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Studies of Pharmacological Interventions and
Pathogenesis of Rheumatoid Arthritis

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Leg Läkare

Stockholm 2002
Till min farmor Karin

och andra patienter med reumatoid artrit
Abstract

Rheumatoid arthritis (RA) is a systemic inflammatory disease primarily affecting the joints. The chronic inflammation frequently results in joint destruction and various forms of physical impairment. T cells are believed to be of importance for the propagation of many cases of RA due to the association with certain types of HLA class II, whose function is to present antigen to the T cell receptor. There is, however, evidence that also macrophages and B cells may be of prime importance in driving the inflammatory process in RA.

In this thesis, an approach has been made to study immune functions in RA during treatment with two different anti-rheumatic drugs, intramuscular gold and tumour necrosis factor-α (TNF)α-blockade with etanercept (a soluble TNFα-receptor), with the goal to learn more about RA pathogenesis. The mechanism of action of intramuscular gold treatment is not known but it has been suggested that gold may shift the immune system towards production of anti-inflammatory cytokines, rather than inducing a general immune suppression. We investigated the cytokine production in vitro in response to gold sodium thiomalate (GSTM), and found a stimulatory effect on monocyte dependent production of the anti-inflammatory cytokine interleukin (IL)-10 along with a decrease of interferon-γ (IFN)γ levels in corresponding supernatants. In concordance with these results, there was an increased IL-10 production during GSTM treatment in RA patients. In addition, the in vitro effect of GSTM on IL-10 production from peripheral blood mononuclear cells (PBMC) predicted development of skin reactions during in vivo treatment with GSTM, with low IL-10 production being associated with appearance of skin reactions. From these studies we conclude that intramuscular gold treatment has cytokine stimulating properties, and the stimulation of IL-10 production might have importance for the therapeutic effect of gold in RA. Moreover, the ability of RA patients to produce IL-10 in response to gold may influence the development of skin reactions.

RA T cells are hyporesponsive when stimulated with microbial antigens in vitro compared to T cells from the blood of healthy subjects. Activated monocytes/macrophages suppress T cell functions, possibly mediated through pro-inflammatory cytokines such as TNFα. We investigated peripheral T cell reactivity in RA patients during etanercept therapy and found an increased T cell reactivity against microbial antigens and collagen type II, an autoantigen. These findings indicate that T cell hyporesponsiveness in RA is, at least partly, TNFα-mediated and that TNFα-blockade may not only suppress but also stimulate certain aspects of antimicrobial immune defence and autoimmunity. The findings thus warrant further consideration of development of autoimmune reactions during TNFα-blockade therapy.

TNFα is also known to stimulate production of matrix metalloproteinases (MMPs), which are upregulated in the inflamed joint and highly associated with development of synovial degradation and joint erosions. During etanercept therapy, serum levels of both MMP-1 and MMP-3 were downregulated in parallel with the reduction of inflammatory parameters. Moreover, pre-treatment MMP-3 serum levels correlated with changes in disease activity during etanercept therapy.

Cytokine promoter polymorphisms are known to be associated with different levels of production of the same cytokine. This observation indicates that also intervention with a cytokine may differ in efficacy depending on genetic variations. Although TNFα-blockade is very efficient in ameliorating diseases activity in most of the treated patients with RA, about one third of the patients do not respond appropriately to this therapy and there are as yet no prognostic markers for clinical response. We analysed whether promoter polymorphisms of pro- and anti-inflammatory cytokine genes correlated with clinical response to etanercept. A combination of alleles conferring a normal TNFα production (-308 T1/T1) and high IL-10 production (-1087 G/G) was associated with good clinical responsiveness to etanercept. Another combination conferring high inflammatory capacity (A2 allele in intron 2 of the IL1RN gene and rare C allele in codon 25 of the TGFB1 gene) was associated with non-responsiveness. Thus, genetic polymorphisms that influence the balance of cytokines that are of relevance for the course of RA seem to be associated with clinical outcome of etanercept therapy. This finding may be of value for further studies possibly promoting the use of cytokine polymorphisms as predictors for response to various biological agents in the future.

ISBN 91-7349-372-4
Jon Lampa 2002
Original articles

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*Journal of Rheumatology* 29(1):21-28

II  Ernestam S, Lampa J (contributed equally), Rogberg S, Rönnelid J, Klareskog L, Hafström I  
Evidence for immunostimulatory effects of intramuscular gold in rheumatoid arthritis; correlation with skin reactions (Submitted)

III  Berg L, Lampa J (contributed equally), Rogberg S, van Vollenhoven RF, Klareskog L (2001) Increased peripheral T cell reactivity to microbial antigens and collagen type II in rheumatoid arthritis after treatment with soluble TNFα receptors  
*Annals of the Rheumatic Diseases* 60(2):133-139

*Rheumatology (Oxford)* 41:484-489

Genetic markers for the efficacy of TNF blocking therapy in rheumatoid arthritis (Submitted)

Note: In papers II and III the first two authors contributed equally to the work
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Front cover picture: Aurosomes (gold-containing granula) in a monocyte. Photo: J Lampa
### Abbreviations

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<td>ACR</td>
<td>American College of Rheumatology</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>AU</td>
<td>auranofin</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
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<tr>
<td>CII</td>
<td>collagen type II</td>
</tr>
<tr>
<td>COMP</td>
<td>cartilage oligomeric matrix protein</td>
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<tr>
<td>COX</td>
<td>cyclo-oxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CYA</td>
<td>cyclosporine A</td>
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<tr>
<td>DAS</td>
<td>disease activity score</td>
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<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DMARD</td>
<td>disease modifying anti-rheumatic drug</td>
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<tr>
<td>ds DNA</td>
<td>double-stranded DNA</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>enzyme linked immunospot assay</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
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<tr>
<td>GPI</td>
<td>glucose-6-phosphate isomerase</td>
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<td>GSTM</td>
<td>gold sodium thiomalate</td>
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<td>HAQ</td>
<td>health assessment questionnaire</td>
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<tr>
<td>HC gp39</td>
<td>human cartilage glycoprotein 39</td>
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<tr>
<td>HEVs</td>
<td>high endothelial venules</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule – 1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL-1 Ra</td>
<td>interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>LEF</td>
<td>leflunomide</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein – 1</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<td>MTX</td>
<td>methotrexate</td>
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<tr>
<td>NSAID</td>
<td>non-steroid anti-inflammatory drug</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PHA</td>
<td>phytohemagglutinin A</td>
</tr>
<tr>
<td>PPD</td>
<td>purified protein derivative</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RF</td>
<td>rheumatoid factor</td>
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<tr>
<td>SASP</td>
<td>sulfasalazine</td>
</tr>
<tr>
<td>SF</td>
<td>synovial fluid</td>
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<tr>
<td>Tbb</td>
<td>Trypanosoma brucei brucei</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFiβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor for metalloproteinases – 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule - 1</td>
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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease primarily affecting the joints. Progressive inflammation may subsequently lead to joint destruction and various forms of physical impairment. During the last decade progress has been made in the understanding of RA pathogenesis. Possible pathogenic pathways have been identified and target molecules, such as TNF$\alpha$, have been pinpointed, thereby allowing the development of efficacious therapeutic strategies. One way to further investigate pathogenesis is through mechanistic studies of pharmacological interventions used in RA. These studies may further increase the understanding of mechanisms that lead to the initiation and progression of the disease and may also provide the basis for development of more targeted therapies with reduced risks of associated side effects in the future.

In this thesis, pharmacological intervention has been studied with the purpose of learning more about the role of different cytokines and immunoactive cells in RA pathogenesis.

Rheumatoid Arthritis

Brief history

RA was first described as a disease entity during the eighteenth century. Initially, clinical observations sought to distinguish the disorder from other prevalent joint diseases, such as gout and rheumatic fever, and emphasized distinctive features, for example, its chronicity, joint deformities, female sex distribution, and disability. The term "rheumatoid" is derived from the greek term “rheumos” which means fluid.

The term “rheumatoid arthritis” was first used by Alfred Baring Garrod in 1859 (243).

Clinical course and epidemiology

Initial symptoms of RA often include joint pain and swelling, generalized fatigue, and stiffness that characteristically occurs after several hours of inactivity. The most frequently affected joints are the proximal interphalangeal joints, wrists, knees, ankles and the metatarsophalangeal joints (212, 3). Recurrent inflammation in the joints subsequently leads to various degrees of joint destruction and disability. In scientific litterature, RA is defined using the revised American College of Rheumatology (ACR) criteria for RA (12) (Table 1). The criteria represent a collection of symptoms and laboratory findings that are prevalent among
RA patients, but none of them should be considered as pathognomonic for the disease, reflecting our lack of understanding of specific aetiologic factors that lead to RA.

**Table 1.** The 1987 American College of Rheumatology (ACR) Classification criteria for RA

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
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<tr>
<td>1. Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.</td>
</tr>
<tr>
<td>2. Arthritis in three or more joint areas*</td>
<td>Soft tissue swelling or fluid (not bony overgrowth) observed by a physician, present simultaneously for at least six weeks.</td>
</tr>
<tr>
<td>3. Arthritis of hand joints</td>
<td>Swelling of wrist, MCP or PIP joints for at least six weeks.</td>
</tr>
<tr>
<td>4. Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas (defined in 2.) on both sides of the body (bilateral involvement of PIP, MCP or MTP joints is acceptable without absolute symmetry) for at least six weeks.</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous nodules over bony prominences, extensor surfaces or in juxta-articular regions, observed by a physician</td>
</tr>
<tr>
<td>6. Rheumatoid factor (RF)</td>
<td>Detected by a method positive in less than 5% of normal controls.</td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>Typical of RA on posteroanterior hand and wrist radiographs; it must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).</td>
</tr>
</tbody>
</table>

* Possible areas: right or left proximal interphalangeal (PIP) joints, metacarpophalangeal (MCP) joints, wrist, elbow, knee, ankle, metatarsophalangeal (MTP) joints.

Patients fulfilling ≥ 4 criteria are classified as having RA.

Patients with more than one diagnosis are not excluded.
The prevalence of RA is approximately 0.5-0.8% (140, 235) and women are two-to-three times more likely than men to develop the disease (102, 156, 239). RA is associated with premature mortality (291, 196) of which the major part represents cardiovascular mortality (291, 24). Moreover, RA is associated with increased risk of osteoporosis, irrespective of treatment (159, 219).

