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RHEUMATOID ARTHRITIS
PHARMACOLOGICAL MODULATION OF CYTOKINES – ASPECTS OF CLINICAL RESPONSE AND ENDOCRINE REGULATION

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Livet forstås baglæns, men må leves forlæns.

Søren Kierkegaard

Till Magnus, Harald och Agnes

ABSTRACT

Rheumatoid arthritis (RA) is a systemic inflammatory disease primarily affecting the joints. In the pathogenesis several cells of the immune systeme and messengers of the immune systeme, cytokines, are important. In this thesis, the cytokine-modulating effects of two different treatments in RA, gold sodium thiomalate (GSTM) and the TNF-antagonist infliximab, are investigated. Further genetic factors potentially predicting clinical response to another TNF-antagonist, etanercept, are presented. Finally, the effect of TNF-antagonists on the hormone levels of the adrenal and gonadal axes is demonstrated.

The mechanism of action of GSTM is not fully understood. We studied 20 patients with RA during treatment with GSTM. The numbers of IL-6-, IL-10- and IFNγ-producing cells measured with ELISPOT were significantly increased after four weeks treatment with GSTM, as was serum concentrations of IL-10. In addition a higher IL-10 production from peripheral blood mononuclear cells was recorded in patients without a subsequent skin rash. There was no correlation between clinical response and cytokine production. In conclusion, GSTM seems to have immunoregulatory properties that may be important for the therapeutic effect in RA.

The effect of TNF-antagonists on cytokine expression in the synovial membrane may be of relevance as additional therapeutic targets may be identified. We studied the effect of the TNF-antagonist infliximab on the synovial expression of TNF, IL-1α, IL-1β, IFNγ and IL-15 with immunohistochemistry. IL-15 is still present in the synovial tissue after treatment with infliximab as a remaining potential target. There was no correlation between synovial expression of IL-15 and response to therapy, but expression of TNF at baseline was exclusively seen in patients with good response to infliximab.

Genetic factors, such as HLADRB1/shared epitope or cytokine gene promoter polymorphisms, relevant to RA, were investigated concerning correlation with clinical response to the TNF-antagonist etanercept. The combination of the alleles –308TNFG/G and –1087IL-10G/G was correlated with a good response, compared with all other combinations. This combination may correlate with a presumed functional phenotype with a low immune response.

Both adrenal and gonadal axes are reported to be downregulated in RA with a decreased responsiveness to inflammatory stimuli. This down-regulation has been suggested to be the effect of proinflammatory cytokines. We studied ACTH, cortisol, DHEAS, LH, testosterone and estradiol during two years treatment with TNF-antagonists. An individual stability in hormone levels was recorded, with no effect of decreased disease activity on this stability. DHEAS increased in females without prednisolone treatment, with a correlation with improved physical function, possibly relevant to other effective treatments. A subset of women with low adrenal hormone levels had a disease onset at a young age. Presuming a stable individual hormonal homeostasis, these low adrenal hormone levels may even precede disease onset.

In conclusion, studies on cytokine-modulating treatment strategies in RA may provide information on mechanism of action and give further insight in potential pathogenetic mechanisms.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Ernestam S*, Lampa J*, Rogberg S, Rönnelid J, Klareskog L, Hafström I
Evidence for immunostimulatory effects of intramuscular gold in rheumatoid arthritis; correlations with skin reactions
Journal of Rheumatology; 2003 Aug; 30(8):1748-55

* = contributed equally

II. Ernestam S, af Klint E, Catrina AI, Sundberg E, Engström M, Klareskog L, Ulfgren AK
Synovial expression of IL-15 in rheumatoid arthritis is not influenced by blockade of tumour necrosis factor
Accepted for publication in Arthritis, Research and Therapy

III. Padyukov L, Lampa J, Heimbürger M, Ernestam S, Cederholm T, Lundkvist I, Andersson P, Hermansson Y, Harju A, Klareskog L, Bratt J
Genetic markers for the efficacy of TNF blocking therapy in rheumatoid arthritis
Annals of the Rheumatic Diseases; 2003 Jun;62(6):526-9

IV. Ernestam S, Hafström I, Carlström K, Werner S, Tengstrand B
Serum markers of adrenal and gonadal activity in rheumatoid arthritis are stable during two years of treatment with TNF-antagonists, irrespective of clinical response
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
CCP	Citrullinated cyclic peptide
CD	Cluster of differentiation
CRP	C-reactive protein
DAS	Disease activity score
DAS28	Disease activity score, calculated on 28 joints
DC	Dendritic cell
DHEAS	Dehydroepiandrostendione sulphate
DMARD	Disease modifying anti-rheumatic drug
EULAR	European Leauge Against Rheumatism
ESR	Erythrocyte sedimentation rate
FcR	Fc receptor
FLS	Fibroblast-like synoviocyte
GH	General health
GSTM	Gold sodium thiomalate
HAQ	Health Assessment Questionnaire
HLA	Human leucocyte antigen
HPA	Hypothalamus-pituitary-adrenal
HPG	Hypothalamus-pituitary-gonadal
hsCRP	High sensitivity C-reactive protein
IFN	Interferon
IL	Interleukin
LH	Luteinising hormone
LHRH	Luteinising hormone-releasing hormone
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NFκB	Nuclear factor kappa B
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
TcR	T cell receptor
Th	T helper
TGFβ	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor α
VAS	Visual analogue scale

1 CLINICAL ASPECTS

1.1 CLINICAL FEATURES AND CLASSIFICATION

Rheumatoid arthritis (RA) is a chronic inflammatory disease with autoimmune features, primarily affecting the synovium of joints, with local symptoms of joint swelling, pain and morning stiffness. The joint inflammation and subsequent cartilage damage and joint destruction contribute to a considerable impairment of physical function.

