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

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Chapter 1

General introduction
Rheumatoid Arthritis

Rheumatoid arthritis is a systemic chronic inflammatory joint disease. The disease affects about 1% of the population worldwide. The reasons why inflammation starts and continues within joints is unknown (1).

The normal joint consists of two bone ends covered with cartilage and connected by a capsule. The inside of this capsule is covered with the synovial membrane (1-3 cell layers thick) and is filled with synovial fluid (SF). In the inflamed joint the synovial membrane is thickened and invaded with cells (macrophages and T-cells), the SF contains many neutrophils, cartilage is destructed by proteolytic enzymes produced by macrophages and fibroblast like synoviocytes (FLS), and in progressed stages also bone is destructed. Eventually, bone destruction leads to deformations and disability.

In the SF and synovial tissue of RA patients a multitude of cytokines can be detected, such as tumor necrosis factor-alpha (TNF\(\alpha\)), interleukin-1beta (IL-1\(\beta\)), IL-6 and IL-8. These cytokines, and especially TNF\(\alpha\) and IL-1\(\beta\) play an important role in the ongoing inflammation. The actions of several cytokines will be discussed later in this chapter. Activated CD4+ T-cells stimulate monocytes, macrophages and fibroblast-like synoviocytes (FLS) present in the joint. When stimulated, these cells produce cytokines and matrix metalloproteinases. These soluble mediators can subsequently stimulate chondrocytes, endothelial cells and osteoclasts present in the synovium. In addition, osteoclasts, chondrocytes and FLS can be stimulated by T-cells through expression of cell-surface molecules. Furthermore, CD4+ T cells stimulate B-cells to produce immunoglobulins such as rheumatoid factor.

Clues for the pathogenesis

It has been observed that infection with *Borrelia burgdorferi* can induce arthritis. Much research has been done to find other infections that provoke RA. However, evidence for the idea that RA is provoked by a preceding infection has not been found. Whether in response to infection or to autoantigens, it is clear that hemopoietic cells play an important role in the disease. There is an unsettled debate about which cells are important in the pathogenesis of RA. Association between susceptibility and severity of disease and certain HLA-DR4/DR1 epitopes (2) provides a strong argument for T-cells, since the primary role for the HLA-DR molecule is to activate T-cells. T-cells are also important in several animal models. Collagen-induced arthritis (CIA), for example, is an antigen-driven model where the antigen is type-II collagen. Recent identification of antibodies against deiminated fibrinogen detected in 70% of early RA patients (3,4) urged others to investigate the T-cell response to deiminated peptides. In mice, transgenic for one of the HLA-DR epitopes genetically associated with RA, deimination of peptides leads to a higher affinity with the MHC molecule and to CD4+ T-cell activation (5). Whether deiminated fibrinogen really is an autoantigen that can cause RA in humans remains to be investigated.

Since macrophages and fibroblast-like synoviocytes (FLS) produce the actual destructive mediators, such as matrix metalloproteinases, these cells are thought to play a central role (6,7). Evidence comes from the abundant expression of cytokines by these cells in synovial tissue, whereas there is hardly any detectable cytokine production by T-cells (8). Analysis of RA synovial tissue demonstrates that monocytes, FLS, and macrophages are activated. There are indications that FLS can act independent from T-cell control in the
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destructive phase of RA (for references see (9)). Furthermore, mutations in the p53 tumor suppressor gene are found in RA patients. Therefore some authors believe that FLS of RA patients behave like transformed cells (7).

There are also indications that RA can develop independent from T-cells. K/BxN T-cell receptor transgenic mice spontaneously develop RA like symptoms (10). Injection of serum from sick mice induces arthritis in healthy mice. The presence of antibodies directed against glucose 6-phosphate isomerase, a glycolytic enzyme, is responsible for the induction of disease (11). A serological test frequently used for the assessment of RA is the determination of rheumatoid factor (RF). RF are autoantibodies directed to the Fc portion of multimerised IgG. RF can be of the IgM, IgG or IgA isotype and are present in 80% of the RA patients. Production of (auto) antibodies by B-cells can be a part of the pathology of RA. However, since B-cells need T-cell help for antibody production and isotype switching, the T-cell could still be the crucial cell type. Interest for the role of the B-cell has also been revived after reports stating improvement of the disease after treatment with B-cell depleting chimeric antibodies to CD20 (12,13).

