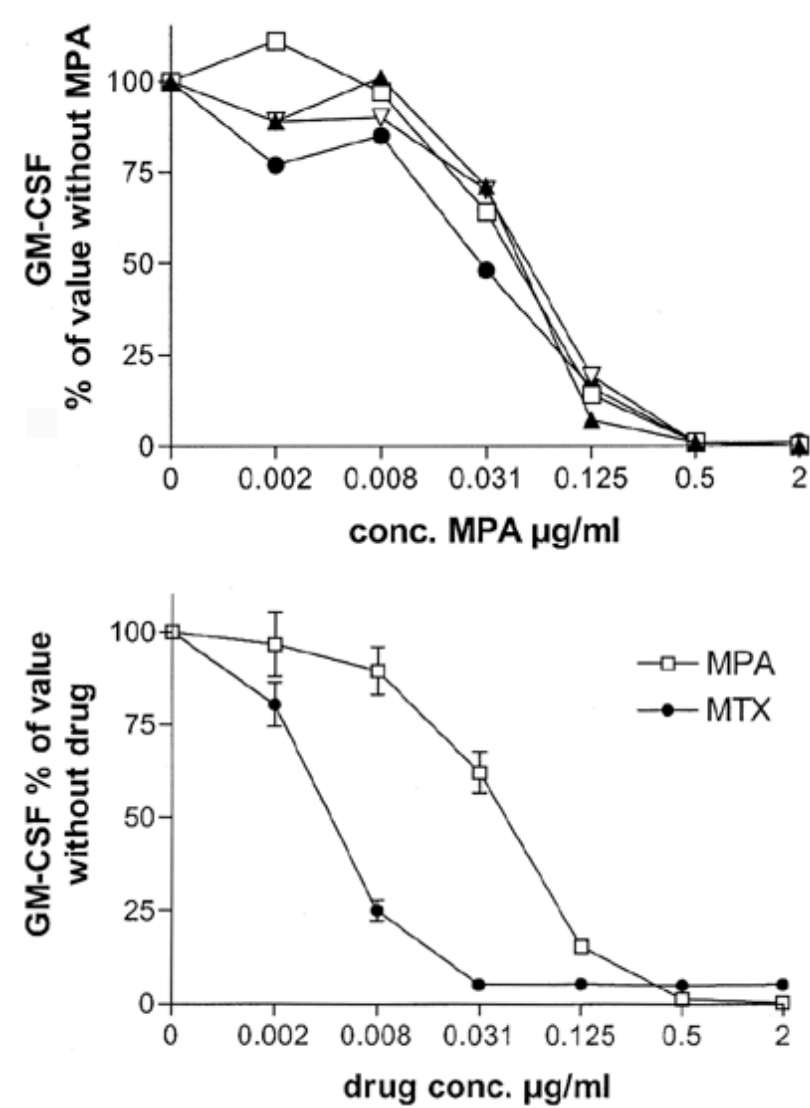


Figure 1

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 37,330-136,050 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug. **A:** The dose-dependent inhibition of GM-CSF production by MPA of 4 of the donors is shown. **B:** inhibition of GM-CSF production by MPA (carré is compared to the inhibition by MTX (*) (mean of 8 donors, $\pm$ SEM).

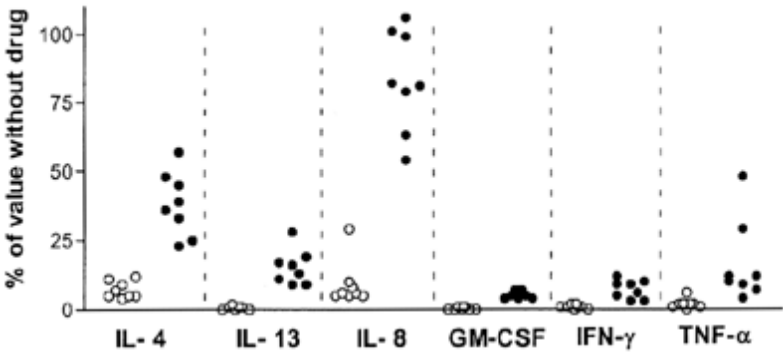
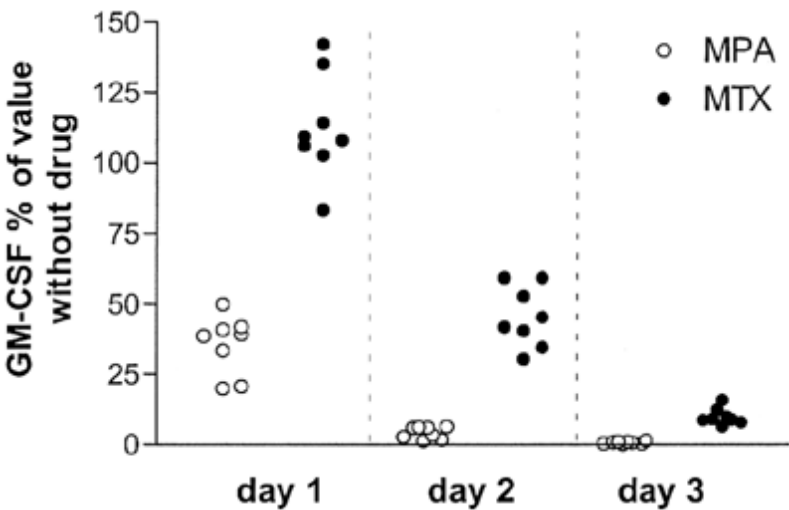