The American College of Rheumatology (ACR) (formerly American Rheumatism Association (ARA)), presented a core set of classification criteria for RA in 1987, based on analyses of 262 patients with established RA (Arnett et al. 1988) (Table 1). These criteria are important tools in research but are also used as clinical guidelines. The sensitivity in early arthritis is, however, low (Harrison et al. 1998; Saraux et al. 2001).

Table 1. The 1987 American College of Rheumatology classification criteria for RA	
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of three or more joint areas	At least three joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth) observed by a physician. The 14 possible areas are the right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle and metatarsophalangeal (MTP) joints.
3. Arthritis of hand joints	At least one area swollen in a wrist, MCP or PIP joint.
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2.) on both sides of the body (bilateral involvement of PIP, MCP or MTP joints is acceptable without absolute symmetry).
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences, extensor surfaces or in juxta-articular regions, observed by a physician.
6. Rheumatoid factor (RF)	Detected by a method positive in less than 5% of normal controls.
7. Radiographic changes	Radiographic changes typical of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).
Patients fulfilling at least four of these seven criteria are classified as having RA. Criteria 1 through 4 must have been present for at least six weeks. Patients with two clinical diagnoses are not excluded.	

1.2 HISTORY

RA was first described as *goutte asthénique primitive* by Landré-Beauvais in 1800 (Landre-Beauvais 2001) and Garrod named the disease rheumatoid arthritis in 1859 (Storey 2001). However, skeletons with changes consistent with RA have been found in pre-Columbian North America (Rothschild et al. 1990), in contrast to archaeological findings elsewhere, suggesting that RA first appeared in North America.

1.3 EPIDEMIOLOGY

The prevalence of RA is 0.4-0.8 % in Scandinavia (Kvien et al. 1997; Aho et al. 1998; Simonsson et al. 1999). At earlier ages women are more frequently affected than men (Symmons et al. 1994), while at later ages, the incidence is the same for men and women (Tengstrand et al. 2004). RA incidence is falling (Doran et al. 2002), particularly in women, and an older age of disease onset is reported in 1990 compared with in 1975 (Kaipiainen-Seppanen et al. 1996).

1.4 ASSESSMENT OF DISEASE ACTIVITY AND FUNCTIONAL DISABILITY

Disease activity in RA is often measured by the composite index Disease Activity Score (DAS) and can be calculated on 28 joints (DAS28) (Prevoo et al. 1995). The index includes number of swollen joints count (SJC), number of tender joints count (TJC), erythrocyte sedimentation rate (ESR) in mm/h and the patient's assessment of general health (GH), measured on a visual analogue scale (VAS) (0-100 mm). The DAS28 is calculated as follows:
DAS28 = 0.56 * sqrt(TJC) + 0.28 * sqrt(SJC) + 0.70 * ln(ESR) + 0.014 * GH.

A simplified disease activity score (SDAI) has been introduced based on the numerical sum of five outcome parameters: TJC and SJC, based on 28-joint assessment, patient's and physician's global assessment of disease activity on a VAS of 10 cm and the level of C-reactive protein (CRP) (Smolen et al. 2003). Recently, a clinical disease activity index (CDAI), a modified variant of SDAI without CRP, was introduced (Aletaha et al. 2005). Both SDAI and CDAI have been presented as complements to DAS28 in clinical practice, where the calculator and the need for recent laboratory values of acute phase reactants may be a limiting factor (Aletaha et al. 2005).

Since Steinbrocker described a four-point ordinal scale for evaluating the severity of disability in RA in 1949, several global disability rating scales have been developed. One of these, the Stanford Health Assessment Questionnaire (HAQ) has been translated into Swedish and validated as the Swedish version of HAQ (Ekdahl et al. 1988). The HAQ score ranges from 0 to 3, where a higher score indicates a higher degree of disability.

1.5 RESPONSE CRITERIA

Currently, two different systems for assessing clinical response in RA are used: the Dutch EULAR response criteria using the DAS28 value (van Gestel et al. 1996) and the American ACR response criteria (Felson et al. 1995). As mentioned above, the SDAI and CDAI have recently been proposed as complementary ways to assess disease activity, particularly in clinical settings (Aletaha et al. 2005). A validation of these criteria in the assessment of clinical responses has also been performed, but so far they have not been used in clinical trials.