Most likely different cell types are relevant at different stages of the disease. Although there is no evidence for antigen driven T-cell activation in RA, T-cells may be important in the perpetuation of inflammation in RA (9,14). A better insight in the function of several cell types will lead to a better understanding of the pathogenesis of RA, which will ultimately lead to better therapies to counteract the disease.

Cytokines

Cytokines are important mediators in the immune system, regulating both innate and adaptive immune response (15). Cytokines are secreted by immune cells upon activation and regulate growth, proliferation, activation, differentiation and cytokines can have chemotactic properties. Other cell types, such as endothelial cells and fibroblasts also secrete cytokines. Various cytokines have similar biological effects, and at the same time a single cytokine can have different effects on different target cells. Cytokines can perform their functions in an autocrine, paracrine or endocrine manner. Cytokines act through binding to specific receptors on their target cells. The expression of these receptors on target cells determines whether or not a cell can respond to that cytokine.

Normally the inflammatory process is tightly regulated by cytokines; there is a balance between cytokines that initiate and maintain the response, (pro-)inflammatory cytokines, and cytokines that suppress the response, anti-inflammatory cytokines. However, during chronic inflammation, such as in RA, the balance between the pro-and anti-inflammatory cytokines is not maintained. Imbalanced production of cytokines can be responsible for the clinical symptoms and tissue damage seen in affected joints of RA patients (16,17). Many cytokines are found in synovial fluid; however, the relevance to the pathogenesis remains unclear. Cytokine expression in synovial tissue is probably more relevant, as this is the site where the inflammatory reaction takes place. Immunohistochemic analysis and the analysis of mRNA expression in inflamed RA tissue shows expression of many proinflammatory cytokines, such as TNFα, IL-1β, IL-6, granulocyte-macrophage-colony-stimulating factor (GM-CSF) and the chemokine IL-8 (8,17,18). A short description of their actions will be given.
Inflammatory cytokines

TNFα and IL-1β are prototype inflammatory cytokines. TNFα is produced by macrophages, monocytes, and T-cells. TNFα can bind to two forms of the TNF receptor, p55 and p75. Both receptors are also found as soluble form (sTNFR). TNFα is a potent inducer of other cytokines, such as IL-1, IL-6, IL-8 and GM-CSF. TNFα can also stimulate fibroblasts to express adhesion molecules and indirectly activate osteoclasts which are responsible for bone degradation (for references see (16)). Intra-articular injection with TNFα results in accelerated onset and more severe arthritis in a mouse model of collagen induced arthritis (CIA) (19). In addition, administration of monoclonal antibodies against TNFα led to attenuation of the disease (20,21). A dominant role of TNFα in the pathology of RA is also likely in man, since therapeutic blockade of TNFα results in major clinical improvement (22-24).

IL-1β is also produced by macrophages and monocytes. IL-1β is a member of the IL-1 family, of which IL-1α, IL-18 and IL-1 receptor antagonist (IL-1RA) are other members. IL-1β can stimulate IL-6 production and the production of chemokines (25). Both TNFα and IL-1β are implicated in cartilage degradation. IL-1β can stimulate the release of matrix metalloproteinases from chondrocytes and fibroblasts (reviewed in ref. (26)). TNFα and IL-1β increase the ratio collagenase (MMP)/ tissue inhibitor of MMP (TIMP), secreted by different cell types in vitro (27). Like TFNα, IL-1β can induce inflammation and cartilage degradation in synovial joints in animal models (28). IL-1 is produced as inactive precursor protein, it is activated upon cleavage by IL-1 converting enzyme (ICE, caspase 1). Intervention with IL-1β signaling can reduce arthritis in CIA models (29). The naturally occurring IL-1 receptor antagonist (IL-1RA) can antagonize the actions of IL-1β. By binding to the IL-1 receptor, IL-1RA prevents binding of IL-1β or IL-1α and thus signaling through the receptor. Consequently, IL-1RA has anti-inflammatory properties and is presently tested for its efficacy to treat RA. Active IL-18 is also derived by cleavage of the precursor form by ICE. IL-18 is primarily produced by macrophages. The relevance of IL-18 in RA was demonstrated by the enhancement of CIA and, on the other hand, by IL-18 knock out mice that have a delayed onset and milder severity of CIA (31, reviewed in (30)). Enhancement of CIA by IL-18 may work via recruitment and activation of neutrophils (31,32). IL-18 production is found in synovial cells in RA tissue and is correlated to the expression of IL-1β and TNFα (30,33). Addition of IL-18 induced the production of several inflammatory cytokines in vitro synovial tissue cultures and IL-18 works synergistically with IL-12 to induce production of interferon-γ (IFNγ) in synovial T cells (30,33).