Assessment

Assessment of disease activity, joint damage, and change with time of these variables are essential tools in monitoring intervention in RA (269). It is considered that simple counts are better than weighted ones, swelling is a better indicator than tenderness, and a 28-joint count performs as well as for example the more comprehensive 53 joint counts (200, 201). Radiographically measured progression is the best method of assessing structural damage associated with the disease (166). However, when measuring effects of therapy, other factors such as inflammation and functional outcome also have to be considered. Different methods of assessment of disease activity are discussed in the methods section, page 32.

Figure 1. Advanced rheumatoid arthritis of the hands—metacarpophalangeal replacement. Chronic synovitis of the wrists and finger joints in long-standing rheumatoid arthritis is seen. Volar subluxation and ulnar deviation at the metacarpophalangeals led to considerable hand dysfunction especially in the more affected dominant right hand, in which metacarpophalangeal joint replacement has been undertaken. Swan neck deformities are present in multiple digits, especially the third to fifth digits of the left hand.

Aetiology

The aetiology of RA remains to be elucidated. Several studies have documented the occurrence of familial clustering of the disease. The concordance of RA is about 15% for homozgyotic twins (5, 232), suggesting that environmental factors in addition to the genetic influence may have an important role for development of the disease. A number of environmental factors have been associated with RA. Among these, smoking is considered most important (97, 233, 120), but breast-feeding, adverse pregnancy outcome, previous blood transfusion and obesity may also be important risk factors (245).

Genetic factors

The major histocompatibility complex (MHC) comprises a gene segment located on chromosome 6 and includes a large number of genes, several of which are involved in the immune system. The human leukocyte antigen (HLA)-DR region is comprised of one nonpolymorphic DRA gene whose polypeptide product, the α-chain, combines with the product of numerous polymorphic DRB genes (β-chain) to form HLA-DR heterodimers. HLA class II molecules are constitutively expressed on antigen-presenting cells such as macrophages, dendritic cells and B cells but also other cells can be induced to express HLA class II molecules under certain circumstances. Several different HLA-DRB1 alleles (HLA-DRB1*0401, *0404, *0408, *0101, *0102, *1001 and *1402) have been associated with RA in a wide range of populations (188). DRB1* 04-positive patients with RA have more progressive erosion compared to DRB1*04-negative patients (281, 270). About 75% of patients with RA have a specific amino acid sequence near position 71 of the β-chain of HLA-DR molecules, the “shared epitope” (91). Several studies report associations between these alleles and severity of the disease (152, 262, 144). Moreover, the association of shared epitope alleles and progression of joint damage may be affected by aggressive disease modifying treatment early in the disease course (144). However, it has been debated whether or not the shared epitope is involved in the pathogenesis in all conditions diagnosed as RA. For example, the frequency of these alleles in RA patients varies globally (209).

Taken together, both genetic and environmental factors contribute to the development of RA. The association with certain HLA-DR alleles indicates that specific immune reactions mediated by T cells are important in RA. However, exogenous inciting agents also seem to be of importance for the development of the disease.
Pathogenesis of RA

It is well recognized that pathophysiological pathways in RA synovium involve communication between a number of different inflammatory cells, for instance T cells, macrophages and resident cells of the joint.

Understanding the pathogenesis of RA is important for developing specific and efficient therapies. Below, I will briefly describe differences between normal and rheumatoid synovium, and define the cells and signalling pathways involved in RA pathogenesis and how these are affected by therapeutical interventions.

The normal synovium

The synovial membrane consists of connective tissue lining the joint capsule. Under normal conditions the synovial membrane is only 1-2 cell layers in thickness (1). Beneath the lining layer there is connective tissue that surrounds fibroblasts, dendritic cells, mast cells and blood and lymph vessels. Few leukocytes are present in normal synovium. Normal synovial lining cells express MHC class II molecules (131, 287) indicating that these synovial MHC class II expressing cells may contribute to a local T cell activation (130, 114).

Rheumatoid synovium

In RA, the synovial fibroblasts proliferate and the synovium is thickened. Massive influx of leukocytes accompanies marked vascular proliferation. The endothelium which comprises these new vessels develops the characteristic structure of high endothelial venules (HEVs) (294). HEVs facilitate the influx of leukocytes from the vascular space into the synovial tissue space through the expression of specific adhesion molecules and chemokine receptors (294, 86). T cells in the synovial membrane are mainly CD4+ cells with a mature, activated, memory phenotype (135). Furthermore, B-cells, monocytes, macrophages, dendritic cells, fibroblasts and mesenchymal cells contribute to the inflammation. In addition, a number of both pro- and antiinflammatory cytokines are expressed in the tissue (49). The cytokine pattern displays a vast heterogeneity between different RA patients, suggesting divergent pathogenic pathways (260).

The pannus is subsequently formed. It is comprised of a mass of synovial lining cells that extend into articular cartilage and bone. RA pannus tissue assumes many characteristics of transformed cells, exhibiting the properties of invasion of cartilage and bone, neovascularization, and oncogene expression (298). High levels of inflammatory mediators
stimulate mesenchymal cells, such as synovial fibroblasts, osteoclasts and chondrocytes to release tissue-destroying matrix metalloproteinases (MMPs), causing further destruction (137, 49). The normal and rheumatoid synovium is displayed schematically in figure 2 and the process of synovial inflammation in RA is summarized in figure 3.

Figure 2. Schematic picture of normal and rheumatoid joint
(Modified from Buckley; Science, medicine and the future: Treatment of RA 1997 July 26;315(7102):236-8 BMJ)
Figure 3. Process of synovial inflammation in RA. Antigen processing and activation of T cells, B-cells and macrophages. Intercellular adhesion molecules (ICAM) and lymphocyte function-associated (LFA) molecules promote migration of blood cells into the tissue. Inflammatory mediators including interleukins (IL), tumor necrosis factors (TNF), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), monocyte and neutrophil stimulating peptides (Mo-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), prostaglandins, and nitric oxide promote this complex inflammatory process. The synovium and T cells are the source of osteoclast differentiation factor (ODF; osteoprotegerin ligand), which binds to osteoprotegerin, thereby stimulating increased osteoclastogenesis and bone loss (90). These mechanisms lead to angiogenesis, synovitis and ultimately tissue destruction. RF cell: Rheumatoid factor cell; TcR—T-cell receptor antigen. TNF-α : TNFα (From Matteson E. Atlas of Rheumatology. Edited by Gene Hunder. ©2002 Current Medicine, Inc.)
**T cells**

The synovium in RA is predominantly infiltrated by mononuclear cells (264) and about 30 to 50% of the synovial cells are T cells, particularly CD4+ (79, 271). These cells exhibit phenotypic signs of activation and express MHC class II, CD69 (131, 4, 61) and CD45 RO (271), which indicates a memory phenotype. The most compelling evidence for the importance of T cells in RA is the association of the disease with certain MHC class II alleles, since the only known function of these molecules is to present antigen to the T cell receptor (TCR) as a first step of T cell activation (49).

T cells derived from synovial fluid (SF) mainly produce IFN\(\gamma\) (170, 204), suggesting that the proinflammatory phenotype of T cells (Th1) dominates in synovial tissue. Moreover, T cell production of IFN\(\gamma\) is enhanced in RA SF compared to that in blood (216). Differentiation of Th1 cells is promoted by IL-12 (107), which is produced by SF macrophages in RA (36, 127). The initiating agent for T cell activation in the joint is not known. One possibility is that joint-derived autoantigens may be presented to autoreactive T cells, thereby conferring proinflammatory actions by the activation of macrophages and B cells. However, despite the abundance of phenotypically activated T cells in RA synovium, the expression of T cell cytokines such as IFN\(\gamma\) is low-grade (261, 271). Thus, there are many issues still to be solved concerning the role of T cells in RA. Are synovial T cells inducers of the pathogenic progress in RA or is their infiltration into the synovium a consequence of an inflammatory process already in action within the joint?

RA T cells have a decreased ability to respond to recall antigens (280, 9, 164) and the mechanisms underlying this hyporesponsiveness are not fully understood. Activated monocytes have known suppressive effects on T cell function through the production of proinflammatory cytokines (56) and hydrogen peroxide (136). It has been speculated that the reduced T cell function in RA is caused by chronic oxidative stress. Thus, SF RA T cells have defects in T cell receptor (TCR) signalling (92, 164), which may be due to decreased intracellular levels of the antioxidant glutathione (92). Moreover, oxidative stress by activated macrophages has been shown to suppress expression of the CD3 zeta chain (CD3\(\zeta\)) of the TCR (190), and downregulation of CD3\(\zeta\) is associated with T cell hyporesponsiveness in T cells in RA SF, but not in peripheral blood (164, 22). Thus these studies indicate that an oxidative environment may have impact on T cell functions. With the introduction of new cytokine-targeted therapies it is possible to study the role of pro-inflammatory cytokines on T cell function in vivo. An increased T cell reactivity to microbial antigens was observed after treatment with the TNF\(\alpha\) blocking agent infliximab (54), indicating that TNF\(\alpha\) has substantial effects on T cell reactivity in RA.
The ultimate study of the role of T cells in RA pathogenesis would demand a specific intervention directed towards T cells followed by a close surveillance of both clinical and immunological effects. An example of this kind of approach are studies of the selectively T cell suppressive agent cyclosporine A, which has been shown to exert effect on clinical symptoms in RA (254), thereby suggesting an importance of T cells in some pathways of RA inflammation. Treatment with monoclonal antibodies against CD4 have resulted in transient disease suppressing effects in some patients (104, 207), but in a number of individuals there was no clinical improvement despite severe CD4 depletion (105, 40). These results are problematic to interpret due to other strong evidence for the importance of CD4+ T cells in RA pathogenesis.

B cells

Activated B lymphocytes are present in the rheumatoid synovium and may accumulate beneath the synovial lining layer or form germinal centers in association with T cells (1). However, the fact that RA may occur simultaneously with inactive or hypoactive B lymphocytes (87, 198) has indicated that B cells may not be essential in the development of all conditions diagnosed as RA.

Rheumatoid factor (RF), discovered by Waaler 1940, is an antibody directed against the Fc portion of human IgG and a high level of RF is a major immunologic abnormality in RA. However, RF is not essential for development of RA (3) and it has been debated whether the production of RF is a cause or an effect of the disease.

Autoantibodies directed against certain cartilage-derived antigen, in particular collagen type II (CII), have been recorded in subpopulations of RA patients (51, 53). CII antibodies are produced in synovial tissue and can transfer arthritis in animal models (292). The latter was also found for antibodies against glucose-6-phosphate isomerase (GPI) (161). Antibodies specific for the stress protein BiP, which is overexpressed in RA synovium, occur in the majority of RA patients (25), and antibodies against other cartilage-derived antigens detected in RA patients include cartilage oligomeric matrix protein (COMP) (283) and human cartilage glycoprotein 39 (HC gp 39) (276). None of the above described antibodies are found exclusively in RA, whereas citrullin-specific antibodies have proven highly specific in this context (222). These antibodies are produced locally in the synovium and thus are likely to be triggered by a citrullinated substrate in the synovial membrane (158). However, there are as yet no reports of the transfer of arthritis through citrullin-specific antibodies.