In the EULAR response criteria good responders are those with a DAS28 improvement of at least 1.2 and an end-point value of <3.2. Moderate responders are patients with 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 of <5.1. A patient is considered to be in remission if the DAS28 is <2.6 (Fransen et al. 2004). The ACR response evaluates a change, calculated as a percentage improvement from baseline. TJC and SJC must improve separately by 20%, 50% or 70%, and in addition three of the following five variables must be improved by 20%, 50% or 70%: ESR or CRP, HAQ score, VAS for patient's assessment of disease activity, physician's assessment of disease activity and patient's assessment of pain. ACR 20%, 50% and 70% responses may then be calculated (Felson et al. 1995). ACR 20% response is in concordance with a moderate response according to EULAR response criteria (van Gestel et al. 1999).

1.6 AUTOANTIBODIES

Rheumatoid factor (RF) is an antibody directed towards the Fc region of IgG antibodies. RF is present in 70% of patients with RA (Wolfe et al. 1991), with an association to worse prognoses and higher frequency of extra-articular disease (De Rycke et al. 2004). RF is also present in other autoimmune and infectious diseases and in healthy individuals with increasing age (Nowak et al. 2005).

Antibodies towards cyclic citrullinated peptide (CCP) have recently been described in RA (Schellekens et al. 2000), with a 98% specificity (van Boekel et al. 2002) and preceding the disease onset by several years (Rantapaa-Dahlqvist et al. 2003). These antibodies are directed towards proteins, where a post-translational modification of an arginine to a citrulline has occurred (Schellekens et al. 1998). Anti-CCP-positive patients have worse prognoses concerning disease activity (van der Helm-van Mil et al. 2005) and radiographic outcome (Forslind et al. 2004).

1.7 DISEASE COURSE, OUTCOME, MORTALITY

In most patients, RA is a progressive disease with joint destruction, subsequent disability and socioeconomic consequences for the individual patient (Scott et al. 2005). There is considerable variation between patients in the disease course, the pattern of joints involved, the presence of autoantibodies and/or extra-articular disease. It has been suggested that RA consists of in fact at least two different diseases (Huizinga et al. 2005). Health-economical calculations have shown that costs, both direct and indirect, are associated with increased disability (Kobelt et al. 2002). Mortality in RA has been reported to be higher in RA patients, mainly attributed to cardiovascular disease (Wallberg-Jonsson et al. 1997; Bjornadal et al. 2002), and particularly in patients with extra-articular disease (Turesson et al. 2002). Other comorbidities are infections (Doran et al. 2002) and lymphomas (Ekstrom et al. 2003).

2 ETIOLOGY

The etiology of RA is still not known. A genetic susceptibility in combination with the influence of environmental factors are probably prerequisites for the onset of RA.

2.1 ENVIRONMENTAL FACTORS

There are consistent data indicating that smoking may contribute to the development of RF-positive, destructive RA in HLADRB1/SE-positive individuals (Uhlig et al. 1999; Stolt et al. 2003). The onset of RA has been associated with mineral oils (Sverdrup et al. 2005), silica exposure (Reckner Olsson et al. 2001), diet factors (reviewed in (Choi 2005)) and blood transfusions (Symmons et al. 1997). In addition, infectious agents such as Epstein Barr virus and parvovirus B19 have been suggested to provoke the development of RA (Kouri et al. 1990; Hajeer et al. 1994), without any consistent evidence.

2.2 IMPACT OF SEX AND SEX HORMONES

More women than men are affected by RA (Symmons et al. 1994), particularly at younger ages. This implicates a plausible role for sex hormones in susceptibility and pathogenesis. In women, peak incidence is observed in the peri-menopausal and postpartum periods (Goemaere et al. 1990; Silman et al. 1992). Furthermore, pregnancy has a protective effect (Silman et al. 1992), which may be explained by the fact that pregnancy induces a shift from a T helper cell type 1 reaction, typical for RA (see below), towards a type 2 reaction (Mishan-Eisenberg et al. 2004). The use of oral contraceptives also has a protective effect, whereas the use of hormone replacement therapy does not (Doran et al. 2004). However, hormone replacement therapy has a beneficial effect in post-menopausal patients with established RA (D'Elia et al. 2003).

2.3 ETIOLOGY – GENETIC FACTORS

In twin studies, a higher concordance for the disease of 15% in monozygotic twins compared with 3.6% in dizygotic twins suggests a genetic contribution to the development of RA (Silman et al. 1993), but a considerable residual environmental effect is also indicated.

The genes, apart from gender, that have been reported associated with an increased disease susceptibility (Gregersen et al. 1987) are located on the short arm of chromosome 6 in the human leucocyte antigen (HLA) class II region of the major histo-compatibility complex, where many immune-regulating proteins are encoded (1999). Some alleles of HLA DRB1, including the DRB1*04 and DRB1*01 alleles, share certain amino acid sequences in the peptide-binding groove of the same three-dimensional epitope, called the shared epitope (SE) (Gregersen et al. 1987). The presence of HLA DRB1/SE influences both susceptibility to RA (Gregersen et al. 1987) and the severity of the disease, especially the development of erosions (Gorman et al. 2004) and the prevalence of extra-articular RA (Turesson et al. 2005). The function of the HLA DRB1/SE molecule, antigen presentation to the T-cell receptor, seems important in RA. Recently a protective amino acid sequence has been proposed as a development of the shared epitope concept (van der Helm-van Mil et al. 2005).