IL-6 is secreted by mainly by monocytes. IL-6 can stimulate B- and T-cell growth and differentiation and induces the production of antibodies. In addition, IL-6 activates osteoclasts. Although considered to be a pro-inflammatory cytokine, IL-6 decreases the MMP/TIMP ratio, resulting in a tissue protective effect (27). Furthermore, IL-6 is not arthritogenic. IL-6 is of importance in the pathogenesis of arthritis, since the onset of CIA is delayed and arthritis is attenuated in IL-6 knock out mice and ever resistant against antigen induced arthritis (34). Furthermore, anti-IL-6 and anti-IL-6 receptor therapy has been shown to work beneficial in RA patients (35,36). IL-6 induces production of acute phase proteins such as CRP and can itself be considered an acute-phase protein. Like CRP it can be measured in human serum as marker for disease activity. Also present in synovial fluid from RA patients is IL-8. IL-8 can be secreted by monocytes and granulocytes, fibroblasts and endothelial cells. It is a chemokine that is chemotactic
for neutrophilic granulocytes. In addition, IL-8 promotes angiogenesis. GM-CSF is produced by T-cells and monocyctic cells. As its name indicates, GM-CSF stimulates growth and differentiation of granulocytes and macrophages, and induces cytokine production in these cells. IL-15 production was found in macrophages in synovial tissue of RA patients. IL-15 is a growth factor for T-cells (reviewed in (30)).

Anti-inflammatory cytokines.
IL-10 is secreted by T-cells and monocytes and most hemopoietic cells express the IL-10 receptor. IL-10 has several functions. Inhibition of cytokine production in T-cells, inhibition of the activation of monocyctic cells, and consequently, inhibition of the co-stimulatory capacity of APC, are anti-inflammatory properties of IL-10. On B-cells IL-10 induces isotype switching, prolonged survival and stimulated proliferation (37) and references herein).
Transforming growth factor beta (TGF-β) is also considered to be anti-inflammatory. TGFβ has a wide variety of functions extending from the regulation of adhesion molecule expression by endothelial cells and leukocytes, to the modulation of macrophage function, and the control of lymphocyte activation and proliferation. TGFβ, injected into an inflammed joint, inhibits cartilage destruction, but increases inflammation (38). IL-4 and IL-13 are produced by T-cells. Both cytokines stimulate B-cells. IL-4 and IL-13 inhibit the formation of T-cells from the inflammatory Th1 subset. IL-4 inhibits the production of IL-6 and IL-8 (for references see (16)).

Treatment of patients with RA
In the last 2 decades therapeutic strategies for RA patients have been changed. Traditionally, RA patients were treated according to the 'pyramid approach'. In the pyramid approach treatment was started with non-steroidal anti inflammatory drugs (NSAIDs), to reduce the pain and inflammation. NSAIDs do not have the potential to prevent progression of the disease. When NSAIDs are insufficiently effective, disease modifying anti-rheumatic drug (DMARDs) are prescribed. DMARDs usually have a delayed onset of action and are able to modify the disease. First relatively mild DMARDs such as hydroxychloroquine was used, followed by stronger DMARDs such as MTX (39). Since the publication of Wilske and Healey on remodeling the pyramid (40), the pyramid approach is abandoned. More aggressive treatment of RA and an earlier start is advocated by many groups. Several randomized trails have been undertaken to prove that direct treatment with DMARDs gives better results than the pyramid approach (41-43). An example of aggressive treatment is the combination of two or more DMARDs. Six months of intensive combination therapy with prednisolone, MTX and sulfasalazine (SSZ, salicylazosulfapyridine) results in a better control of the disease and lower rate of radiologic progression, than SSZ alone (44). This effect sustained during the follow up 4 to 5 years later, independent of the therapy following the combination therapy (45). Efficiency of a drug can be measured by evaluation of different clinical parameters, reflecting the activity of inflammation present in the patient, such as tender or swollen joint count (TJC and SJC), or erythrocyte sedimentation rate (BSE). A combination of these and more parameters is included in the DAS28. Furthermore, rheumatologists can determine radiologic progression of the disease by scoring visible bone erosions on X-rays of hand an feet.
There are many DMARDs used for treatment of RA. Many DMARDs originate from other disciplines. Nevertheless, these drugs are effective against RA. A short overview of several DMARDs will be given here, the effects of these drugs on cytokine production will be addressed in another section of this introduction.