Selective B cell-blockade using antibodies against CD20 has recently been proven therapeutically successful in a small study of treatment-refractory RA patients (289), thereby suggesting that B cells have a pathogenic role, at least in some patients with RA.
Monocytes and Macrophages

Macrophages are numerous in the inflamed synovium and at the cartilage-pannus junction. They express MHC class II, pro-inflammatory cytokines such as IL-1, IL-6, IL-10, IL-13, IL-15, IL-18, TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as chemokines, chemoattractants and metalloproteinases (128, 41, 33, 89, 66). Interaction between macrophages and fibroblasts or T cells appears to be important in rheumatoid inflammation. For example, co-culturing of human synovial fibroblasts and monocyte cell lines has been shown to induce cartilage degradation in *in vitro* model systems (224) and direct contact between monocytes and macrophages may enhance production of cytokines and metalloproteinases (142, 143). Interestingly, this type of non-specific interaction does not require viable T cells (78). Moreover, the proinflammatory cytokine IL-15 causes T cells to enhance the production of TNFα by macrophages in a contact-dependent reaction (168). Therapeutical strategies aiming at suppression of monocyte/macrophage function include methotrexate and corticosteroids (128). Moreover, experimental anti-macrophage therapy proven to be effective in RA is leukapheresis with depletion of activated blood monocytes (182). Therapies aimed at blocking proinflammatory cytokines produced by macrophages are discussed below.

Antigen presentation

T cells recognise antigens as peptides bound to MHC molecules. The most efficient antigen presenting cells (APC) are the dendritic cells (DC), and these are enriched in rheumatoid synovium (195). Monocytes and/or macrophages, B cells and synoviocytes may also function as APCs (218). Soluble antigens are ingested by APC and subsequently presented on the surface by HLA II. This complex may engage with the TCR and activate T cells into several pro-inflammatory functions. It is known that macrophages derived from RA SF are phenotypically activated (16, 205) and are more efficient antigen presenters than macrophages of peripheral blood (130, 205, 288). As mentioned above, certain HLA-DRB1 genes are highly associated with RA. It has been suggested that arthritogenic antigens may be presented by RA-associated HLA-DR molecules to activate self-reactive antigen-specific T cells and initiate synovial inflammation. Cartilage-derived collagen type II (CII) as well as other connective tissue matrix proteins such as gp39 have been suggested to play a role in disease propagation in this context (132, 58, 276). CII and COMP may induce autoimmune arthritis in animals (253, 43) and T cells reactive to CII have been isolated from the SF (186, 149) and peripheral blood (23, 15) of RA patients. However, it is not clear whether these proposed autoantigens are involved already from the initiation of disease or only at a later stage when cartilage degradation is already evident.
Intercellular communication, cytokines and matrix metalloproteinases

The cells in rheumatoid synovium communicate via a network of molecules, among which the cytokines are of particular importance. Cytokines may bind to receptors of target cells, usually leukocytes, and cause biological effects such as cell proliferation and release of other cytokines. The degree of expression of these cytokines displays a vast heterogeneity between different individuals with RA, as demonstrated in studies of synovia (260). However, it is generally believed that in RA the balance swings in favour of pro-inflammatory as compared to anti-inflammatory cytokines. Below, I will briefly describe cytokines that have been suggested to be of importance in RA pathogenesis.

Pro-inflammatory cytokines

Tumour necrosis factor (TNF) $\alpha$

TNF$\alpha$ is considered to be a central cytokine in the pathogenesis of RA. It is known to exert a number of proinflammatory actions, including the stimulation of production of IL-1, IL-6, IL-8, GM-CSF, MMPs and prostaglandin E2 (49, 65). A summary of the effects in RA is shown in figure 4.

Figure 4. Effects of TNF$\alpha$ on immuno-active cells of the rheumatoid synovium.
TNFα is expressed at many sites within the synovial membrane, including the cartilage/pannus junction (108, 50). In RA SF, elevated levels of TNFα and soluble TNF receptors have been reported (221, 55, 210).

In addition, TNFα in combination with IL-1 is a potent inducer of synovitis (98). It is known that overexpression of TNFα causes development of chronic arthritis (123). Moreover, lymph node TNFα production precedes clinical synovitis in experimental arthritis (181, 223) and administration of anti-TNFα suppresses collagen induced arthritis (CIA) (284, 252). In human cell cultures, anti-TNFα administration has been shown to block IL-1 production (32). Blockade of the actions of TNFα have proven efficacious for disease suppression in RA (71, 175) and there are currently two agents approved in clinical practice. These are infliximab and etanercept, both of which have proven more efficacious than MTX in the retardation of radiographic progression in early RA (146, 19). Another TNFα-blocking antibody, D2E7 (adalimumab) (118) has also proven efficient in recent clinical trials (18) and is in the pipeline for clinical use.

**IL-1**

Together with TNFα, IL-1 is an important mediator of bone resorption and cartilage destruction (11). IL-1 is mainly produced by activated macrophages upon direct cellular contact with activated T cells. Interestingly, this mechanism is inhibited by apolipoprotein A1 (109). Once produced, extracellular IL-1, membrane-associated (IL-1α) or soluble (IL-1β) bind to IL-1 receptors. Binding to the functional IL-1 receptor (IL-1R1) is controlled by two different molecules, IL-1RII and IL-1 receptor antagonist (IL-1Ra), the latter being more effective in inhibiting IL-1 activities.

Animal studies have confirmed proinflammatory actions of IL-1. Injection of IL-1α and IL-1β into knee joints of rabbits resulted in severe inflammation within hours (194, 99), which could be blocked by IL-1Ra (100). Moreover, IL-1 Ra knockout mice have been shown to spontaneously develop inflammatory arthritis with many features similar to RA (103). In RA, IL-1Ra is approved as an agent retarding both inflammation and joint destruction (34, 52).

**IFNγ**

IFNγ is produced mainly by activated T cells and natural killer cells in response to immune and/or inflammatory stimuli such as IL-12 and has inhibitory actions on the production of IL-4 and the development of anti-inflammatory T cells (Th2) (241).
IFNγ exerts important macrophage activating actions and is the most potent inducer of MHC class II expression on mononuclear cells. Although T cells are abundant in the rheumatoid synovium, IFNγ is expressed in very small amounts (261). However, T cells derived from RA SF produce IFNγ spontaneously (170, 216). The production of IFNγ by synovial T cells may trigger the recruitment and activation of macrophages and DC (78). Moreover, IFNγ has been shown to increase expression of TNFα receptors on synoviocytes (10).

**Regulatory cytokines**

**IL-10**

IL-10, formerly known as cytokine synthesis inhibitory factor (CSIF), is an immuno-regulatory cytokine produced by T cells (76, 174) and monocytes / macrophages (66). It has inhibitory effects on several proinflammatory cytokines such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, TNFα, IFNγ and GM-CSF (76, 282, 77, 66). IL-10 is increased in RA serum and SF (61), downregulates MHC class II expression and also inhibits T cell proliferation (66). Moreover, IL-10 has inhibitory actions on Ig secretion by peripheral blood mononuclear cells (PBMC) through suppression of the accessory cell function of monocytes (202). In animal models, IL-10 has been shown to exert potent anti-inflammatory effects. CIA may be suppressed by administration of exogenous IL-10 (263, 193) and through systemic IL-10 gene transfer (74). However, clinical trials with recombinant IL-10 in RA were inconclusive (240) and therefore further studies may be needed to elucidate the pathophysiological role of IL-10 in RA.

**IL-4**

IL-4 is a T-cell derived anti-inflammatory cytokine which inhibits the production of several pro-inflammatory cytokines, i.e. IL-1β, TNFα and IL-6 (251). IL-4 also reduces bone resorption *in vitro* (171) and systemically injected IL-4 suppresses the chronic destructive phase in streptococcal cell-wall-induced arthritis in rats (8).
Cytokines with dual roles in arthritis

IL-6

IL-6 is an IL-1-inducible protein produced by T cells and monocytes and is also spontaneously produced by cultured fibroblast-like synoviocytes (93). IL-6 is elevated in both SF (93) and serum (106) of RA patients. It is the major factor regulating acute phase responses from the liver, and also exhibits lots of other proinflammatory properties, i.e. the stimulation of immunoglobulin synthesis in B cell lines and the differentiation of cytotoxic T lymphocytes. In RA, IL-6 activity in serum correlates with serum levels of C-reactive protein (CRP), α1-antitrypsin, fibrinogen and haptoglobin (106). IL-6 knockout mice do not develop antigen-induced arthritis (28) and blockage of IL-6 receptor ameliorates CIA in mice (247). Moreover, promising results of IL-6 receptor blockade in RA have been reported recently (184).

On the other hand, IL-6 has also been shown to express anti-inflammatory actions. Several studies have demonstrated IL-6-stimulated production of tissue inhibitor of metalloproteinases (TIMP)-1, suggesting a protective effect on cartilage degradation (227, 228, 265). These studies thus suggest a dual role for IL-6 in RA pathogenesis.

Matrix Metalloproteinases (MMPs)

MMPs are secreted mainly from monocytes/macrophages and are capable of degrading a variety of extracellular matrix protein components including the collagens, proteoglycans, fibronectin and laminin (290). There are at least 19 known human MMPs that can be divided into four groups: the collagenases, the stromelysins, the gelatinases and the membrane-type MMPs (MT-MMPs). The most important MMPs in RA are stromelysin 1 (MMP-3) and collagenase (MMP-1). Increased levels of MMP-3 have been detected both in serum (297) and SF (113) of RA patients. Highly significant correlations between matched samples suggests that serum MMP-3 is mainly derived from the synovium (296). Moreover, a correlation of serum levels of MMP and disease parameters (211, 111, 124) and joint destruction (199) has been observed. The main inhibitors of MMPs are the tissue inhibitors of matrix metalloproteinases (TIMPs), a class of low molecular weight proteins that form noncovalent high affinity complexes with active MMPs. TIMP-1 is increased in RA serum (296) and SF (113). TNFα and IL-1 constitute the most potent cytokines for the induction of MMPs (157).
Gene regulation of cytokines

A number of functionally relevant polymorphisms that are assumed to be of importance for the balance of pro- and anti-inflammatory mechanisms have recently been identified. Polymorphic sites located in the promoter regions for several of the cytokines involved in RA pathogenesis are depicted in table 2. Genotypes associated with minimal or maximal potentials for inflammatory responsiveness may be associated with different clinical features. For example, the allele TNF2 has been related to a higher \textit{in vitro} production of TNFα than TNF1 (286), (138). The influence of these TNF alleles was also investigated in combination with other selected alleles including the homozygous state of A at −1087 of IL10 (low-producer IL-10 genotype). In combination with TNF2 this IL-10 genotype has previously been shown to associate with high risk for heart transplant rejection (256), suggesting a functional importance of this genotype combination \textit{in vivo}. Thus polymorphisms in the regulation of cytokines that are important in RA pathogenesis may not only affect the natural course of the disease, but also the response to therapy.