Figure 2

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 mug/ml MPA (o) or 2 mug/ml MTX (*). Cytokine production was measured by ELISA after 4 days. Without addition of a drug, values for the different donors ranged from: 165-2,500 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 IFN-gamma and 470-10984 pg/ml TNF-alpha. The value on the y-axis are percentages of the production in the absence of the drug.



	<p>Figure 3</p> <p>The kinetics of GM-CSF inhibition by MPA and MTX.</p> <p>WB of 8 healthy donors was stimulated by anti-CD3/anti-CD28 mAB without a drug or in the presence 2 mug/ml MPA (o) or 2 mug/ml MTX (*).</p> <p>At the indicated times, 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 between 370-1,635 pg/ml at day 1, 2,925-15,940 pg/ml at day 2 and 20,840-95470 pg/ml at day 3.</p>

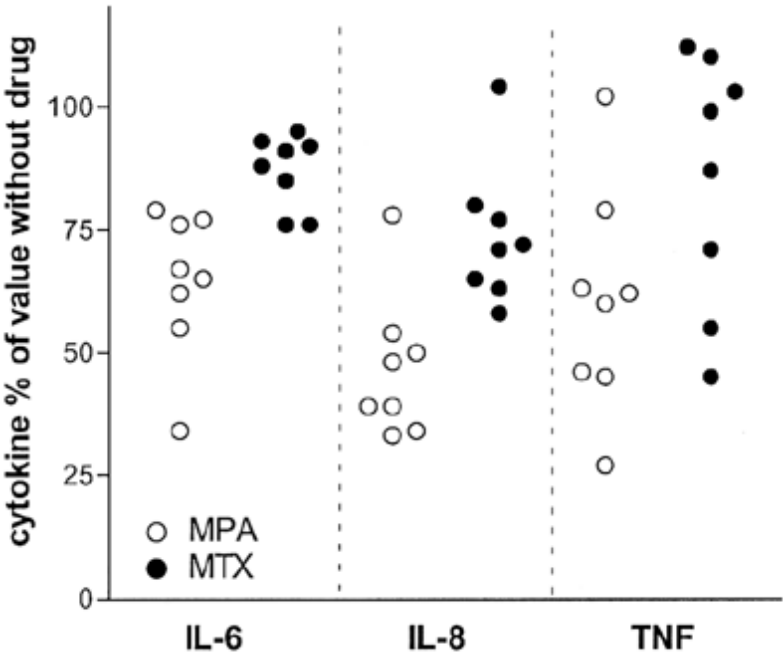


Figure 4

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 2 mug/ml MPA (o) or 2 mug/ml MTX (*). After 24 hours, the production of cytokines was measured by ELISA. The production of IL-6 ranged between 1,853-3,780 pg/ml, IL-8 between 5,257-26,030 pg/ml and TNF-alpha between 270-1,053 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug.