**DMARDs**

Gold has been used for treatment of many diseases for centuries. In 1935 the first trial in RA was reported with an improvement of 70-80% and more followed. There are several forms of gold available for either intramuscular or oral administration. Even though gold reduces inflammation and delays radiologic progression, it is seldom used nowadays. Chloroquine (CQ) and hydroxychloroquine (HCQ) are antimalarial drugs. These 4-aminoquinoline derivatives differ from each other by one hydroxyl group at the end of the side chain. Antimalarials are successful in combination therapy, for example with MTX (46). In spite of the fact that antimalarials can decrease inflammation, they can not delay radiologic progression. SSZ was frequently used for the treatment of inflammatory bowel disease. SSZ was rediscovered for treatment of RA in the eighties and is capable of delaying radiologic progression (47). SSZ is most effective in combination with MTX and HCQ (46). Cyclosporin A (CsA) is often used to prevent graft rejection after transplantation. CsA inhibits T-cell activation by inhibition of transcription factor NF-AT, which leads to immunosuppression. Effect on radiologic progression is also observed. CsA is more efficient in combination with MTX than alone (48). However, the use of the drug is limited due to toxicity. A relatively new DMARD is leflunomide (N-(4-trifluoromethylphenyl)-5-methy1isoxazol-4-carboxamidine). Its efficacy was shown in placebo controlled studies in human (49,50). Leflunomide is a reversible inhibitor of dihydro-orotate-dehydrogenase, a rate limiting enzyme in the de novo pyrimidine synthesis. This action is unique among DMARDs; therefore its potential lies in combination with other drugs for example with MTX (51), because MTX inhibits purine synthesis and a late step in pyrimidine synthesis. MTX is considered as one of the most powerful DMARDs. MTX was developed for treatment of malignancies. MTX delays radiologic progression and diminishes inflammation. MTX has become the gold standard for RA treatment. Many other and newer DMARDs or combinations of DMARDs are compared to the efficacy of MTX alone. Another drug that inhibits purine synthesis is mycophenolic acid (MPA, mycophenolate mofetil). Its efficacy in RA treatment has been investigated (52,53).

In 1949 Hench et al. published that administration of cortisone to a patient with RA had favorable results, thereby discovering the therapeutic effects of glucocorticoids (54). Glucocorticoids have immediate and strong anti-inflammatory effects. These days the use of glucocorticoids is advocated for early aggressive treatment in combination with other DMARDs (reviewed in (55)). The mechanism of action of glucocorticoids is complex and involves regulation of gene transcription, via the transcription factors AP-1 and NF-κB. These days there are several recombinant proteins available for treatment of RA, these proteins are sometimes called biologicals. Anti-TNF therapy is of most importance in the clinic. Anti-TNF was used after the realization that TNFα is one of the key players in the pathogenesis of RA. Notwithstanding the spectacular efficacy of anti-TNF therapy a major problem is that it is expensive and therefore only prescribed when other therapies fail.
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The effect of anti-rheumatic drugs on cytokine production.
Understanding the role of various cytokines can lead to a better insight into the pathogenesis and perpetuation of diseases such as RA. Knowledge of the role that certain cytokines play has lead to the development of successful anti-cytokine therapies such as anti-TNFα treatment. It is known that many conventional DMARDs have an effect on cytokine production. In the following section several commonly used anti-rheumatic drugs are described with a focus on their effects on cytokine production.

Gold
Many reports have described the interference of gold compounds with monocyte and macrophage derived cytokines induced by bacterial products (56-58). However, there is a debate on which cytokines are inhibited and which are not. In rheumatoid synovial cells stimulated with IL-1β, incubation with gold decreases the production of IL-6 and IL-8 but not of GM-CSF (59). Inhibition was due to the fact that gold interferes with the DNA binding of NF-κB (59,60).
In a more recent report it is observed that in vitro incubation of patients or normal PBMC with gold results in an increased number of cells producing IL-6 and IL-10 measured by ELISPOT. Remarkably, the level of these cytokines in the supernatant was unchanged (61). Possible involvement of NF-κB was not investigated in this paper.