The effects of sex and the HLA DRB1/SE molecule, however only explain about two thirds of the total genetic contribution (Deighton et al. 1992). Other genetic factors have been reported to

influence susceptibility to RA, such as Fcγ receptor III polymorphism, with a phenotype with increased autoantibody production associated with a higher disease susceptibility (Kastbom et al. 2005). Also a corticotropin-releasing hormone (CRH) gene polymorphism has been suggested as a contributing factor to RA susceptibility (Baerwald et al. 2000).

Lately it has been suggested that a gene environmental interaction exists between HLADRB1/SE and smoking in RF-positive patients (Padyukov et al. 2004), a combination that increases the risk more than just their additive effect.

3 PATHOGENESIS

3.1 INNATE AND ADAPTIVE IMMUNE SYSTEM

Although the etiology of RA is not yet solved, new targeted therapies have provided increased insight into the pathogenesis. Several components of both the innate and the adaptive immune system are involved in mediating tissue inflammation and damage in RA. Below some basic concepts of the immune system are introduced as a background. Rheumatology, section 2 (Hochberg et al. 2003), serve as a general reference.

The normal and fundamental function of the immune system is protection from infectious diseases. The skin and mucous membranes lining the respiratory and gastrointestinal tracts are physical barriers. When these barriers are penetrated, the first line defence is innate immunity, with an immediate but unspecific response to invaders in order to eliminate the pathogen or at least to control it. The adaptive, specific immunity is then activated to clear the infection and to render a lifelong immunological memory. The innate and adaptive immunities have overlapping functions and communicate through cytokines, special messengers of the immune system. Cytokines important in RA and with relevance to this thesis, are reviewed in (Dinarello et al. 2002), and are listed in Table 2.

3.2 THE INNATE IMMUNE SYSTEM

A pathogen is initially recognised by macrophages and mast cells, whereby soluble cytokines and chemokines are produced. Vasodilatation and altered permeability of the capillaries take place due to the effect on endothelial cells. The increased permeability of the capillaries results in an influx of fluid and cells into the tissues. Macrophages and neutrophils, important phagocytic cells, are recruited. Macrophages act as phagocytes, producing free radicals, nitric oxide and proinflammatory cytokines. In addition they serve together with dendritic cells (DC) as antigen-presenting cells, inducing the adaptive immune system. Neutrophils cause tissue damage in different ways. Cytotoxic granulae contain different cytotoxins that eliminate the phagocytosed invader and the production of reactive oxygen species mediates a destructive oxidative burst. Acute phase reactants, like C-reactive protein, are produced in the liver and contribute to activation of the complement system that damages the membranes of pathogens or facilitates clearance. The result is local inflammatory signs of redness (rubor), heat (calor), swelling (tumor) and pain (dolor), the four classic signs of inflammation, described already by the Roman physician Celsius. Later a fifth sign, loss of function (functio laesa), very relevant in RA, was added.

3.3 ADAPTIVE IMMUNE SYSTEM

The adaptive immune system recognizes an endless number of specific antigens and distinguishes self from non-self. When a response of the adaptive immune system has occurred, a lifelong immunological memory is induced. The adaptive immune system can be classified into two parts: humoral immunity and cell-mediated immunity. B cells produce specific antibodies, mediating humoral immunity by recognizing and eliminating antigens. Cell-mediated immunity is mediated by T cells with T cell receptors (TcR), recognising antigens that are presented by antigen-presenting cells, such as macrophages and dendritic cells. There are two subpopulations of T cells, CD4+ T helper cells, described belowin further detail, and CD8+T cytotoxic cells, that kill self-cells that have been altered by a virus or cancer. Some CD8+ T cells are memory T cells with the ability to respond again to the same antigen.

3.3.1 Antigen recognition

Immature DC specialised for antigen uptake by the presence of scavenger receptors such as the Fcγ receptor and Toll like receptors (TLR) are scattered in the body. DCs function as antigen-presenting cells in capturing antigens, both self and non-self, in the periphery. The DC thereafter migrate to the lymph node where the DC present the antigen to a T cell in the context of a MHC class II (Sarkar et al. 2005). If a self antigen is taken up, unresponsiveness, i.e. tolerance, of the T cell is promoted. Immature DCs are activated during maturation if in addition "danger signals" are signalled through TLRs on DCs. "Danger signals" occur when exogenous ligands from bacteria and viruses bind to TLRs, but can also be endogenous ligands signalling about cell damage such as mRNA, hyaluronic acid and cytokines produced during inflammation, such as IL-1 and TNF (Matzinger 2002). TLRs signal through different pathways, among them nuclear factor kappa B (NFκB), and regulate gene expression of proinflammatory cytokines, for example IL-1 and TNF.

3.3.2 T cell activation

Immature DC capture antigens and mature upon inflammatory stimulation, as desribed above. Mature DC migrate to lymph nodes and present antigen in the context of MHC and costimulatory ligands to T cells, whereby an adaptive immune response is triggered and a T cell differentiation starts (Sarkar et al. 2005). The MHC class II-peptide complex and TcR interact forming a strong binding that initiates T cell activation. As a second signal co-stimulatory ligands (CD28 on the T cell, B7 on the APC) bind and IL-2 production and T cell differentiation starts. After some days CTLA4 appears on the surface of the T cell, binding strongly to CD28, producing an inhibitory signal and T cell activation ceases.