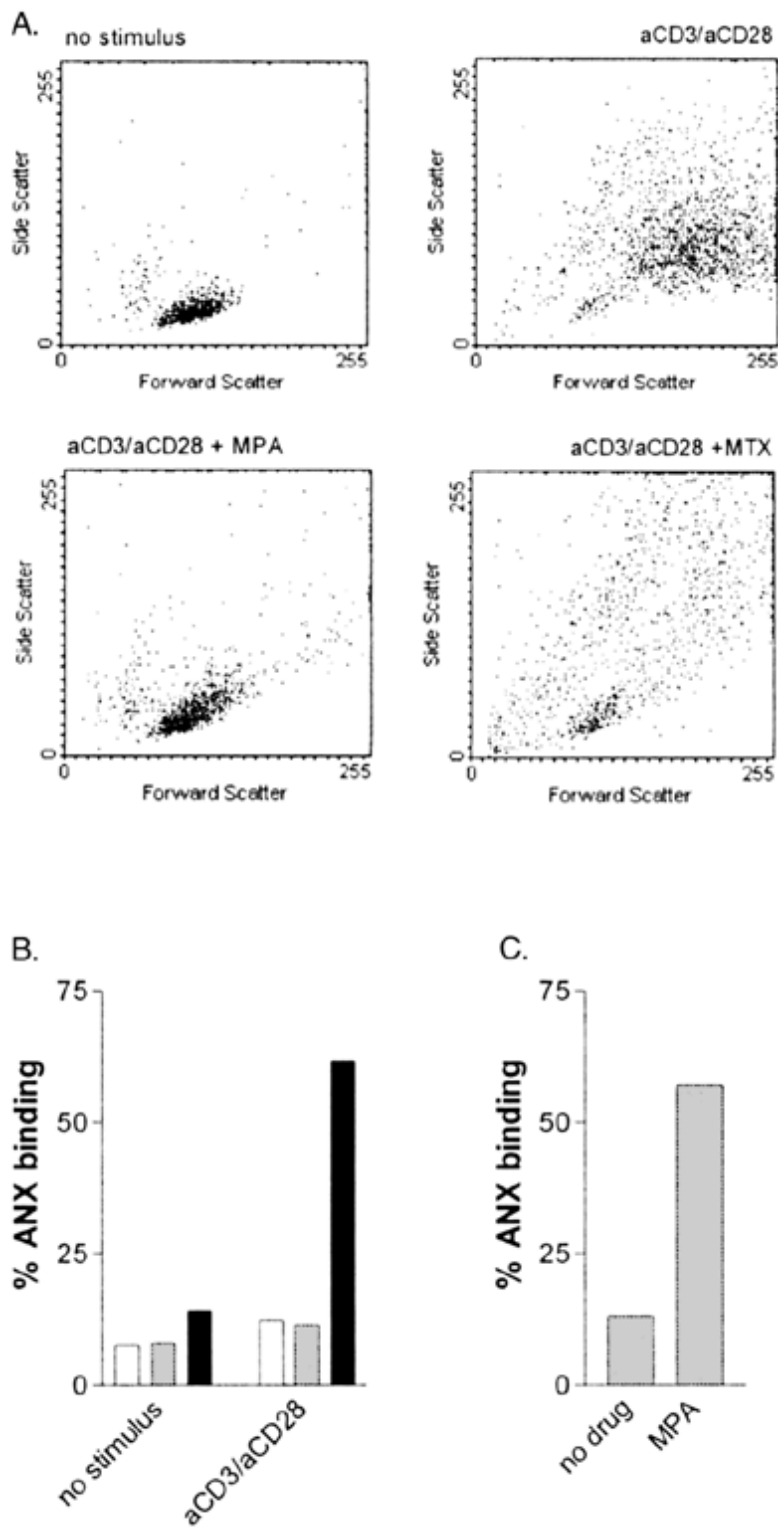


Figure 5

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. **A:** 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). **B:** 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/anti-CD28 (right three bars) in the presence of; no drug (white), MPA (gray) and MTX (black). Similar results were obtained in 3 separate experiments. **C:** 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 or with 2 mug/ml MPA. At day 6, cells were stained with annexin-V and were analyzed on the FACS. The percentage of annexin-V binding cells within the CD2 population is depicted on the Y-axis. Similar results are obtained in 2 separate experiments.

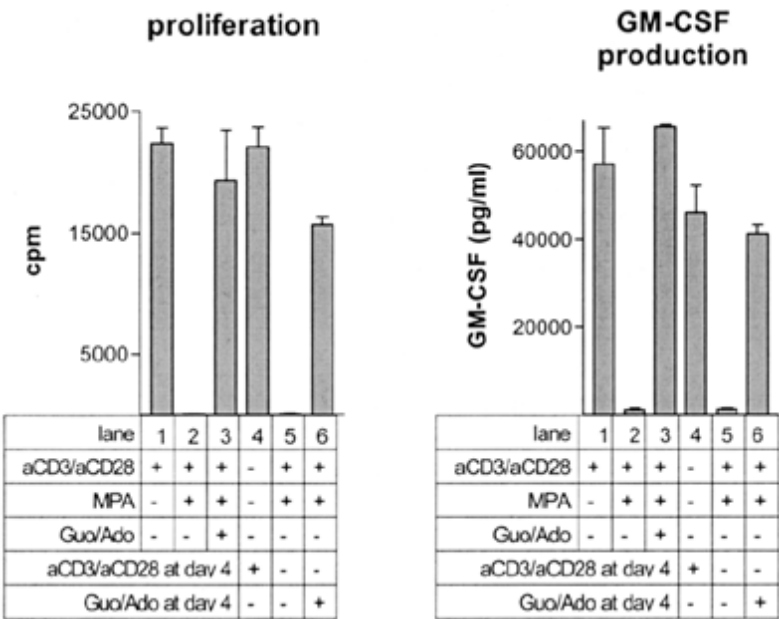


Figure 6

Adenosine and guanosine reverse the effects of MPA.