Table 2. Cytokine gene polymorphisms with suggested pro- and anti-inflammatory potential

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Chromosome</th>
<th>Polymorphism position</th>
<th>Alleles</th>
<th>Functional role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFA</td>
<td>6</td>
<td>-308</td>
<td>G=TNF1, A=TNF2</td>
<td>Normal production of TNF</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upregulation of TNF production in different types of cells</td>
<td>(286)</td>
</tr>
<tr>
<td>IL10</td>
<td>1</td>
<td>-1087</td>
<td>G</td>
<td>GG genotype is associated with upregulation of IL-10 production in lymphocytes</td>
<td>(257)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>AA genotype is associated with downregulation of IL-10 production</td>
<td></td>
</tr>
<tr>
<td>TGFB1</td>
<td>19</td>
<td>+915, codon 25</td>
<td>G</td>
<td>Normal production of TGFβ1</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>Downregulation of secretion of TGFβ1 from the peripheral blood leukocytes</td>
<td></td>
</tr>
<tr>
<td>IL1RN</td>
<td>2</td>
<td>Intron 2</td>
<td>A1=4 repeats, A2=2 repeats</td>
<td>&quot;Normal&quot; allele</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upregulation of IL-1Ra production in sera and downregulation in saliva</td>
<td>(192)</td>
</tr>
</tbody>
</table>
Interference with the cytokine pattern

Apart from methods blocking cytokine actions and the administration of recombinant cytokines, there are other methods that can be used to influence cytokine patterns in animal models as well as in RA.

In animal models of arthritis it has been shown that infection with the parasite Trypanosoma brucei brucei (Tbb) will suppress CIA (162). This indicates that the joint inflammation may be modulated by immune mechanisms related to immune responses towards an external antigen. Moreover, it was recently showed that mucosal administration of a bacterial antigen prevented CIA, partly by enhancing the activity of regulatory T cells (151). It has also been speculated that a change of the balance from a Th1 to Th2 dominated immune pattern would be advantageous for suppression of inflammation in RA and this approach has also been tried in animal models. Thus, immunization with the strong Th2 inducer alum downregulates CIA (163), suggesting an importance of these mechanisms at least in this animal model of arthritis. However, gold compounds that have many similarities to alum do not suppress CIA (165, 237).

Several studies have demonstrated a decreased prevalence of allergic diseases in patients with RA. It is suggested that the prevalence of hay fever in patients with RA is significantly lower than it is in appropriate controls (277). Moreover, in another study the incidence and prevalence of atopy was lower in patients with RA than in controls (3.5 versus 16.2%) and the cumulative incidence of atopy was significantly lower in patients with RA (7.5%) than in controls (18.8%) (7). These studies support the hypothesis that the occurrence of an immune response different from that apparent in RA might be beneficial by inhibiting Th1-mediated immunity. As pointed out earlier, IL-12 is a potent inducer of Th1 cell development and IFNγ-production. In attempts to enhance anti-tumour cellular cytotoxicity, IL-12 has been administered experimentally to treat different forms of cancer. It was then noted that IL-12 treatment in a woman with metastatic cervical cancer led to a severe exacerbation of her RA (191). There is also evidence for the induction of autoimmunity during treatment with the strong Th1 inducer IFNα (215), (119), (112). Moreover, van der Graaff et al showed that the baseline Th1/Th2 ratio in peripheral blood correlated with the disease activity score in RA patients after 9 months treatment with disease modifying antirheumatic drugs (DMARDs) (266).

Altogether these studies suggest that an induction of a change in the cytokine pattern in RA, preferably Th2-dominated, may modulate the disease causing mechanisms of RA.
Pharmacological therapy in RA

For a long time, treatment of RA was initiated with non-steroidal anti-inflammatory drugs (NSAIDs) followed years later by the use of DMARDs, often after clinical and radiographic evidence of joint destruction was demonstrated (273). During the last decade, treatment regimens have turned to a more aggressive approach using DMARDs early in the disease course, thereby providing better impact on long-term outcome. Below, I will give a brief description of the drugs used for symptom relief and disease retardation in RA and the pharmacological treatment guidelines of RA today.

DMARDs

This term includes a variety of drugs with the mutual principal effect of retarding disease progression in RA. These agents have a variable grade of toxicity and continuous monitoring with blood samples is often recommended. The most widely used DMARDs and biological agents for treatment of RA are presented in table 3. The regimen of early administration of DMARDs in the disease course has proven more efficacious than earlier treatment strategies with regard to clinical signs and symptoms (267, 180), radiographic progression (37), mortality (139) and quality of life (267). Combination therapy with different DMARDs has shown even better results on disease progression than did monotherapy (185, 176). The combinations proven to be efficacious in randomised controlled trials include methotrexate and other drugs, i.e. sulfasalazine (67), sulfasalazine and chloroquine (176), cyclosporin A (255), etanercept (274) and infliximab (146).

Table 3. DMARDs and biological agents for treatment of RA

<table>
<thead>
<tr>
<th>DMARDs</th>
<th>Biological agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimalarial: Chloroquine phosphate or Hydroxychloroquine</td>
<td>Etanercept</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>Infliximab</td>
</tr>
<tr>
<td>Sulfasalazine (SASP)</td>
<td>Adalimumab</td>
</tr>
<tr>
<td>Cyclosporin A (CYA)</td>
<td>IL-1Ra</td>
</tr>
<tr>
<td>Auranofin (AU)</td>
<td></td>
</tr>
<tr>
<td>Aurothiomalate – Intramuscular gold (GSTM)</td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td></td>
</tr>
<tr>
<td>Alkylating agents</td>
<td></td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td></td>
</tr>
<tr>
<td>Leflunomide (LEF)</td>
<td></td>
</tr>
<tr>
<td>CPH 82 (Reumacon®)</td>
<td></td>
</tr>
<tr>
<td>D-Penicillamine</td>
<td></td>
</tr>
</tbody>
</table>
Pharmacological treatment guidelines

Based on a number of longitudinal clinical and epidemiological studies current clinical guidelines for RA treatment emphasize 1) the need for early diagnosis, 2) identification of prognostic factors, and 3) early aggressive treatment.

It has become apparent during recent years that aggressive treatment early in RA has an important impact on radiographic progression (29, 242, 145) which is an important prognostic factor for long-term outcome (68, 166). Other poor prognostic features include the early onset of synovitis, joint erosions, high baseline HAQ-score and rheumatoid factor positivity (3, 68).

The general recommendations for treatment of RA therefore include early administration of a DMARD such as MTX. If this treatment is not sufficient for disease control, combinations of DMARDs described above or TNFα-blockade are recommended for disease suppression. A summary of the current recommendations for RA treatment is displayed in figure 5.

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![Figure 5. Treatment strategies in early RA](image)

Abbreviations: I: Insufficient; NT: Not tolerated.
* MTX/SASP/Antimalarials or MTX / CYA  ** SASP or SASP/Antimalarials or GSTM or LEF
There is an ongoing discussion if TNFα-blockade should be considered earlier in the disease course and not only for patients who respond inadequately to other DMARDs. However, reports of the association of TNFα inhibition with reactivation of tuberculosis and possibly other infections (275) warrant further studies of long-term safety before these drugs can be recommended as first-line agents. Another therapeutical question currently discussed is if it is possible to identify predictive markers for response to DMARDs and biological agents such as TNFα-blockade.

As opposed to DMARDs, NSAIDs have not proven efficacious in affecting the disease progression of RA, but nevertheless provide fast relief of pain and stiffness. The mechanism of action of these drugs is inhibition of constitutional cyclo-oxygenase (COX)-1 and induced COX-2 in varying proportions (172).

Glucocorticoids are used for suppression of more severe disease symptoms and can be given intraarticularly, orally or by parenteral administration. Several recent studies have reported the benefits of low dose corticosteroid treatment (129) (or an initially high dose, followed by fast tapering ((176), (29)) on the development of radiological damage in RA. However, glucocorticoids have long-term effects which are sometimes more severe than RA itself. These include diabetes, hypertension, excessive weight gain, cataract (82) and osteoporosis (150).

**Pharmacological intervention as a way to study the pathogenesis of RA**

As pointed out before in this thesis there is a vast diversity in the clinical symptoms expressed by different patients diagnosed with RA (49). It is likely that these disparities represent different disease mechanisms and diversity in the target cells and cytokines involved in pathogenesis. Moreover, there is a wellknown heterogeneity in the group of RA patients concerning the clinical response to the same DMARDs by deterioration of disease symptoms, retardation of erosions and the development of side-effects. Therefore, besides studying the production and presence of different cytokines and other markers in RA to learn more about RA pathogenesis, another way is to use a pharmacological approach, i.e. to study the mechanistic effects of drugs used for disease suppression. For some anti-rheumatic drugs we know some of the mechanisms, for example cyclosporine A, which has a distinct T cell suppressive effect (254) and the new targeted therapies blocking action of IL-1 and TNFα. For other agents, such as intramuscular gold, it is not known exactly how they
exert their anti-rheumatic action. Still, the concept of using anti-rheumatic drugs for studies of RA pathogenesis offers several possibilities. Firstly, studies of changes of the cytokine pattern during therapy may give information about the processes involved in RA pathogenesis that are possible to influence. Moreover, using this approach it may be possible to relate immunological mechanisms reflected by changes in cytokine patterns to the clinical symptoms and response to treatment. Secondly, the immunological causes of side-effects of the drugs may be studied. This may provide knowledge on how to treat these side-effects in an ultimate way and possibly also to define individuals at risk of developing certain undesired effects of one drug or a drug combination. Thirdly, using genetic approaches, it would be possible to find predictive markers not only for responsiveness to the drugs, but also for the presence of side-effects.