3.3.3 T cell differentiation and Th1/Th2 concept

When T cells are activated, differentiation and subsequently proliferation take place. CD4+ T cells differentiate into subclasses. Type 1 T helper cells (Th1) develop in the presence of IL-12 and are characterized by cytokine production of IFNγ, IL-2 and TNF, stimulating cell-mediated immunity, typically a delayed hypersensitivity reaction, and autoimmunity. IL-2 and IFNγ are produced of Th1-cells, mediating an activation of macrophages, which subsequently produce the proinflammatory cytokines TNF and IL-1. Type 2 T helper cells (Th2) are characterized by the production of IL-4, IL-5 and IL-10, producing humoral immunity (Mosmann et al. 1986). RA and other autoimmune diseases are considered as Th1-driven with an insufficient downregulating Th2-response (Miossec et al. 1997; Schulze-Koops et al. 2001).

3.3.4 Clinical aspects of Th1/Th2 concept

Normally there is a balance between these two subsets of T cells which are altered during disease, where a shift towards Th1 is recorded in autoimmune diseases such as RA, with IFNγ as a driving cytokine inhibiting proliferation of Th2 cells. On the other hand a shift in the balance towards Th2 is reported in atopic diseases, with IL-4, IL-5 and IL-13 as dominating cytokines, resulting in IgE production and eosinophil activation. Indeed, there are several case-control studies indicating a lower prevalence of atopic diseases, in particular hay fever, in RA (Verhoef et al. 1998; Hartung et al. 2003) but patients with early RA have not been studied. A considerable recall bias concerning the time before disease onset as well as ongoing immunomodulating treatments, may then influence the results. Moreover, a considerable Th1 component of inflammation is reported in asthma, in particular in exacerbations (El Biaze et al.

2003), implicating that models like this must be interpreted with cautiousness. Models like this are simplifications, cytokines may have different effects depending on target cells and receptor factors (Yadav et al. 2003).

3.3.5 Regulatory cells

The adaptive immune system with T and B cells is capable of responding to endless different antigens, both self and non-self. Tolerance towards self is regulated through different mechanisms. The opposite of tolerance is autoimmunity with surviving self-reactive cells, resulting in autoimmune diseases. DCs are responsible for regulation of the innate and adaptive immune response (Lanzavecchia et al. 2001) and in keeping both central and peripheral tolerance, (reviewed in (Quah et al. 2005)). Peripheral tolerance is also kept by another type of cells, T regulatory cells, (reviewed in (Gregori et al. 2005)). In the absence of inflammation and danger signals, immature DCs take up an antigen in the periphery, remain immature, and migrate into the lymph node. CD4+ cells are then differentiated into type 1 T regulatory cells (Tr1), with ability to produce IL-10 and TGFβ (Cottrez et al. 2000) and CD25+ T regulatory cells that suppress both a Th1 and Th2 response in a cell-to-cell contact mechanism (Setoguchi et al. 2005). IL-10 inhibits maturation of DCs (Steinbrink et al. 1997). Altogether, immunoregulation by DCs and T regulatory cells is effectuated in many ways.

3.4 CYTOKINES

Cytokines, which are important in the pathogenesis of RA and relevant to this thesis, are presented in Table 2, (reviewed in (Dinarello et al. 2002)). Cytokines are small proteins/glycoproteins 5–50 kDa produced by different cells including macrophages, fibroblasts and lymphocytes. They function as chemical messengers of the immune system, signalling from the innate to the adaptive immune system in infections, inflammation and trauma. More than a hundred different cytokines have been described. Similar functions can be mediated by more than one cytokine, sometimes in a synergistic way. Cytokines signal through specific receptors on target cells of the immune system and exert their effect mainly locally, in a paracrine (effect on a neighbouring cell) or autocrine (effect on the cell that produced the cytokine in a feedback loop) way, and modulate inflammatory responses. Nevertheless increased levels of cytokines may also be detected in the circulation in RA as well as other inflammatory and infectious diseases (Hack et al. 1989; Robak et al. 1998), and in this setting work more as a hormone (Turnbull et al. 1999). As an example, IL-6 acts on distant organs such as the liver, in regulating acute phase responses with production of CRP (Fey et al. 1990), and the hypothalamus (Mastorakos et al. 1993) in regulating the activity of the hypothalamus-pituitary-adrenal (HPA) axis. Cytokines can be arranged as monomers, homodimers and homotrimers as TNF. On the surface of the target cells specific, high-affinity receptors are located and the biological action of the cytokine is executed through binding to them. The receptor can be divided in three domains, the extracellular ligand binding domain, the transmembrane domain and the intracellular signalling domain. The extracellular part of a cytokine receptor can be shed and released in the extra-cellular space as a soluble receptor. These shed receptors may have inhibiting or enhancing effects, depending on the concentration of the cytokine and the cytokine receptor (Van Zee et al. 1992). Production of TNF from monocytes/macrophages or other cells starts with an immunological or bacterial signal mediated by for example TLRs, followed by activation of intracellular signal transduction pathways. NFκB, map kinase, P13 kinase and JAK/STAT are signal transduction pathways that regulate many genes that are involved in signal transduction in inflammation, (reviewed in (Morel et al. 2004)). Transcription factors are produced and they bind to different regulatory DNA sequences. The binding results in the enhancing or silencing of regulatory steps that decide the transcript of the gene (Kremer et al.

