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

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Chapter 5

A differential effect of MPA on monokine production, evidence for induction of IL-1β production

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CHAPTER 5

Abstract

Mycophenolic acid (MPA) is an immunosuppressive drug used for the prevention of organ rejection after transplantation. We have investigated the effect of MPA on cytokine production by monocytes. MPA inhibited both SAC- and LPS- induced production of TNF, IL-8 and IL-6. LPS-induced IL-1β production was increased by MPA, whereas SAC induced IL-1β production was not affected. Elevated IL-1β production was not due to an increase of mRNA levels. In the presence of Z-VAD MPA does not increase IL-1β production. Therefore we suggest that MPA increases IL-1β processing rather than its secretion.
Introduction
The drug mycophenolate mofetil (MMF) or its active compound mycophenolic acid (MPA) is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) (1). It is widely used as an immunosuppressive drug. The immuno-suppressive properties have been well established for prevention of rejection after organ transplantation (2-4). Recently it has attracted interest as therapeutic agent for other diseases as well, because MPA has shown to reduce lupus nephritis in mouse models and in humans (3,5,6).
We have previously reported that MPA profoundly inhibits cytokine production after T-cell stimulation (7). MPA achieves this inhibition by preventing T cells to go into cycle at the G1/S transition (7,8). In this article we have extended our research on the mechanism of action of MPA by investigation of the effect of MPA on monocytes, with a focus on IL-1β production.
IL-1β is a pro-inflammatory cytokine produced by activated monocytes and macrophages. IL-1β acts on many cell types and has many functions. It can increase lymphocyte responses, induce fever and increase expression of adhesion molecules (9,10). As a consequence, IL-1β is implicated in various diseases such as rheumatoid arthritis and inflammatory bowel disease (11). IL-1β is synthesized in the cytosol as inactive 31 kD protein without a signal peptide. It is secreted as biologically active 17 kD mature form. Cleavage of pro-IL-1β occurs by IL-1β converting enzyme (ICE, also called caspase-1) concurrently with secretion (12,13). The mechanism by which IL-1β is cleaved and secreted has only recently been elucidated (14,15). Stimulation of monocytes or macrophages with LPS results in pro-IL-1β synthesis, which is neither secreted nor processed to the mature form. Secretion and processing follows after a secondary stimulus with ATP, via stimulation of the purinergic P2X7 receptor (14,16-20). The P2X7 receptor belongs to a family of ATP-gated ion channels. Upon binding to extracellular ATP the channel opens and is permeable for ions, upon longer stimulation a pore is formed that is permeable for larger ions and low-molecular-weight solutes (reviewed in reference (21)). P2X7 receptors are predominantly expressed on cells of the immune system, in contrast to the other P2X family members, which are more widely expressed. Stimulation of the P2X7 receptor with ATP leads to mature IL-1β production in LPS primed macrophages and monocytes (17,18). Blockade of the receptor with a monoclonal antibody results in inhibition of mature IL-1β production by monocytic THP-1 cell line after LPS and ATP (22). Further evidence for a role of the P2X7 receptor in production of IL-1β comes from P2X7 knock out mice. Macrophages of these mice are unable to make mature IL-1β upon stimulation with LPS and ATP (19).
The present study demonstrates that the anti-inflammatory drug MPA inhibits the production of IL-6, IL-8 and TNF after monocyte stimulation. However surprisingly IL-1β production is stimulated by MPA when monocytes are stimulated with LPS. This could have consequences for the application of MPA as an anti-inflammatory drug.

Materials and methods.
Heparinised blood collected from healthy volunteers was used for whole blood (WB) cultures and for isolation of peripheral blood mononuclear cells (MNC). 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. MNC were isolated from freshly drawn blood and separated over a Percoll gradient (d=1.078, Pharmacia Fine Chemicals AB, Uppsala, Sweden). MNC 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 96 wells flat bottom plates (Nunc, Roskilde, Denmark) or in 1 ml in 24 well plates (Nunc) for mRNA isolation and immunoprecipitation. Stimuli and drugs were freshly diluted in culture medium supplemented with FCS and directly added to the cell culture. The duration of the cultures is indicated for each experiment.

**Antibodies, stimuli and drugs.**

To stimulate the cells we used LPS (100 pg/ml, derived from *Neisseria Meningitidis*, a gift from Dr. J. Poolman, RIVM, Bilthoven, the Netherlands) and Staphylococcus aureus cells (SAC, Pansorbin, 1:4000, Calbiochem, La Jolla, CA). The drug MPA (GibcoBRL, Grand Island, NY) was freshly diluted in culture medium from a 2.5 mg/ml stock and used at 1µg/ml. Z-VAD (Alexis, Lausen, Switzerland) was dissolved to 40mM in methanol and used at concentration of 40µM. At a 1:1000 dilution methanol has no effect on the cells.