In this thesis, an approach has been made to study the mechanistic effects in RA during treatment with two different DMARDs, intramuscular gold and TNF\(\alpha\) blockers. The value of intramuscular gold in RA has been confirmed in controlled studies, both as to the improvement of disease activity (80, 2) and also in the reduction of cartilage destruction (231). Numerous gold-containing compounds have been used for treatment of RA and the parenteral administered gold sodium thiomalate (GSTM) is most widely used. Moreover, intramuscular gold appears to be one of the best, if not the best, DMARD for induction of long-lasting remission of the disease, even after withdrawal of the drug (206, 94). This among other features makes GSTM attractive as a model agent for the possible induction of a switch of the immune response in RA, conferring established low grade or absence of inflammation. As described earlier, TNF\(\alpha\) is a central cytokine in RA inflammation and its action is blocked by very efficient therapies. This makes mechanistic studies of TNF\(\alpha\)-blocking treatment important for the investigation of pathogenic pathways in RA.

**Intramuscular Gold**

**Pharmacokinetics**

The peak concentration of GSTM in serum is 7 µg/ml six hours after intramuscular injection but the serum concentrations during maintenance therapy is lower, between 0.75 µg/ml and 1.25 µg/ml (238). There is no relation between steady-state gold serum concentration and effect (84). After injection, there is a separation of the gold atom and the thiomalate moiety (116), leaving the gold atom free to bind to serum proteins. Thus the majority of injected gold
is protein-bound (90% or more) (64, 27). Several studies have shown the synovial uptake of gold, with higher concentrations observed in inflamed tissue (27, 278). However, it is not known whether or not the synovial membrane is the actual effector site for gold. The half-life of GSTM after intramuscular injection is about 5 days, followed by a slow excretion, preferentially via the urine (27, 85, 88). Adverse reactions, presenting predominately as dermatitis and stomatitis are more common than for other DMARDs (206, 94).

Effects on cytokines

The mechanism of action of intramuscular gold in RA is still unclear. In vitro studies have shown that GSTM has suppressive effects on T cell (226) and monocyte (101) function, as well as downregulation of several pro-inflammatory cytokines (95, 17). The relatively frequent presence of dermatitis and the presence of eosinophilia and increase in IgE (35) often preceding the beneficial effects of the drug, suggest that gold may alter the cytokine drive in RA in favour of more allergic immune reactions.

There are several reasons to study GSTM in order to understand certain features of RA pathogenesis. Firstly, RA is considered to be Th1 dominated. As described above, the properties of inducing rashes and dermatitis in a substantial portion of the treated patients makes GSTM likely to cause an allergic type of immune response, possibly mediated by the production of Th2 cytokines. Secondly, these studies may provide more knowledge about a possible correlation between clinical parameters and cytokine patterns. For example, it has been shown that transfer of spleen cells from rats treated with gold may ameliorate arthritis in recipient rats (42). In another experiment GSTM induced an upregulation of IL-4 mRNA accompanied by vasculitis in rats, suggesting a shift of the T cell population to the Th2 phenotype with production of anti-inflammatory cytokines (203). These observations make it reasonable to believe that, instead of suppressing immune functions by general inhibition of cytokine production, gold might have divergent effects on the production of both pro- and anti-inflammatory cytokines in RA.

Anti TNFα therapy

The TNFα blocking agents currently in clinical practice are infliximab, a chimeric monoclonal anti-TNFα comprising a human IgG1κ antibody with a mouse Fv of high affinity and neutralizing capacity (133), and etanercept, an engineered p75 TNFR dimer linked to the Fc portion of human IgG (173). The clinical efficacy of these agents has been described earlier.
The mechanisms of action considered to be most important during TNFα-blockade are suppression of the proinflammatory cytokine cascade at the site of inflammation and reduced recruitment of inflammatory cells from blood to the rheumatoid joint. CRP and IL-6 levels in serum decline rapidly during infliximab (71) as well as etanercept therapy (69) and in a study of 8 patients, the synovial expression of TNFα was reduced after treatment with infliximab (259).

Interestingly, in this study patients with the highest baseline TNFα synthesis achieved the best clinical responses. Moreover, infliximab has been shown to reduce the joint expression of E-selectin and VCAM-1 (246) and also the immunohistological expression of chemokines such as IL-8 and MCP-1 (250). Etanercept effects on cytokines have not been studied to the same extent as for infliximab. According to different studies, between 60 and 70 per cent of the etanercept treated patients achieve ACR20 response (19) and data on infliximab reveals similar results (154). This means that about one third of patients do not respond appropriately to these therapies. Thus, there is an increasing need to further understand the molecular basis of this heterogeneity and to identify predictive markers for clinical response.

**Figure 6.** Inflamed synovium from a patient with active RA and insufficient response to conventional DMARDs. Arthroscopy of the knee joint. *(Photo: J Lampa)*

**Figure 7.** Arthroscopy of the same knee joint after treatment for eight weeks with etanercept. The synovial capsule shows no signs of macroscopic inflammation. *(Photo: J Lampa)*
Aims

To study the mechanistic effects of anti-rheumatic therapies in RA with the goal of obtaining more knowledge about pathogenic mechanisms involved in the development of RA.

To study the relation between mechanistic effects of anti-rheumatic drugs and clinical efficacy of the drugs as well as the development of side-effects.

To study the possibility of finding genetic markers of efficacy of TNFα-blocking treatment.
Patients and Methods

Patients

In papers II, III, IV and V, all patients investigated met the American College of Rheumatology classification criteria for RA (12). In paper I, all patients but one (diagnosed with seronegative polyarthritis) met these criteria for RA. Informed consent was obtained from all patients before sampling.

Control subjects used in the investigations in papers I and III were either laboratory personnel or blood donors without any history or present signs of rheumatoid disease. All the studies were approved by the local ethic committee.

DAS 28 response score

The composite index "Disease Activity Score" (DAS), using a 28 joint score (DAS28) (201) includes number of swollen joints, number of tender joints, patients’ global assessment of disease activity measured on a 0-100 mm visual analogue scale (VAS) and erythrocyte sedimentation rate (ESR) and creates a score ranging from 0-10. According to this index, high disease activity is defined as DAS28 > 5.1 and low activity as DAS28 < 3.2. Good response is defined as an improvement of at least 1.2 and an end-point DAS28 value of <3.2. Moderate response is defined as either an improvement of at least 1.2 independent of the attending DAS28 value or an improvement of at least 0.6 in combination with an end-point DAS28 < 5.1 (268). This response score was used in papers II, IV and V.

ACR response score

Clinical response to therapy (papers III, IV and V) were assessed using the ACR response score (75) which includes the fulfillment of the change of total number of tender joints, swollen joints and 3 of 5 of the parameters ESR, CRP, assessment of global pain, global disease, HAQ-score and the Doctor’s assessment. ACR 20 represents a definition of improvement in RA that is suggested to correspond closely to the rheumatologists’ own impressions of patient improvement and also powerfully discriminates between active and placebo treatment (75). However, it does not include measures of joint damage and ACR 20% response during one year does not ensure that treatment has stopped the progressive joint destruction within 5-10 years (197). Moreover, it is important to consider that these response criteria are mainly defined for application in therapeutic studies, and not for evaluation in a practical clinical context. In clinical practice, mainly patients that are defined as non-responders by both ACR20 and DAS28 criteria are subject to change of therapy. In
paper V a combination of DAS 28 and ACR response criteria was used in which non-responders to treatment were defined according to failure to fulfil any of these criteria.

**Measurement of disability**

The Swedish version of the Stanford Health Assessment Questionnaire (HAQ) (70) is a self-reporting instrument measuring disability of daily life activities. The created score for the disability index ranges from 0 to 3, where a higher score indicates a higher degree of disability (81).

**Cell isolation**

In papers I-III, PBMC were isolated using density gradient separation (Ficoll) and used for cultures or ELISPOT analyses. The analyses are described in the respective papers.

**ELISPOT for measurement of cytokine production**

The ELISPOT technique (225, 62) allows detection of cytokine production at a single cell level. The procedure is described schematically in figure 8. All ELISPOT measurements in this thesis were performed using ELISA plates and the reliability of this method as compared to the use of nitrocellulose plates has been shown by Rönnelid et al (217). The ELISPOT is 10-200 times more sensitive than ELISA (248). The experience of performing ELISPOT is substantial in our lab, and so far non-specific stimulation has only been observed concerning TNFα spot data, but not for IL-6, IL-10 or IFNγ.

**Figure 8.** Principles for the ELISPOT method for enumeration of cytokine-secreting cells (From J Rönnelid, thesis: Reactivity to collagen type II and C1q in rheumatic diseases, 1997)
ELISA

ELISA measurements were performed for the determination of cytokine content in cell supernatants (I and III) and in serum (II), and MMP and TIMP-1 levels in serum (IV). The different ELISA kits are described in each paper.

Antigen stimulation of cell cultures

Antigens used for stimulation of cell cultures in paper III were purified protein derivative (PPD) 10 µg/ml, killed whole influenza virus diluted 1:1000 and chick CII 100 µg/ml. Mitogen used in papers I and III was phytohemagglutinin A (PHA). PHA concentrations and antigen stimulation procedures are described in papers I and III. Proliferation in response to CII is difficult to measure in human PBMC, see Berg et al (23). One reason for the poor proliferative response to CII may be a partial tolerisation to CII affecting proliferative but not cytokine responses (155). We therefore chose to measure production of IFN$\gamma$, which is primarily produced by T cells, as a sign of T cell reactivity to CII, PPD and influenza. The levels of IFN$\gamma$ were measured in cell culture supernatants after 3 and 7 days.

Genotyping

DNA was extracted from EDTA blood using a modification of the method described (6). The polymerase chain reaction (PCR) is a very specific, primer-directed enzymatic amplification of specific target DNA sequences (179).

Primers used for the analysis of polymorphisms of TNF$\alpha$, IL-10, IL-1Ra, TGF-β and TNFR1 are described in paper V. HLA typings were performed by using DR low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) and according to a methodology previously described (187).

Statistical analyses

In papers I - IV differences between groups were analyzed using the Mann-Whitney U test, and analyses for matched pairs were performed using Wilcoxon’s signed rank test. In paper II, Kruskall-Wallis test was performed to analyse the relation between cytokine production and clinical response. In paper IV, correlations between variables were assessed using the Spearman rank correlation test. In paper V, Fisher’s exact test was used to determine whether there was a random association between the observed alleles in the two studied populations or not. p<0.05 was considered significant.
Results and Discussion

As pointed out earlier, one way to study the role of various immunological and inflammatory reactions including the balance between pro- and anti-inflammatory cytokine drives in RA is to investigate certain immune functions during treatment with anti-rheumatic agents. In this thesis an approach has been made to study the effects of two anti-rheumatic drugs, intramuscular gold and the soluble TNFα receptor etanercept.