Antimalarials
CQ and HCQ are antimalarial drugs often used for the treatment of RA. Onset of clinical improvement is very slow, maximal improvement may not be achieved before 6 months of treatment (62). Antimalarials are often used in combination with other DMARDs such as MTX, which generally improves the efficacy (46,63). These agents accumulate, and probably perform part of their actions, in the acidous lysosomes and golgi complex, where many enzyme reactions are inhibited. This may affect various pathways of inflammation. It is observed that NK cell activity is inhibited and lymphocytes of CQ treated patients show reduced responsiveness to PHA. Both agents inhibit the production of inflammatory cytokines produced by monocytes and by T cells (36,64). Recently the mechanism by which CQ decreases TNFα production has been attributed to the inhibition of mitogen-activated protein (MAP) kinase signaling (65). Inhibition of NF-κB activation was not observed, nor was TNFα inhibition caused by the actions of CQ in the lysosomes (66). The MAP kinase pathway is not only required for TNFα transcription, it can be used for the transduction of many signals, therefore inhibition of this pathway may also lead to various other cellular effects reported for CQ. Because of the resemblance of the drugs, HCQ can work via a similar mechanism however, this has not been reported.

Sulfasalazine
In the colon SSZ is degraded into 5-aminosalicylic acid and sulfapyridine by the colonic bacteria. Interestingly several reports show that these compounds often do not have the same properties as SSZ (67). Similar to the drugs described before, the immunomodulatory action of SSZ is not clear. Most papers have focussed on the effects of the drug on macrophages. SSZ inhibits IL-12 production in macrophages stimulated with LPS in vitro (68). In vitro, macrophages pretreated with SSZ (in vitro and in vivo) stimulated T-cells to produce more
IL-4 and less IFNγ (68). Inhibition of macrophage activation has also been observed, leading to decreased stimulation of T-cells (69,70). A reason for decreased macrophage activity could be that SSZ induced apoptosis (71). Furthermore, SSZ was found to inhibit translocation of NF-kB to the nucleus in a cell line and in macrophages (68,72). However, the role of NF-kB is disputed by others who found inhibition of leukocyte accumulation in both wild-type and in NF-kB knock out mice. They claim that the effects of SSZ are mediated by increased adenosine release (73).

**Cyclosporin A**
CsA is a T-cell specific drug. CsA binds to an immunophilin and this complex can inhibit calcineurin. Calcineurin is a phosphatase required for activation of transcription factor NF-AT and its translocation to the nucleus. Several cytokine genes are not activated in the presence of CsA. One of the cytokines genes regulated by NF-AT is IL-2. In the presence of CsA the IL-2 gene is not transcribed and IL-2 is not produced. Consequently proliferation of surrounding T-cells can not be induced. Another cytokine under regulation of NF-AT is IFNγ. Intracellular concentration of IFNγ is decreased in CD4 T-cells from patients treated with CsA (74).

Some cytokines are indirectly inhibited by the intracellular actions of CsA. M-CSF for example is decreased by the depletion of IL-2 since IL-2 or IL-2R agonist could restore the production in the presence of CsA (75). In a study with RA patients, changes in cytokine levels from baseline to 16 weeks of treatment were compared between one group that received CsA in combination with MTX and a group that received MTX alone. Circulating levels of IL-2, IL-12, TNFα, IFNγ were reduced in the combination group, IL-10 was elevated compared to the group that received only MTX (76).

Another calcineurin inhibitor, FK506, also inhibits T cell proliferation (77). In addition, FK506 inhibits TNFα and IL-1β production by PBMC after anti-CD3/CD28 stimulation (78). The use of FK506 for RA is experimental; positive clinical results are described in a phase II trial (79).