**Cytokine measurements.**

Mature IL-1β, IL-6, IL-8 and TNFα (PeliKine-compact, Sanquin Reagents, Amsterdam, the Netherlands) was measured according to the manufacturers 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 buffer (HPE, Sanquin Reagents). Plates were washed 5 times (PBS, 0.02% Tween 20) and incubated for 30 minutes with streptavidine 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 to the wells and was read at 450 nm.

For measurement of total IL-1β (pro IL-1β + mature IL-1β), sheep anti-IL-1β polyclonal Abs were used (S77, a kind gift of Dr. S Poole, NIBSC, Hertfordshire, UK). 10 µg/ml all-1β was coated and 1 µg/ml biotin conjugated all-1β was used. As standard curve we used rIL-1β from the pro-IL-1β ELISA kit. To measure intracellular proIL-1β the supernatant was removed from the cells and replaced by an equal volume of culture medium and cells were lysed by repeated freezing and thawing.

**Quantitative PCR.**

1x10^6 MNC (0.5 x 10^6/ml) were cultured as described above. After 6 hours supernatant was harvested and tested for cytokine production. Cells were lysed in Trizol LS (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturers protocol. Subsequently cDNA was synthesesised using Superscript and oligo-d(T) primers (Invitrogen). PCR was performed on a LightCycler machine (Roche Molecular Biochemicals, Mannheim, Germany) using a FastStart DNA master SYBRgreen I kit (Roche). The primer sequence for IL-1β was forward: GGATATGGAGCAACAAGTGG, reverse: ATGTACCAGTTGGGGAACTG and for IL-8 forward: TTGCAGGCTTCTCTG
ATTTC, reverse: AACTTCTCCACAACCCTCTCG and for β2-microglobulin (β2M) forward: CCAGCAGAGAATGGAAAGTC, reverse: GATGCTGCTTACATGTCTCG.

Relative amount of target (IL-1β or IL-8) mRNA was calculated according to the formula for relative quantification with an external reference (β2M) described in the LightCycler manual: 

\[ E_{\text{CpT(calibrator)\text{-CpT(sample)}}} = E_{\text{CpR(sample)\text{-CpR(calibrator)}}}. \]

In which CpT stands for crossing point of target and CpR for crossing point of reference and E for the PCR efficiency that was set on 2. An unstimulated sample was used as calibrator.

**IP and Western Blot.**

S77 polyclonal antibody was coupled to CNBr-activated sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) following the instructions by the manufacturer and stored in PTA (PBS, 0.1% Tween, 0.01% NaN₃). Immunoprecipitation was carried out in 500µl sample (supernatant or lysed cells) in presence of 0.1% Tween O/N at 4°C. Sepharose was washed 3 times with PTA and 2 times with PBS and diluted in sample buffer (NuPage, Invitrogen, Groningen, the Netherlands). Electrophoresis and Western blot were carried out using the NuPage system (Invitrogen). Blots were blocked for minimal 1 hour in 0.01M Tris, 0.15M NaCl, 0.05% Tween, (TBST), 5% skimmed milk powder plus 2% sheep serum and subsequently stained with biotinylated S77 polyclonal antibody (1 µg/ml) in TBST 0.5% skimmed milk powder. After washing 3 times 10 min in TBST the blot was incubated with streptavidin-HRP (1:1000, Amersham biosciences, Buckinghamshire, England) for at least 30 min in TBST, 0.5% skimmed milk powder. The blot was developed with ECLplus (Amersham).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prizm software. Wilcoxon signed rank test was used to determine differences in cytokine production in figure 1.

**Results**

**Effect MPA on monocyte cytokine production.**

In vitro production of monokines can be induced by SAC or LPS stimulation of a WB culture. MPA decreases IL-6, IL-8 and TNFα production induced by SAC by about 50% (figure 1a). This reduction by MPA is significant. IL-1β is not significantly reduced by MPA. Likewise, LPS-induced production of TNFα, IL-6 and IL-8 is decreased by MPA. Surprisingly IL-1β production induced by LPS is clearly increased in the presence of MPA (figure 1b). Similar results were obtained using MNC (not shown).

Figures 1a and 1b show cytokine production as a percentage of the production without MPA. In figure 2 the actual amounts of IL-1β production of the 8 donors are depicted after LPS and SAC stimulation. It shows that SAC is a more efficient stimulus for IL-1β production than LPS and that MPA increases LPS-induced IL-1β levels to those obtained by SAC stimulation.