The influence of intramuscular gold treatment on cytokine production in RA

Paper I represents a study of cytokine production from PBMC \textit{in vitro} in response to GSTM (Myocrisin\textsuperscript{®}, 1 ml comprised of 20 mg GSTM and 20 µg mercury nitrate). Incubation with GSTM induced a dose-dependent increase in the numbers of IL-10 producing cells. IFNγ was decreased in corresponding supernatants. Depletion of the monocytes resulted in completely abolished GSTM-induced IL-10 production, suggesting that the latter was monocyte dependent. Moreover, IL-6 production was dose-dependently increased by GSTM. TNFα production was not affected by GSTM. The results were similar in both RA patients and healthy controls.

Next, a prospective study of cytokine production in RA patients during GSTM treatment was performed (paper II). Both spontaneous production of IL-10 \textit{ex vivo} and serum levels of IL-10 were increased after 4 weeks of treatment, concording with the findings in paper I. IL-6 production was also increased after 4 weeks of treatment and was sustained after 12 weeks.

Our main conclusion from these studies is that GSTM may act as an immunostimulator, considering both the direct effects on PBMC \textit{in vitro} and during gold treatment in RA \textit{in vivo}. There are no earlier data on the stimulation of IL-10 production \textit{in vitro} with GSTM, but Lacki et al reported 1995 in a cross-sectional study an increase of circulating IL-10 levels, whereas IL-6 levels were decreased in GSTM treated patients compared to controls (141).

The results concerning IL-10 production in papers I and II suggest that GSTM may act in RA not only by direct suppression of inflammatory cells such as monocytes, but also through stimulation of anti-inflammatory cytokine production. This conclusion is further supported by the fact that in paper II the increase in IL-10 was already apparent after four weeks’ treatment, suggesting that the early stimulation of IL-10 production may be the cause, and not the result of the anti-inflammatory effects of GSTM in RA. Control experiments show the
same effects on IL-10 production for GSTM per se as for Myocrisin® (Jon Lampa, unpublished observations), thereby excluding the possibility that the small amounts of mercury in the preparation should be of importance for the study results.

IL-10 is known to express inhibiting properties on the production of several cytokines including T cell-derived cytokines such as IFN\(\gamma\) (77). The in vitro results of decreased IFN\(\gamma\) levels with GSTM incubation (paper I) support these studies. The stimulation of IFN\(\gamma\) production recorded in paper II during the first 4 weeks of GSTM treatment was unexpected. However, it was shown in paper III as well as in earlier studies that IFN\(\gamma\) may increase in the early phase of DMARD treatment (17). One explanation for these results could be the change of trafficking of inflammatory cells to the joints, as gold downregulates adhesion molecule expression (183),(96).

GSTM-induced IL-10 production was monocyte-dependent (I) and there was no effect of GSTM on TNF\(\alpha\) production. The selective effect on certain cytokines was also reported earlier, when GSTM in concentrations up to 100 µg/ml could not inhibit IL-1 production from macrophages (208, 160). It is thus likely that GSTM has specific stimulatory effects on monocytes, whereby these cells increase their production of IL-6 and IL-10, possibly initiating anti-inflammatory actions reflected by the downregulation of IFN\(\gamma\) in vitro (I).

It is known from earlier studies that gold is taken up by monocytes and stored in the lysosomes, forming so called aureosomes (189), and several studies have also demonstrated other alterations in monocyte morphology and function after incubation with GSTM, (258, 39). In other studies, monocyte phagocytosis of bacteria has been shown to induce IL-10 production (272) and it is thus possible that the phagocytosis of gold granula per se could induce IL-10 production from the monocytes in paper I. Paradoxically, incubation with GSTM may reduce the phagocytic activity of monocytes (39, 258). The increase in IL-6 production after incubation with GSTM (I) and during treatment supports the hypothesis that GSTM acts as an immunostimulator of monocytes. Previous in vitro studies of GSTM effects on IL-6 production have reported the inhibition of IL-1β-induced IL-6 production from synovial cells (134, 295). Prospective in vivo studies have yielded disparate results, however. Madhok et al reported decreased levels of IL-6 in serum after 24 weeks treatment with gold (153) whereas one year later the same group did not detect any significant effects on spontaneous or LPS-induced IL-6 production from RA PBMC during gold treatment (59). Another prospective study detected a decrease of IL-6 serum levels during treatment with two DMARDs during 12 months, but made no distinction as to whether this decrease was induced by intramuscular gold (n=9) or by methotrexate (n=11) (244). It has been pointed out in several studies that IL-6 may have anti-inflammatory properties. Thus, among others Shingu et al have shown IL-6 stimulated production of TIMP-1,
suggesting a protective effect on cartilage degradation (38, 227, 228, 265). The stimulating effect of GSTM on IL-10 production suggests that gold may stimulate monocytes into anti-inflammatory actions \textit{in vivo}. The possibility that GSTM-induced IL-6 from monocytes may act against inflammation in this context can thus not be excluded.

A hypothesis for the action of intramuscular gold in RA can now be proposed. Gold is injected intramuscularly and after separation of the thiomalate group and the gold atom (116), the latter is bound to albumin (26). A rapid equilibrium between serum and SF is obtained (83). Gold-albumin complexes may be ingested by monocytes/macrophages in the blood and tissue through phagocytosis. The exact action site for gold is not known, although gold has been shown to accumulate in predominantly inflammatory tissue such as the inflamed synovial membrane (278). Gold stimulation or phagocytosis of gold-albumin complexes may induce IL-6 and IL-10 production in both blood and in tissues. It is possible that IL-10 may mediate some of the GSTM effects known from \textit{in vitro} studies, i.e. downregulation of adhesion molecules (183, 96), the inhibition of fibroblast proliferation (160) and the inhibition of pro-inflammatory cytokine production (148). Moreover, GSTM suppress TNF\textsubscript{\alpha}-induced NF-kappa B-activity (30) and interestingly, the action of I-kappa B-kinase (117), possibly mediated by modification of a cysteine-sulphydryl group necessary for the activity of this enzyme. Subsequently the inflammatory cell trafficking to the joints may diminish which lead to a reduced number of monocytes and macrophages in the synovial membrane during GSTM therapy, demonstrated by Yanni \textit{et al} (293). Moreover, since several studies have shown a cartilage protective effect of IL-6, the increase in IL-6 production from monocytes may have importance for retardation of erosions.

**Gold and skin reactions**

A total of 10 of the 20 RA patients treated with GSTM (paper II) developed skin reactions. Seven of these withdrew IMG treatment as a result of their skin reactions. The remaining three patients with mild skin reactions continued with a lower dose IMG without aggravated dermatitis. The frequency of dermatitis and the need to discontinue the therapy in one-third of the patients is in accordance with earlier reports (147). Our study could not confirm earlier observations that patients with dermatitis respond more advantageously to gold than those without (44). Interestingly, when PBMC were incubated with GSTM before initiation of treatment, a higher increase in IL-10 production was detected for PBMCs from patients who later did not get dermatitis than from patients with subsequent skin reactions. These results support earlier findings of increased IL-10 production correlating with protection for dermatitis in animal models (234) and in cross-sectional studies of nickel-allergic and non-allergic
subjects (46). Further supportive for a protective role of IL-10 concerning development of allergic immune responses is provided from studies on IL-10 knockout mice expressing aggravated cutaneous inflammatory responses (21) and the fact that IgE production can be inhibited by IL-10 (115, 13).

One patient in our study (paper II) reacted immediately with a severe dermatitis after the first GSTM injection. Interestingly, PBMC from the same patient expressed a four-fold increase of IFN\(\gamma\) production in response to gold in vitro (Jon Lampa, unpublished observations). This pattern was not evident in any other patient in the study. It is thus possible that this reaction to gold was T cell-mediated. Interestingly, it has been reported earlier that GSTM-treated patients who develop dermatitis have gold-reactive T cells (279). Similar effects have been reported for nickel-allergic subjects when exposed to this metal (236). Thus the gold dermatitis may reflect a T cell-mediated delayed type of hypersensitivity reaction to gold. In this context it is very interesting that the patients in paper II with a high capability for inducing IL-10 production from their monocytes were less likely to develop skin reactions during GSTM treatment.

Taken together, it may be speculated that IL-10 produced by monocytes interacting with GSTM may hamper T cell-mediated proinflammatory actions both in general (with the suppression of IFN\(\gamma\)-production), and may also suppress T cell responses specific for GSTM, which may otherwise lead to delayed hypersensitivity reactions and dermatitis.

**TNF\(\alpha\) and T cell function**

In paper III T cell reactivities in RA patients and healthy controls were investigated during etanercept treatment. We could detect an increased baseline production of TNF\(\alpha\) in RA PBMC compared to controls and the number of cells spontaneously producing IFN\(\gamma\) was increased after four, but not eight weeks' treatment with etanercept. T cell reactivity to PPD, influenza, and CII was increased after four and sustained after eight weeks treatment. The increased baseline production of TNF\(\alpha\) in RA PBMC supports earlier reports of a higher activation state of peripheral monocytes in RA than in controls (229).

It is known that blocking of the action of TNF\(\alpha\) leads to decreased peripheral monocyte activity, as indicated by a decrease in serum levels of TNF\(\alpha\), IL-1 Ra, IL-6 and soluble TNF receptor (47). Moreover, a recent report demonstrated an increased apoptosis of synovial macrophages during etanercept treatment in vivo (45). Since activated monocytes suppress T cell function a decreased peripheral monocyte activity may be one explanation for the enhanced activation and reactivity of peripheral T cells during etanercept treatment. Thus we may conclude that the hyporesponsivity in RA T cells is, at least partly, TNF\(\alpha\)-
mediated. In this context it is also interesting that the oxidative burst from macrophages induced by microbial antigen ex vivo is augmented during etanercept-treatment (177). Another mechanism underlying the increased peripheral T cell reactivity and IFNγ production during etanercept treatment might be an increase in the number of circulating lymphocytes, possibly due to a decrease in ICAM-1 expression in synovial endothelial cells, leading to reduced extravasation of activated peripheral T cells. Similar effects have been reported in infliximab-treated patients (57).

The finding of increased T cell reactivity to the autoantigen CII during TNFα-blockade may have implications for care in clinical use of these drugs. By blocking TNFα peripherally, these drugs may alter the function of antigen-presenting cells and increase TCR signaling of autoreactive T cells (275).

Increased serum levels of antibodies to double-stranded DNA have been reported in some RA patients during TNFα-blockade (72, 48, 275) and there have also been reports of a transient lupus-like syndrome during this treatment (48). Recently a case report was presented with onset of TNFα-blockade for juvenile RA possibly being associated with development of multiple sclerosis (MS) (230). Although TNFα-blockers cannot penetrate the blood-brain barrier to neutralize TNFα in the brain, these drugs may exacerbate MS by augmented peripheral T cell reactivity (275).