PBMC are cultured with the additions indicated. At day 8, 3H-thymidine incorporation and cytokine production were assessed. Similar results were obtained in 4 separate experiments. Error bars indicate the SEM of triplicate cultures.

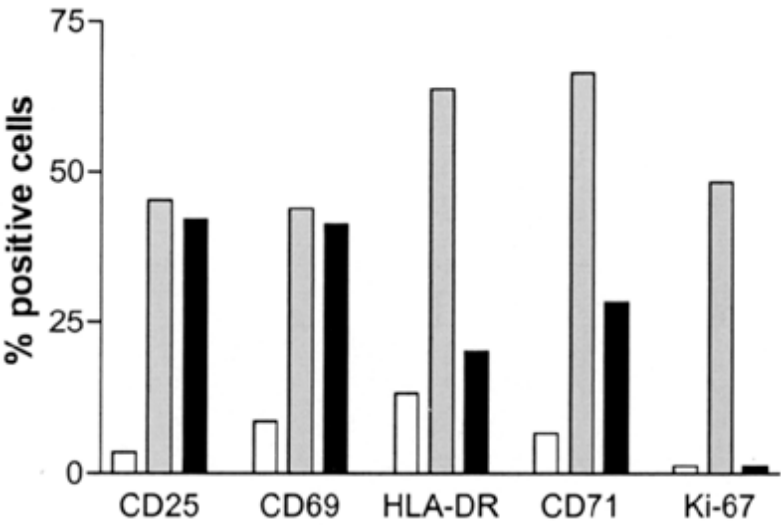


Figure 7

The effect of MPA on activation markers.

PBMC 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 were obtained in 3 separate experiments.

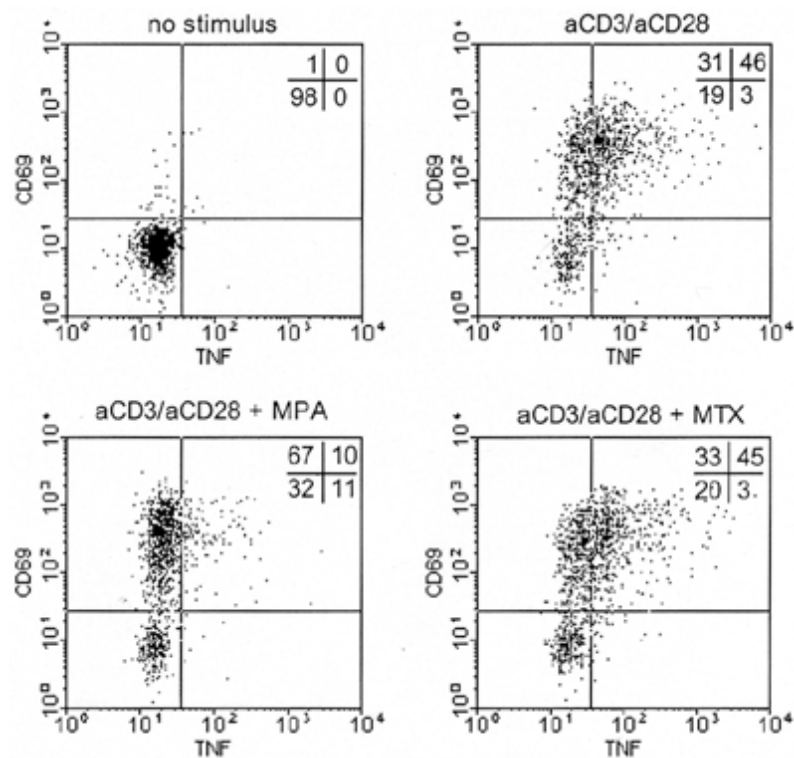


Figure 8

Intracellular TNF-alpha 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-alpha after permeabilisation. Non-stimulated PBMC (upper left panel), PBMC stimulated with anti-CD3/anti-CD28 (upper right 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-alpha staining on the X-axis. The percentage of cells in each quadrant is indicated in the upper right corner of every plot. Similar results were obtained in 3 separate experiments.

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