To investigate whether changes in cytokine protein levels were a reflection of mRNA levels we have quantified IL-1β and IL-8 mRNA. Quantification of IL-8 mRNA was used as representative of the cytokines produced after LPS stimulation that are inhibited by MPA. Because the anti-coagulant heparin used in WB cultures can interfere with enzymes used in PCR and cDNA synthesis, we chose to use MNC instead. In contrast to the findings with IL-1β protein in the supernatant, we found an decrease in relative amount of IL-1β
mRNA in MPA treated MNC, when stimulated with LPS (figure 3). Relative IL-8 expression in LPS stimulated MNC is also decreased in MPA treated MNC (figure 3). Without LPS stimulation expression of IL-1β and IL-8 mRNA was less than 100 pg/ml (not shown).

**The effect of MPA on intracellular IL-1β.**
The IL-1β ELISA used for the experiments depicted in figure 1 and 2 measures the mature form of IL-1β only. To investigate whether MPA enhances the processing and secretion of proIL-1β we used an ELISA that can detect both mature- and pro-IL-1β. With this ELISA IL-1β was measured in supernatant and in cells (figure 4). As found before, MPA increased (mature) IL-1β production in the supernatant. In line with the notion that MPA might enhance processing of IL-1β, addition of MPA leads to a decrease of (pro)IL-1β in the cells.

![Figure 1. Effect of MPA on cytokine production after monocyte stimulation. WB (1:10) of 8 healthy donors is incubated for 24 hours with 1:4000 SAC (a) or 100 pg/ml LPS (b) with and without MPA. Cytokine production is depicted as percentage of the production in the absence of MPA. * p < 0.05, ns = not significantly different from production without MPA. Ranges of cytokine production after SAC stimulation in the absence of the drug are: IL-6 from 1453 - 5080 pg/ml, IL-8 from 8103 - 55810 pg/ml, TNF from 1197 - 4667 pg/ml and IL-1β from 1290 - 6020 pg/ml. After LPS stimulation ranges are: IL-6 from 1800 - 3860 pg/ml; IL-8 from 4650 - 26100 pg/ml; TNF from 220 - 1100 pg/ml and IL-1β from 580-2160 pg/ml.]

![Figure 2. Effect of MPA on IL-1β production. WB (1:10) of 8 healthy donors is incubated for 24 hours with 100 pg/ml LPS or 1:4000 SAC in the absence of MPA or presence of MPA. Mature IL-1β production of each donor is depicted, each donor is represented by a symbol.]
Figure 3. mRNA expression in the presence of LPS and MPA. Quantitative PCR was performed on PBMC that were cultured for 8 hours in the presence of LPS with or without MPA. Relative amount of IL-1β mRNA expression was calculated after the amount of mRNA in the samples was normalised on β2M mRNA expression. The amount of mRNA expressed after LPS stimulation was set to 100%, the percentage of mRNA expression in the presence of LPS and MPA is depicted on the y-axis. Error bars represent the SEM of several PCR reactions performed on samples of three independent experiments. IL-1β production without LPS was not detectable in ELISA, when stimulated the increase by MPA was 547%, 238% and 224% of the value with LPS alone in three experiments (from 265 to 1449 pg/ml, from 1085 to 2580 pg/ml and from 1236 to 2774 pg/ml respectively.

Effect of Z-VAD on IL-1β production.

The experiments described indicate that the increase of mature IL-1β after LPS stimulation in the presence of MPA is caused by increased processing, secretion or both rather than increased transcription or translation. To discriminate between secretion and processing a broad range caspase inhibitor, Z-VAD, was used to inhibit ICE activity. As expected mature IL-1β is not detected in the supernatant of LPS-stimulated cultures in the presence of Z-VAD (figure 5). Production of other cytokines was not affected by Z-VAD (not shown). Supernatants were also tested in the ELISA that detects both pro and mature IL-1β. With this ELISA IL-1β was detected in the supernatant in the presence of Z-VAD. Indicating that the pro form is secreted, as described previously (18,23).

There is no difference in IL-1β production in the presence of Z-VAD between cultures incubated with or without MPA (figure 5). Immunoprecipitation and Western blot analysis of these supernatants confirms that in the presence of Z-VAD only proIL-1β is secreted into the supernatant of LPS stimulated cultures. In the presence of Z-VAD there is no difference in proIL-1β secretion between cultures incubated with or without MPA (figure 6). Suggesting that MPA only influences the processing but not the secretion of IL-1β.
Figure 4. **Effect of MPA on mature and pro IL-1β production.** Total IL-1β production was measured with an ELISA detecting both in mature and pro IL-1β. Total IL-1β was detected in supernatant and cells as described in the materials and methods section. PBMC were cultured O/N with LPS in the presence and absence of MPA. In the absence of LPS cytokine values were not detectable. Error bars indicate the SEM of duplicate cultures. This is a representative of several experiments performed.

![Graph showing effect of MPA on IL-1β production](image)

Figure 5. **Differences between mature and total IL-1β production after Z-VAD treatment.** Mature and total (mature and pro) IL-1β were measured in supernatant. PBMC were cultured O/N with LPS in the presence and absence of MPA and/or 40µM Z-VAD.