**TNFα effects on MMPs**

Among its several pro-inflammatory properties, TNFα induces MMPs which contribute to bone resorption and cartilage destruction. In paper IV, MMP-3 serum levels were found to be downregulated during etanercept treatment in parallel with the reduction of inflammatory parameters, i.e. CRP and ESR. No consistent changes in TIMP-1 serum levels were observed, while ratios of MMP-1 and MMP-3 to TIMP-1 were reduced following etanercept treatment. These data concord with earlier reports of infliximab effects on MMP expression (31). Serum levels of both MMP-3 and MMP-1 correlate with radiological joint destruction (60). Downregulation of MMPs during TNFα blocking treatment may thus reflect the ability of TNFα blocking therapy to prevent further development of joint damage. Interestingly, no changes in the synovial expression of MMPs and TIMP-1 during etanercept therapy were detected. The biological significance of this finding is uncertain and need further evaluation. One alternative explanation could be the low number of patients subjected to arthroscopy in the study and/or lack of specificity between the recognition of active and inactive forms of MMPs.
MMP levels and clinical outcome

In paper IV, MMP-3 serum levels at baseline correlated with changes in clinical disease activity during etanercept therapy. There was no correlation with DAS28 or ACR response criteria in this respect, however. Interestingly, pre-treatment serum levels of MMP-1 did not correlate with inflammatory parameters and the etanercept action on MMP-1 was independent of the suppression of inflammation. This finding corresponds to recently published data, in which patients with little clinical response to infliximab therapy nevertheless exhibited retardation of joint destruction compared to the control group (146). Moreover, clinical activity in RA is not always correlated with joint destruction and damage may develop in some patients with clinically low activity in their joint disease (178, 169). In our study, serum levels of TIMP-1 before treatment correlated with both CRP and ESR but were not affected during etanercept therapy despite a general decrease in all inflammatory markers investigated. This finding suggests that TIMP-1 is mainly influenced by mechanisms other than those involving TNFα.

Predicting clinical response to TNFα blocking treatment

In paper V our aim was to analyse if polymorphisms that are known to influence production of cytokines involved in the pathogenesis of RA, also could influence the responsiveness to TNFα-blockade with etanercept. 123 patients with active RA were treated with etanercept and response rates were determined after 3 months using ACR20 and DAS28 response criteria. 24 patients (20%) were defined as non-responders from their failure to fulfil any of the ACR20 or DAS28 response criteria. None of the recorded alleles alone was significantly associated with responsiveness to therapy. Analysis of HLA-DR polymorphisms also failed to show any association between response and HLA-DR alleles known as shared epitope (SE) genes (data not included in paper V). In addition, combinations of alleles were analysed to delineate genotypes associated with minimal respective maximal potentials for inflammatory responsiveness. The combination of TNFA1 and IL10 G/G alleles relates to a low inflammatory reactivity and has proven functionally important in other conditions (256). This combination was associated with a good response to etanercept (figure 9). Conversely, another combination conferring high inflammatory capacity (A2 allele in intron 2 of IL1RN and C allele in codon 25 of TGFB1 gene, see table 2) was associated with a poor response to the same drug.
Mass significance problems in this type of analysis are common and difficult to resolve. In paper V we tried to diminish these problems in two ways. Firstly, a combination of the ACR20 and DAS28 response criteria was used in the way that is considered most appropriate in daily clinical practice, i.e. defining as “responders” all those who fulfil either of the two alternative response criteria. Secondly, genetic analyses were only performed for a limited number of alleles or combination of alleles which we considered functionally most relevant. Several earlier studies have reported an association between these selected genetic markers and susceptibility and clinical course in other inflammatory conditions (167, 256, 192, 220). Our results support the hypothesis that both TNF and IL-10 are functionally involved in the mechanisms leading to response of TNFα-blocking treatment. Hypothetically, individuals with constitutive high TNFα expression may have developed compensatory mechanisms possibly resulting in TNFα not being the most important cytokine for coordination of inflammation in these patients. Together with a normal IL-10 production, conferring no additional restraining effects on inflammation, this may result in an insufficiency of TNFα inhibition to further suppress inflammation in these patients. Interestingly, in the only reported genetic study on efficacy of TNFα-blockade an association was found between a relatively rare haplotype in the lymphotoxin A gene and a lower response to infliximab in a group of Crohn's disease patients (249).

Our results indicate that certain functionally important cytokine gene polymorphisms may be associated with clinical responses to etanercept, but caution is obviously warranted in transferring those into algorithms that can be useful for predicting responses to etanercept in the clinic. The polymorphisms that were investigated represent only a minor part of the existing polymorphisms, and these were chosen according to previously documented functional and/or clinical significance.

![Figure 9](image)

**Figure 9.** Response to etanercept therapy in RA patients with different genotypes (combinations of promoter polymorphisms of TNFA (T1 and T2 alleles) and IL-10 (A and G alleles)). Out of 23 patients with the combination of T1/T1 G/G all but one responded to etanercept therapy after 3 months. The remaining single patient became a responder after 6 months. Numbers of responders and non-responders are indicated inside each column. P < 0.05 between T1/T1 G/G and the other combinations.
General Discussion

It is generally considered that immune mechanisms are of importance for pathogenesis and disease severity of RA. In order to learn more about RA pathogenesis, we have used pharmacological approaches as tools for mechanistic studies. In general, it is of value to consider a number of things when using pharmacological intervention for pathogenic studies.

Firstly, it is important to consider the timing of immunological mechanisms in relation to clinical effects of the studied drug. For intramuscular gold, the anti-rheumatic effect usually comes into action not earlier than after 12 weeks (206, 231). We chose to study the early immunological mechanisms of GSTM, before the clinical effect had come into action, thereby making the actual effects of the drug per se difficult to interpret. Already after 4 weeks treatment with GSTM a modulation of the immune responses was detected (an increase of IL-10 and IL-6 production in peripheral blood (II)). Moreover, several earlier studies have reported the decline of IL-6 levels in serum 6 months after the initiation of GSTM treatment, and this is well concording with GSTM having an IL-10 stimulating effect that downregulates IL-6 production. Conversely, when studying the actions of GSTM after 4 weeks, we found that GSTM treatment resulted in an augmentation of spontaneous IL-6 production, lasting for 12 weeks (II).

For TNFα-blockers, the time to clinical action is much shorter than for GSTM (about 1-2 weeks) and for infliximab it is known that IL-6 levels decrease rapidly already the first 24 hours after treatment (47). We did not study the early, pre-clinical immunological mechanisms during etanercept treatment, but after 4 and 12 weeks treatment.

Secondly, it is important to differ between methodologies when measuring cytokine production in various compartments. As pointed out in the method part of this thesis, ELISPOT detects direct, spontaneous cytokine production by PBMC in contrast to ELISA of serum, detecting the net concentration of a cytokine. Thus in paper II there was an increase in the number of IL-6 producing cells during GSTM treatment, whereas the IL-6 serum levels remained unchanged during this time (II).

Thirdly, it is important to bear in mind that diverse effects on various parameters may be observed in different compartments. This means that one effect in peripheral blood may reflect the opposite effect in the joint compartment. As an example, gold found in the blood is almost exclusively bound to albumin and the proportion of blood leukocytes with digested gold in aurosomes is minimal (26). However, in tissue, gold may be found intracellular in aurosomes (189) and we have also observed this in monocytes in vitro (see front cover.
picture of this thesis). We can thus not rule out that the effects of gold are different in tissue, where a possible phagocytosis of gold would lead to different effects than in blood. In papers II and III the increase of IFN$\gamma$ production in vivo was increased during treatment with gold and etanercept, respectively. It may be speculated that at this stage the treatment had begun to exert its effect on synovial adhesion molecules. This would result in a diminished trafficking of leukocytes to the inflamed joint and thereby increase of the lymphocyte number in peripheral blood. Thus, the increase in IFN$\gamma$ in papers II and III may be explained by this phenomenon, but regarding IL-6 and IL-10 production in paper II these mechanisms may not be applicable, since an increased production was detected also when incubating with GSTM per se (I).

What is then the importance of the increased IL-10 production during GSTM treatment? The significance of IL-10 as a modulator of RA inflammation has been described earlier in this thesis. As an example, neutralization of endogenous IL-10 with anti-IL-10 antibodies increased the production of TNF$\alpha$ and IL-1 in RA synovial cultures (121). Interestingly, IL-10 has been shown to potentiate IL-4 induced IgG4 production (115). Kiely et al have reported that although GSTM hampers IgG production in general, the inhibiting effects on IgG4 is less marked (125). Thus this modulating effect by GSTM could be mediated by IL-10. We could confirm a decrease of IgG levels during GSTM treatment, but in this experiment IgG subtypes were not measured (II, data not included). An alternative pathway for IL-10 action is the induction of T regulatory cells (214) conferring TGF$\beta$-production and other immunosuppressive mechanisms.

The patients with skin reactions in paper II had in common that their monocytes were suppressed in their GSTM-induced IL-10 production. The hypothesis that IL-10 may protect from gold-induced rash concords with earlier studies of other metals and allergies as well as studies of IL-10 knockout mice. Moreover, IL-10 may suppress delayed type of hypersensitivity reactions, perhaps involving T cells reactive for gold. The possible importance of gold-specific T cells in gold dermatitis is further supported by studies showing that HLA-DR5 may be a possible predictor for gold-induced skin reactions, with HLA-DR7 as a protective genotype (213). Another genetic approach is identification of polymorphisms in certain cytokines. For example, microsatellite markers TNF$\alpha$b5 and TNF$\alpha$b5 in the TNF$\alpha$ gene, were associated with gold skin reactions (73). Taken into a clinical perspective, polymorphisms of the IL-10 gene, conferring different levels of IL-10 production, may also be of importance in this respect. In addition, a protein known as metallothionein may play an important role in the elimination of GSTM and thus high activity of this protein may be associated with a diminished effect of gold (110). To date, little is known about possible functionally relevant polymorphisms of this protein, but this may be an interesting pathway in
the search of markers for the prediction of clinical response and/or side-effects of intramuscular gold treatment.

Moreover, stimulation of monocytes to produce anti-inflammatory cytokines, like IL-10, may be a therapeutic possibility for the future. Thus, it may be conceivable to develop new drugs that specifically stimulate monocytes in the selective production of anti-inflammatory cytokines, and with less toxicity than the gold compounds of today.