**Leflunomide**
The primary action of leflunomide is the inhibition an early step in pyrimidine synthesis. This was demonstrated by the fact that the anti-proliferative effect could be antagonized by the addition of uridine or cytidine to the cell cultures (see references in (80)). At high concentrations of leflunomide this was only partially effective. A reason for this is given by the observation that leflunomide can stimulate TGFβ production and inhibits IL-2 production in vitro. Both actions may lead to suppressed immune reactions (81). Two recent reports demonstrate that leflunomide inhibits IL-6, IL-1β, TNFα, MMP-1 and MMP-3 production in cultures of human synovial tissue cells (82,83). Furthermore leflunomide is a potent inhibitor of the transcription factor NF-kB. In an open study 24 weeks of treatment with leflunomide and MTX of patients already on MTX therapy at the start of the study leads to a lower plasma concentration and a reduction in mRNA expression of several chemokines (MCP-1, TARC, and MDC) in PBMC (84).

**Glucocorticoids**
Glucocorticoids are often used in the treatment of rheumatic diseases. The production of many inflammatory cytokines is downregulated by glucocorticoids. Glucocorticoids bind to
intracellular glucocorticoid receptors and this complex can bind to glucocorticoid responsive elements on the DNA, affecting transcription either positively or negatively. But interaction between glucocorticoid-receptor and transcription factors may also be important. In mice, inhibition of cytokine production induced by TNFα by dexamethasone seems to be dependent on NF-κB activation, whereas in human FLS, taken form RA patients, inhibition by dexamethasone (Dex) seems to be independent of NF-κB (85-87). Production of IL-10 was increased in PBMC isolated from RA patients, 42 days after treatment with Dex (88). Both IL-4 and IFNγ production by PBMC were decreased by in vitro addition of Dex. However, the ratio IL-4/IFNγ increased, resulting in an immunosuppressive effect (88). Inhibition of collagenase expression by glucocorticoids has been ascribed to interaction between the glucocorticoid receptor and transcription factor AP-1 (89).

Mycophenolic acid

The immunosuppressive drug mycophenolate mofetil (MMF) or its active compound MPA is a product of several penicillium species. MPA is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) (90). Inhibition of IMPDH leads to inhibition of guanosine synthesis and subsequent guanosine depletion from the cells which leads to impaired DNA synthesis (91). The immunosuppressive properties of MPA have been well established for prevention of rejection after organ transplantation (92-94). Recently MPA has attracted interest as therapeutic agent for inflammatory diseases, because it has shown to reduce lupus nephritis in mouse models and humans (93,95,96). Nagy et al. have shown that MPA inhibits superantigen-induced cytokine production in human T cells whereas it had no effect on LPS- or mitogen-induced cytokine production (97). In the mouse, in vitro as well as in vivo, MPA inhibited TNFα and IFNγ production whereas it did not affect IL-6 production (98). Furthermore, no effect on IL-2 and IL-10 mRNA expression was seen after ConA stimulation of mouse cells (99). In human cells MPA blocked lymphocyte proliferation at the G1/S transition (100,101). It has been reported that MPA blocks proliferative responses in human T cells but that IL-2 production is not changed (99,102,103).

Therapeutic proteins

Strategies to inhibit TNFα

TNFα has been identified as a major player in the pathogenesis of RA. Successful blockade of TNF signaling in mouse models (29,104) have led to the development of anti-TNF therapies. At this moment there are two different approaches available to inhibit the action of TNFα; by administration of anti-TNF monoclonal antibodies or administration of soluble TNF receptors. Both approaches are clinical effective (23). Anti-TNF therapy in combination with MTX is more effective than MTX alone (24,105,106). Several groups have investigated the effect of anti-TNF Ab treatment on cytokine levels (reviewed in (33)). Treatment with a single anti-TNF Ab infusion results in a rapid decline of serum concentrations IL-6 and IL-1β. The extent of the decrease differs between the studies (107-109). An increase of TNFα was noted, for up to 30 days with a high dose of anti-TNF Ab (108). Furthermore decreases in IL-8 and other chemokines have been described after histological analysis of synovial tissue (109).
Strategies to inhibit other cytokines

IL-1 blockade has been attempted by administration of IL-1RA. Placebo-controlled trials demonstrate clinical improvement (110,111). The combination of anakinra and MTX yields better clinical results than MTX alone (112). Invasion of the synovial membrane by mononuclear cells is reduced after 24 weeks of treatment (113).

In an open trial, anti-IL-6 receptor Ab (MRA) was efficient in reducing RA (114), larger randomized trials are currently performed by the same group and by others (115). Endothelial growth factor VEGF was reduced in patients using IL-6R Ab (116). Beneficial results with anti-IL-6 have also been reported (35). Polyclonal anti-IFNγ Abs have also been observed to be beneficial in RA in a small placebo controlled trial (117). All other pro-inflammatory cytokines involved in RA are considered to be potential targets.