2005). Also at the post-transcriptional level, regulation may occur (Anderson et al. 2004). The membrane-bound cytokine is cleaved by an enzyme. Shed receptors (described above) and receptor antagonists, for example IL-1 receptor antagonist, provide additional local regulation. The circulating cytokine level and the local concentration are thus products of a very delicate multi-step regulation.

Table 2. Cytokines in RA, relevant to this thesis, reviewed (Dinarello et al. 2002)

Cytokine	Produced from	Effect	
IFNγ	Th1-cells, NK-cells	Stimulates MHC expression, antigen presentation, inducing macrophage activation. Inhibits IL-4 and Th2 responses.	Proinflammatory Th1
IL-1	Macrophages, monocytes, endothelial cells, T & B cells	Stimulates release of MMPs from fibroblasts and chondrocytes. Bone destruction. Induce TNFα-production.	Proinflammatory
IL-2	T cells	T cell growth factor, stimulates IFNγ production from T cells and TNF production from monocytes.	Proinflammatory
IL-4	Th2 cells	Stimulates IL-1ra production, inhibits IL-1, IFNγ, TNF production B cell growth factor, isotype switch towards IgG1 and IgE	Anti-inflammatory Th2
IL-6	T cells, B cells, monocytes and fibroblasts	Regulate innate immunity, differentiation of cytotoxic T cells, B cell growth factor, induction of immunoglobulin production, acute phase reaction	Pro- and anti-inflammatory
IL-8	Various cells	Chemoattractant to neutrophils.	Proinflammatory
IL-10	Monocytes, macrophages, DCs, T regulatory cells, T cells, B cells	Immunoregulation. Suppress antigen presentation through downregulating MHC class II expression, inhibit synthesis of Th1 cytokines, prevent DC maturation, B cell growth factor, antibody prod.	Anti-inflammatory Th2
IL-12	DCs, monocytes, macrophages	Th1 inducer. Induces T cells, IFNγ & TNF production.	Proinflammatory Th1
IL-15	Macrophages, FLS, endothelial cells	T cell growth factor.	Proinflammatory
IL-17	T-cells	Enhance production and effect of IL-1 and TNF.	Proinflammatory
TGF-β	Platelets, activated macrophages, T regulatory cells	Regulate the inflammatory response and differentiation of T regulatory cells, promote wound healing, fibrosis and angiogenesis	Pro- and anti-inflammatory
TNF	Monocytes, macrophages	Inflammatory central modulator, induce acute phase reactants induce IL-1 production, bone destruction, apoptosis	Proinflammatory Th1

3.4.1 TNF

TNF is a potent inducer of the inflammatory cascade, produced predominantly by monocytes and macrophages. Synthesis of TNF and other proinflammatory cytokines is triggered in response to bacterial toxins, immune complexes, complement activation and other cytokines, i.e. IL-1, IL-2, IFNγ and TNF itself. TNF is first expressed as a membrane-associated molecule, mediating inflammatory and cytotoxic effect through cell-to-cell contact. This molecule is cleaved by TNF-converting enzyme to a secreted monomer that mediates local and systemic inflammatory and cellular immune responses.

Two different membrane-bound receptors for TNF are described: the 55 kDa or TNF Receptor (TNFR) 1, constitutively expressed in most tissues, and the 75 kDa TNFR2, expressed most in lymphoid cells (Bazzoni et al. 1996). Both receptors exist in soluble form, when an extra cellular domain derived from the membrane bound receptor is cleaved from the membrane (Reddy et al. 2000). These soluble receptors act as natural inhibitors of TNF, preventing TNF from binding to the membrane-bound receptors (Kohno et al. 1990), but may in addition also have an enhancing effect. The shed receptors with the bound cytokine are cleared in the kidney (Bemelmans et al. 1994). TNFR2 can only be activated by membrane-bound TNF, whereas TNFR1 is activated by both soluble and membrane-bound TNF. At low soluble TNF concentrations TNFR2 may serve as a ligand passer for TNFR1. TNFR2 is more inducible, expressed mainly in hematopoetic derived cells, whereas TNFR1 is constitutively expressed in most types of cells.

Three TNF monomers bind to the membrane-bound receptor forming a trimer and a subsequent intra-cellular signal is produced. The binding is with high affinity and nearly irreversible to the TNFR1, whereas the rate of dissociation to the TNFR2 is higher. Two main intra-cellular pathways may be activated by TNF, both receptors' signal through activation of NFκB and TNFR1 induces in addition an apoptotic signal through caspase-8 (Darnay et al. 1997).

TNF is a powerful inducer of cell growth, differentiation and apoptosis, mediated through stimulation of IL-1 expression and other proinflammatory cytokines, leukotrienes, prostaglandins and nitric oxide (reviewed in (Dinarello et al. 2002)).