The hyporesponsiveness of RA T cells has been discussed earlier in this thesis. The finding of an increased T cell reactivity during treatment with TNFα-blockade (III) further supports the importance of TNFα on T cell function in vivo. With the exception of infections with intracellular bacteria such as mycobacterium tuberculosis and also some opportunistic organisms (275), there is presently little evidence supporting an increased presence of other infections associated with TNFα-blocking treatment (154, 274). The results of an increased T cell reactivity to recall microbial antigens after etanercept treatment in paper III may partly explain this paradox.

Our finding of increased T cell reactivity to CII suggests that increased reactivity of potentially disease inducing T cells may indeed also occur during treatment with etanercept. This could reflect that non-specific T cell actions in RA propagation may not be of uttermost importance, but is uncertain since we do not know anything about the activation state of the T cells in the rheumatoid joints of these patients. On the other hand, an increased autoreactivity of T cells may also be limiting for additional disease suppressing effects of TNFα-blockers. It is possible that additional targeting of T cells would cause an even better effect of TNFα-blockers in suppression of disease propagation in some patients. Animal studies have shown that TNFα-blockers and monoclonal antibodies to CD4 have synergizing effects in ameliorating CIA in mice (285). Considering these results one possible treatment strategy of RA would include TNFα-blockers in combination with a T cell suppressive agent, for example cyclosporine A. Such a combination would hypothetically be even more efficient than TNFα-blockers alone, acting on TNFα and suppressing the effects of potentially disease propagating auto-reactive T cells in RA.

It has also been suggested that an inappropriate clinical response to TNFα-blockers may be associated with another cytokine, for example IL-1, driving the inflammatory processes instead of TNFα. Treatment with IL-1 Ra has proven efficient in RA (34), (52), but as yet there are no reports of IL-1Ra treatment in non-responders to TNFα-blocking treatment. The aim of inhibiting the inflammatory mechanisms from different angles has been implicated in combinations of TNFα -blockade and IL-1Ra in animal studies with the result of more efficient therapies (20). Studies of the combination of IL-1Ra and TNFα-blocking treatment in
RA are ongoing and in aiming at increased efficacy, also combinations between TNFα blockers and other biological therapies may be considered. Our studies of GSTM indicate that IL-10 may be of importance in RA pathogenesis and therapies directed at raising IL-10 levels should be further evaluated. Interestingly, Kim et al showed greater efficiency with viral IL-10 treatment in combination with soluble TNFα receptors in CIA than TNFα-blockade alone (126). Possibly, combination therapy between TNFα-blockade and recombinant IL-10 would also be useful in RA by utilising different ways to decrease inflammation (122). IL-10 may for example suppress T cell-mediated effects, possibly leading to augmented disease suppression than for TNFα-blockers alone. The discussed combinations between cyclosporine A or IL-10 and TNFα-blockers may however be associated with higher risks of infections and these kinds of studies would need careful monitoring.

Another new treatment strategy based on our findings would be the ability to genetically define which patients would respond to a certain anti-rheumatic agent. The findings of genetic correlation with clinical response during etanercept treatment may thus give knowledge for further studies, possibly promoting the use of cytokine polymorphisms as predictors for response to various biological agents in the future.

In conclusion, the studies in this thesis have contributed with some further understanding concerning the pathogenesis of RA. IL-10 may be of importance both as a protective cytokine for disease mechanisms in RA, indicated by the gold studies, but also for the protection of gold-induced side-effects. We already know that TNFα is of importance in disease progression, but also for T cell function in vivo and also, as shown by our studies, for the suppression of development of autoreactive T cells. In this context, it is important to consider that the disease processes in RA include complex interactions between different immunoactive cells and cytokines with dual roles for disease propagation and immune defence mechanisms, some of which are probably not presently identified. The observations in this thesis warrant further care in the future utility of all biological and cytokine-targeted therapies, as possible dual effects unknown today may influence both clinical efficacy and safety of these therapies.
We shall not cease from exploration
and the end of our exploring
will be to arrive
where we started
and know the place
for the first time

T. S. Eliot
(1942)
Acknowledgements

I would like to express my sincere gratitude to all colleagues, friends and my family who have in different ways contributed to this thesis. In particular, I would like to thank:

Professor Lars Klareskog, my main supervisor, for providing excellent working facilities in the rheumatology lab, for sharing some of your immense knowledge in rheumatology and immunology and for your devotion in research. Also for the ability to always create a friendly atmosphere and for a remarkable engagement in the writing of this thesis, for example that you skipped a stag-party (svensexa) to read it carefully and provide final comments!

Johan Rönnelid, my second supervisor, for your never-ending enthusiasm, for your vast knowledge on immunology and immunological methods and for constructive comments and enthusiastic help whenever I ask for it. Also for being a good friend, a nice travelling companion to China and for sharing cultural interests.

Siv Rogberg, for excellent technical assistance, for considerable efforts especially in the second gold study, for fruitful discussions on immunological methods and for your personal support.

Johnny C Lorentzen, for being my first co-supervisor at the lab, introducing me to immunological methods, and later for fruitful discussions and fun during conference trips.

Leonid Padyukov, for sharing some of your vast knowledge in the genetic field and for the enthusiasm in teaching me genetic methods.

Associate Professor Ronald F van Vollenhoven, for sharing some of your immense knowledge in rheumatology, for fruitful discussions, collaboration and for nice moments at the “clinic piano”

Ann-Kristin Ulfgren, for nice collaboration and devoted work in the exploration of biopsies.

My former co-authors; Louise Berg and Anca I Catrina for good collaboration.

Associate Professor Ingiäld Hafström and Sofia Ernestam, Huddinge University Hospital, for the recent fruitful collaboration.

Erik af Klint, for sharing an interest in arthroscopy, for fruitful discussions and many laughs and for sharing the situation of being a PhD student and a physician.

Johan Bratt, Huddinge University Hospital, for inspiring discussions and for nice collaboration in the TNFα genetic study.

Professor Thomas Skogh, Professor Marita Troye-Blomberg and Associate Professor Johan Frostegård for encouragement at my half time seminar.

The “human rheumatology lab”; in particular Eva Jemseby, for keeping order of blood samples in an extraordinary way; Tina Trollmo and Vivianne Malmström, for creating a nice atmosphere in the lab and with hope for future collaboration in functional T cell studies; Duoija Cao, for valuable help with ELISPOT measurements; Therese Wallerskog and Andreas Fasth; Good luck with your PhD studies!

Eva Lindroos and Marianne Engström for excellent technical help with the biopsies and Lotta Aveberger for the joy of combining lab work and singing together.

My all-the-way-to-PhD-companions: Pernilla Englund, Lena Svelander, Adla Bakri Hassan and Barbro Holm. Good luck to you all!

All other old and new friends and colleges at the rheumatology lab for sharing the research for improvement of rheumatic patients:

Ulf Andersson, Anders Bucht, Carina Sehlsstedt, Yajuan Gao, Dung Dang Thi Ngoc, Erik Sundberg, Ewa Burek, Gerður Gröndal, Eva Lindroos, Jenny Huang, Helena Erlandsson Harris, Hong-Wei Xu,
Ingela Andersson, Karin Lundberg, Karin Palmblad, Lars Mattsson, Lars Ottosson, Åsa Müssener-Bostrom, Linn Horvath, Liselotte Backdahl, Marie Dahlström, Monica Ek, Malin Liljestöm, Anders Ericsson-Dahlstrand, Åsa Jansson, Guozhong Fei, Riikka Kokkola, Jenny Hui Huang, Helene Alexander, Gabriella Dombos, Anna Cederholm, Cecilia Grundtman, Sevim Barbasso, Stina Salomonsson, Ulrica Ribbhammar, Peter Hjelmström, Alexander Espinosa, Elisabet Welin-Henriksson, Pla Tegnér, Heidi Wahamaa, Annika van Vollenhoven and Mona Esbjörnsson-Liljedahl

Associate Professor Bob Harris, for valuable linguistic help with the manuscripts and thesis.

Associate Professor Bo Ringertz, the former head of the Department of Rheumatology, KS, for personal support during my clinical and research work, for providing space for clinical studies and for sharing an interest in a cappella music.

Associate Professor Ralph Nisell, the present head of the Department of Rheumatology, for the continuation to create a friendly and research stimulating atmosphere in our clinic.

Staffan Lindblad, for inspiration in research and clinical work and for teaching me arthroscopy.

Elisabet Svenungsson and Iva Gunnarsson, for good support during the past years, the thesis writing and for future collaboration. Elisabet also for being an inspiring clinical mentor.

Associate Professor Per Larsson, for clever comments on manuscripts.

Associate Professor Ingrid Lundberg, for support during my clinical work the last years and for being such a nice person.

Gunnel Bemerfeldt, for all kinds of assistance and for sharing my interests in jazz music and movies.

The nurses and other staff at D10 and the Rheumatology outpatient clinic, in particular Rosmari Rönqvist, Ann-Mari Malmborg, Inger Hallman and Jill Gustafsson, for excellent and enthusiastic help with blood samples from the study patients

Sonja Brannemark, Elin Lindblad and Inga Lodin for help with the clinical data files.

All the other old and new colleges and staff at the Department of Rheumatology for sharing daily work, lunches and conversations: Alf Elman, Ann-Kari Lefvert, Anders Harju, Birgitta Nordmark, Brigitte Dupré, Christina Dorph, Esbjörn Larsson, Tomas Lerndal, Eva Bornefalk, Johan Askling, Lars Vestersköld, Lena Björnadal, Marie Wahren-Herlenius, Maryam Dastmalchi, Petra Rällstrand, Thorunn Jonsdottir, Tomas Zweig, Ulf Nyman, Göran Karlsson, Annica Nordin, Marie Svensson, Gunilla Holmstedt, Veronica Bushati and Marius Wick

Colleges and collaborators outside the Dep of Rheumatology, especially Dr Catarina Olivestedt, Dep of Clinical Immunology, KS, for help with the RF data in the Enbrel-genetic paper and Patrick D W Kiely in London, for an interest in gold studies and stimulating discussions.

All patients who have contributed to the studies in this thesis.

All friends outside the lab who have shared a lot of fun, including musical activities, sports and just having a good time.

My friends in the a cappella group, Vocal Selection, for all the years of singing and joy.

My relatives in Göteborg, for showing interest in my work.

My mother, Majlis, for love, support and a sincere interest in everything I am doing.

My late father, Kent, who unfortunately did not live to follow me through medical and PhD studies, but whose love and positive view of life I bear with me.

And Ulrika, the love of my life, for sharing my interests in medicine, research and music, for always being encouraging and for making life joyful!
“To Do Is To Be” -- Socrates

"To Be Is To Do" -- Plato

"Do Be Do Be Do" -- Sinatra
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