Strategies to deplete inflammatory cells

Efficacy of several anti-CD4 antibodies have been tested in open and placebo controlled trials (118-120). The outcome of these trials varies. Despite depletion of CD4+ cells no significant clinical improvement was found (119,120). In another trial beneficial effects have been reported, although these effects were not related to the decrease in CD4+ cells (118). Analysis of long term beneficial effects of an relatively non-depleting anti-CD4 mAb was stopped due to 'unacceptable CD4 lymphopenia' according to the authors (121). Effects on cytokine production were not investigated in these trials. Blocking T-cell function rather than deleting T-cells is another approach to diminish inflammatory responses. Currently under investigation is CTLA4-Ig that blocks costimulation via CD28 on the T-cell.

Also under investigation is the effect of B-cell depletion with anti-CD20 mAbs (rituximab) in two open trials improvement of clinical parameters was reported (12,122). Deletion of macrophages has been considered. Therapeutic benefits of local deletion and systemic deletion are investigated (122,123)

MTX

MTX (amethopterin, 4-amino 4-deoxy-N10-methylpteroyl-glutamic acid) is a folate antagonist. It was developed together with several other anti-folates more that 50 years ago for the treatment of malignancies (124). The target of MTX proved to be dihydrofolate reductase (DHFR) (125). Folic acid is a water soluble vitamin that can not be synthesized by the human body, hence dietary intake is essential. Folic acid is one of the many folate forms that are part of the folate metabolism. The main folate form in plasma is 5-methyl tetra-hydrofolate (THF). Folates, and also antifolates such as methotrexate, can enter the cell via the reduced folate carrier (RFC) or the folate receptor (FR). RFC has a greater affinity for MTX than for folic acid for the FR the reverse is true (126,127). Like MTX, folic acid (5-formyl-THF) is mainly transported by the RFC. Once MTX enters the cell MTX is rapidly polyglutamated by folylpolyglutamyl synthetase. This can have several advances. First polyglutamates are preferentially retained intracellular and second, there is evidence for an increased affinity to folate-dependent enzymes (128), reviewed in (129). While MTX-polyglutamates were detected in liver cells and erythrocytes, their presence in lymphocytes has not been convincingly demonstrated (130,131).

Several THF forms serve as one carbon donors for reactions in the purine and pyrimidine synthesis and methionine-homocysteine metabolism. Inhibition of DHFR by MTX results in
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the depletion of reduced folates available for these reactions. Besides the inhibition of DHFR, MTX is responsible for the direct or indirect inhibition of several other folate-dependent enzymes involved in the de novo pathway of DNA synthesis such as thymidine synthetase (132), AICAR transformylase (133), and amidophosphoribosyltransferase (134).

In 1951 the related drug aminopterin was found to inhibit cell proliferation in psoriasis and RA (135). But it was not until the 1980s that MTX was tested in placebo controlled trials for the treatment of RA (136-138). MTX is usually given in a weekly dose of 7.5-20 mg and administered either orally or by parenteral injection. Despite frequent adverse reactions to MTX, serious toxicity has been rarely reported. Gastro-intestinal problems, such as nausea or diarrhea, are most frequently seen (139). Folic acid and sometimes folinic acid supplementation are given to reduce these side effects. Indeed, there is less discontinuation through adverse effects of MTX treatment in patient groups treated with folic acid and folinic acid and only a slight increased dose of MTX was given to obtain similar responses in the patient groups (140). MTX is effective in 80% of the RA patients, it is unknown why some patients do not respond to therapy. In order to improve efficacy, decrease side effects and identify those patients that will not respond, elucidation of the mechanism of MTX is essential.