3.5 SYNOVIA

3.5.1 Normal synovium

The synovium covers all non-cartilage intra-articular surfaces. The normal synovial lining layer consists of only 1-3 loosely organised, avascular cell layers, and is not supported by any basement membrane. Two cell types in the intima are identified by electron microscopy: macrophage-like synoviocytes and fibroblast-like synoviocytes (FLS) (Barland et al. 1962). Macrophages express MHC class II, CD 163, CD68 and FcγRIIIa (Bhatia et al. 1998). In normal synovia they are a minority, whereas in RA synovia up to 80% may be macrophages. FLS do not express MHC class II or CD68 and have no phagocytic ability. FLS produce synovial fluid produced from filtered plasma. In normal joints the synovial fluid just lubricates the cartilage parts of the joint. Lower down, in the synovial sublining, there are only a few cells and scattered blood vessels. The functions of synovial tissue in healthy individuals are maintenance of an intact tissue surface, lubrication and nutrition of the cartilage.

3.5.2 RA synovium

In RA the synovial tissue is characterised by a prominent inflammation (Tak et al. 2000), where both *FLS* and synovial macrophages contribute. The synovial hyperplasia may have several causes (reviewed in (Mor et al. 2005)). Several growth factors driving fibroblast proliferation are over-expressed in the synovia and FLS have mutations in proliferation-regulating proteins, among them the tumour suppressor gene p53, and these mutations are frequent at sites with cartilage damage (Seemayer et al. 2003). Furthermore, an impaired apoptosis with more long-lived cells has been suggested as RA FLS express high levels of Bcl-2, consistent with an anti-apoptotic phenotype (Perlman et al. 2000). IL-15, produced from FLS and synovial macrophages, supports further increased Bcl-2 expression and cell survival (Kurowska et al. 2002). FLS respond to and produce a wide range of inflammatory mediators, such as proinflammatory cytokines and pro-angiogenic factors. Signal transduction pathways, like NFκB and MAP kinase-related pathways, are activated in FLS (reviewed in (Mor et al. 2005). A direct contact between IL-15-producing RA FLS and T cells promotes T cell activation (Miranda-Carus et al. 2004).

The proliferating synovial tissue subsequently forms a pannus that invades the cartilage and bone. FLS contribute significantly to cartilage degradation through the production of enzymes such as matrix metalloproteases (MMPs). TNF, IL-1α and growth factors induce MMP expression in FLS (MacNaul et al. 1990; Shingu et al. 1993). Bone destruction is mediated mainly by osteoclasts, derived from macrophage precursor cells influenced by osteoprotegrin ligand (OPGL), produced by FLS and receptor activator of NFκB ligand (RANKL) (reviewed in (Mor et al. 2005). FLS stimulated with TNF may also directly invade bone (Pap et al. 2003).

Macrophages are also abundant in the inflamed thick synovial tissue in RA. They express MHC class II and produce several proinflammatory cytokines, such as IL-1 (Miyasaka et al. 1988; Ulfgren et al. 2000), IL-6 (Okamoto et al. 1997), TNF (Firestein et al. 1990; Chu et al. 1991; Ulfgren et al. 2000), IL-15 (McInnes et al. 1996) and IL-18 (Gracie et al. 1999). They also produce some proteolytic enzymes, but their matrix-degrading ability is modest in comparison with FLS. *DCs* are present in the synovial tissue arranged with lymphocytes (van Dinther-Janssen et al. 1990), immature DCs are detected in the lining, and mature DCs exclusively in lymphocyte infiltrates (Page et al. 2002). DCs with the capacity to produce a great amount of proinflammatory cytokines are also present in the synovial tissue (Cavanagh et al. 2005) as well as TLRs (Roelofs et al. 2005).

T cells are present in the sublining of the synovial tissue in RA. T cells express the surface marker CD3 and either CD4 or CD8. The majority in the synovial tissue are CD4+. A few *B cells* are present in the synovial membrane in RA. The formation of antibodies to immune complex are common in RA and immune complexes with anti-citrulline specificity are detected in synovia in murine models (Lundberg et al. 2005).

In the synovial tissue *neutrophils* are rare, but they are demonstrated in the cartilage-pannus junction (Liu et al. 2004) and are abundant in the synovial fluid (Pillinger et al. 1995), where the survival has been suggested to be prolonged due to an impaired ability to undergo apoptosis (Edwards et al. 2004). Neutrophils are recruited to the synovial fluid by chemoattractants (C5a, IL-8) and immune complexes. Neutrophils contribute to cartilage destruction through the release of lysozomal enzymes and oxygen radicals.

4 IMPACT OF INFLAMMATION ON ENDOCRINE REGULATION OF ADRENAL AND GONADAL HORMONES

4.1 INFLAMMATION AND THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

IL-1 and IL-6 are described to influence the adrenal axis at all levels, from the stimulation of corticotropin-releasing factor in the hypothalamus to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland and stimulation of cortisol and dehydroepiandrosterone sulphate (DHEAS) production from the adrenal cortex, (reviewed in (Turnbull et al. 1999). This is quite appropriate during acute stress, such as a traumatic injury and sepsis. On the other hand, TNF has been described to have a stimulating effect on the central parts of the axis, but an inhibiting effect on the periphery (Jaattela et al. 1990).