![Graph showing differences between mature and total IL-1β production](image)

Figure 6. **Immunoprecipitation of both forms of IL-1β from cells and supernatant.** Pro and mature IL-1β are immunoprecipitated from supernatant and lysed cells with polyclonal anti-IL-1β coupled sepharose and analysed on western-blot as described in materials and methods. The blot was stained with the same polyclonal Ab. Culture conditions are indicated in the figure. Positions of pro IL-1β and mature IL-1β are indicated with a black and white arrow respectively. In lane M proteins of the sea-blue marker are indicated with their size in kDalton.

![Western blot showing immunoprecipitation of both forms of IL-1β](image)
Discussion.

We demonstrated that MPA inhibits the production of inflammatory cytokines induced by SAC and LPS with the exception of IL-1β. MPA increases the production of LPS-induced mature IL-1β and does not influence SAC-induced IL-1β production. The increase of mature IL-1β protein levels induced by MPA was not caused by the increase in mRNA expression. Like IL-8 mRNA expression the expression of IL-1β mRNA was decreased to 65-70% of the expression induced by LPS alone.

In many recent reports it has been described that LPS induces only intracellular proIL-1β accumulation in monocytes and macrophages but does not induce much mature IL-1β secretion. For secretion of IL-1β from LPS treated monocytes, a secondary stimulus is necessary. This second stimulus can be given by multiple agents such as nigrecin, ATP or cell injury (16,17,24).

Recently, induction of secretion of IL-1β by ATP has received much attention. There are indications that this induction is a result of the activation of the P2X7 receptor by ATP (14,18-20). As described previously (13) we do find considerable amounts of mature IL-1β in the supernatant after 6 to 24 hours stimulation with LPS alone. We do not see an increase of mature IL-1β secretion after an 80 minute ATP stimulation of our LPS primed cells (data not shown). This discrepancy can reflect a difference in culture conditions used (25).

As described before (26,27) we show that SAC is a better stimulus for the production of mature IL-1β than LPS. Furthermore, we showed that, in contrast to LPS-induced IL-1β production, SAC-induced IL-1β production is not increased by MPA. Because the intracellular activation pathways of LPS and SAC are different (28), it is possible that SAC is a better stimulus for the processing and subsequent secretion of IL-1β than LPS is. Indeed, stimulation with SAC at this concentration leads to complete secretion and processing of pro IL-1β. Therefore MPA does not have an additional effect on the production of mature IL-1β induced by SAC stimulation.

Stimulation of LPS primed monocytes with ATP in the presence of the caspase 1 inhibitor, Y-VAD, leads to secretion of pro IL-1β in the supernatant (18,23). These results indicate that the effect of ATP on the secretion of IL-1β is independent from the effect of IL-1β processing and thus that secretion is independent from processing. Our results point to an effect of MPA on the processing of pro IL-1β only. When processing of IL-1β is inhibited by Z-VAD, MPA does not increase the amount of pro IL-1β in our cultures. Only pro IL-1β is found in supernatants after Z-VAD treatment and it is the same amount for both LPS- and LPS plus MPA-treated cultures. This suggest that the secretion process is not affected by MPA.

The role of apoptosis in the maturation of IL-1β is not clear. Maturation can be induced by apoptosis (24). However, the induction of apoptosis is not necessary for the production of IL-1β as has been shown for LPS and ATP-induced IL-1β production (14). Another argument against the induction of apoptosis in the involvement of maturation of IL-1β is that the active form of caspase-3, one of the caspases responsible for the execution phase of apoptosis, is not detected after LPS plus ATP stimulation (29). Although MPA can induce apoptosis in activated cells (30,31) MPA does not induce apoptosis in initially resting (T-)cells (7). Therefore it is not expected that possible activation of ICE by MPA is a result of the induction of apoptosis in these cultures.
We conclude from these results that MPA can induce the processing of IL-1β but not its secretion. We think that MPA does so by activating ICE, although we do not present direct evidence for this hypothesis. Activation of ICE by MPA is remarkable and should be further investigated. Especially since ICE has been considered as a new target to suppress inflammation. Besides processing IL-1, ICE also cleaves IL-18 another pro-inflammatory cytokine that induces IFNγ production. Mice treated with a caspase inhibitor or deficient in ICE are protected against experimental mucosal inflammation (32,33). The same ICE inhibitor is currently under investigation in osteoarthritis models (34).

We report an IL-1β inducing effect of the immunosuppressive drug MPA in vitro. In view of the development of new immunosuppressants that inhibit the generation of mature IL-1β, one can question the use of MPA as anti-inflammatory drug. However, whether MPA can also induce mature IL-1β in vivo or whether this feature of MPA has clinical consequences remains to be investigated.

References