Although many target enzymes of MTX are known, there is a debate on the mechanism by which MTX works in a low, weekly dose. Several reports on the effects of cytokine production appeared. Immunohistochemical analysis of synovial biopsies show that IL-1β and TNFα, but not IFNγ and IL-1β were reduced after MTX treatment (141). In bone marrow derived mononuclear cells but not in peripheral blood derived mononuclear cells (PBMC) of RA patients, MTX enhanced IL-1RA secretion and inhibit IL-1β secretion in in vitro cultures (142). Since the cells were not stimulated for cytokine production, IL-1β production levels are very low in this report. Barrera et al. show that a single dose of MTX given to RA patients leads to a decrease in IL-1β production and not in TNFα production in PBMC, but not in WB, when the cells are stimulated with LPS, ex vivo, 48 hours after intake of MTX (143). A lack of effect on TNFα expression after LPS stimulation by MTX, in vitro, has also been demonstrated at transcriptional level in macrophages isolated from healthy individuals (71). Patients responding to 24 weeks of MTX treatment had higher levels of IL-10 after LPS stimulation ex vivo than non responding patients (144). These reports show that there is no consensus about the effect of MTX on monocyte derived cytokines. None of the reports use similar methods or measures similar cytokines, therefore it is not possible to draw a conclusion.

Circulating cytokines have been measured. Inflammatory cytokines such as TNFα, IL-6 and IL-1β, are higher in RA patients than in healthy subjects. Soluble cytokine receptors (TNFR p55, p75 and IL-2R) are measured as a reflection of the activation of the immune system in a patient. Unfortunately, besides the reduction of serum IL-6 there does not seem to be an unequivocal effect of MTX treatment on these cytokines and soluble receptors (145-147).

Modulation of T cell cytokine production by MTX also received attention. Ex vivo experiments show that, after more than 6 months of MTX treatment, the percentage of TNFα-producing CD4+ T cells declined, while an increase of IL-10-producing CD4+ cells was found (148). Similarly, in vitro stimulation of PBMC with PHA in the presence of MTX, leads to decreased mRNA levels of IL-2 and IFNγ, whereas mRNA levels of IL-10 and IL-4
increased under these conditions (149). Murine spleen cells cultured in the presence of MTX show a reduction in IFNγ and TNFα levels (98) Mice treated with MTX produce less IFNγ in one study (98) but not in another (150). In the latter TNFα production is reduced on protein level but not on mRNA level. In SLE mice, IL-1 and TNFα were decreased by MTX, whereas IL-2 and IFNγ were increased, resulting in a restoration of cytokine levels as in control mice (151).

The group of Cronstein has hypothesized that MTX inhibits inflammation because its use results in intracellular accumulation of intermediates of the purine synthesis leading to an increase of extracellular adenosine (152). Indeed adenosine has several immunosuppressive properties such as inhibition of neutrophil degranulation (153) and inhibition of expression of adhesion molecules (reviewed in (152)). Furthermore, in monocytes, adenosine increases IL-10 production, whereas it decreases TNFα production (51,154). Evidence for this hypothesis comes from animal models. A combination of two non-selective adenosine receptor antagonists, theophylline and caffeine, reversed the therapeutic effect of MTX in a rat adjuvant arthritis model. Furthermore, MTX decreased lymphocyte accumulation at inflamed sites in wild type mice but not in adenosine receptor knock-out mice (138). Others argue that the main target of MTX are T-cells, since MTX induces apoptosis in activated T-cells (155,156). However, induction of apoptosis by MTX has been disputed (134,157). MTX was found to be cytostatic, halting the cells in the G1 phase of the cell cycle (134).

Scope of this thesis.
The scope of this thesis is to elucidate the mechanism of action of low dose methotrexate in RA. We have used an in vitro whole blood system to study the effects on cytokine production. A better insight into the mechanism of action is important for the improvement of efficacy of the drug and for the reduction of the side-effects and toxicity that MTX causes in some patients. Our research has focussed on effects of MTX on cytokine production by various immune cells. Besides MTX, we have also investigated the effects on cytokine production of another drug that inhibits DNA synthesis, MPA.

In the first chapters of this thesis the effects of MTX and MPA on in vitro cytokine production are investigated. In chapter 2 effects of MTX are investigated. The mechanism of inhibition of cytokine production by MTX and MPA is studied in chapter 3. Effects of adenosine and MTX are compared in chapter 4. In chapter 5 the effect of MPA on IL-1β production by monocytes is described. In vitro effects of MTX on cytokine production are compared to the clinical outcome of RA patients in chapter 6 in order to be able to predict the response to MTX treatment before or soon after the beginning of treatment. Finally, in chapter 7 a new method for MTX measurement in plasma is described. With this method bio-active MTX can be measured. The results of the previous chapters are discussed in chapter 8.

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