In patients with RA, the HPA axis has been described as inappropriate normal in response to an inflammatory stimulus (Straub et al. 2001). In response to a stressful event the response in the HPA axis is lower than in controls concerning the cortisol whereas no difference was recorded concerning ACTH (Eijsbouts et al. 2005). An inflammatory process such as arthritis may thus not be appropriately downregulated (Straub et al. 2001). Low cortisol and DHEAS levels are seen in young women with RA, also preceding disease onset (Masi et al. 1984; Masi et al. 1999). Theoretically these women may be at a higher risk of developing RA when their HPA axis does not respond to an inflammatory or stressful stimuli.

DHEAS is described to be low in female patients with RA (Feher et al. 1984; Masi et al. 1999; Imrich et al. 2005), particularly in young women, where low levels may also precede disease onset (Masi et al. 1999). Also in other autoimmune diseases, Sjögren syndrome (Valtysdottir et al. 2003), SLE (Derksen 1998) low DHEA and DHEAS levels are reported. A negative correlation with inflammatory markers has been suggested in RA (Tengstrand et al. 2003), but the contrary has also been reported (Nafziger et al. 1998). In healthy women, on the other hand, there is no correlation between DHEAS and inflammatory markers (Sowers et al. 2005).

4.2 INFLAMMATION AND THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Centrally in the HPG axis luteinising hormone-releasing hormone (LHRH) is produced in the hypothalamus, which activates the anterior pituitary gonadotropic cells to produce luteinising hormone (LH). In men LH stimulates testosterone production from the Leydig's cells of the testis. LHRH and LH are controlled by a negative feedback of sex hormones at the hypothalamic levels.

Androgens suppress both humoral and cellular immune responses. Estrogens exert immunoenhancing activities. In low doses (physiological), estrogens enhance the cellular immunity/RA (Th1 cytokine network) and in high doses (pharmacological) the humoral/SLE (Th2 cytokine network), (reviewed in (Salem 2004)).

TNF, IL-1 and IL-6 inhibit sex hormone production in the gonads and stimulate aromatase activity leading to increased conversion of androgens to estrogens in the periphery (Straub Cutolo 2001).

TNF administered intravenously to healthy men induces an immediate increase in LH and a transient decrease in testosterone after 4 hours, when LH was already normalised (van der Poll et al. 1993). IL-6 to healthy men decreases testosterone without any effect on LH (Tsigos et al. 1999). In critically ill patients, e.g. sepsis, testosterone levels decrease (Vogel et al. 1985; Woolf et al. 1985), and TNF and IL-1 increase (Damas et al. 1989; Calandra et al. 1990).

In RA, low testosterone levels have been reported in a cross-sectional study (Tengstrand et al. 2002) where low testosterone levels are correlated with high disease activity (Gordon et al. 1988). High estradiol is also reported in male patients with RA and correlates to high ESR (Tengstrand et al. 2003).

5 THERAPY

5.1 THERAPY – GENERAL ASPECTS

While the etiology of RA is unknown, studies on the effect of different therapies may provide clues as to the pathogenesis and help to identify new targets for therapy.

A decade ago it was established that an early start with disease-modifying anti-rheumatic drugs (DMARDs) in patients with RA is of great importance in inducing remission (Mottonen et al. 2002) and in preventing future joint damage (Egsmose et al. 1995; van der Heide et al. 1996). In today's practice methotrexate is the most common drug (Pincus et al. 2003), but combination therapies are frequent and more efficient than monotherapy (O'Dell et al. 1996; Choy et al. 2005), with only slightly increased toxicity (Choy et al. 2005). Recent data favour an early start of combination therapy (Suresh et al. 2005), with intensive management and remission as a treatment goal (Grigor et al. 2004). In patients who fail to respond to conventional DMARDs and in selected high-risk patients with early rheumatoid arthritis treatment with TNF-antagonist are recommended (Furst et al. 2005; Goekoop-Ruiterman et al. 2005). In absence of contraindications an additional treatment with low-dose glucocorticoids also seems beneficial (Svensson et al. 2005). In the following paragraphs only therapies relevant to the articles included in this thesis are addressed.

5.2 GLUCOCORTICOIDS (GC)

Glucocorticoids, initially a discovery of Hench and Kendall (Hench et al. 1949) have been used in RA for more than 50 years. Side-effects such as osteoporosis, infections and diabetes are, however, feared (Lukert et al. 1990; Saag et al. 1994). Only recently the beneficial role of low-dose treatment with GC together with DMARDs in early arthritis was demonstrated (Kirwan 1995; Svensson et al. 2005). GCs exert their role on specific cytoplasmatic receptors and induce changes in lymphocyte function, Fc receptor suppression and an inhibiting effect on proinflammatory cytokines through downregulation of NFκB, being an example of a "dirty drug" with multiple unspecific effects. The advantage is, however, that many patients are responders and a remission frequency after two years of 55% when combined with DMARDs was recently reported in early RA, compared with 33% in GC-naive patients (Svensson et al. 2005). In the future selective glucocorticoid receptor agonists may be developed with more selective inhibition of NFκB, without simultaneous transactivation of gluconeogenesis enzymes (Song et al. 2005).

5.3 GOLD SODIUM THIOMALATE (GSTM)

5.3.1 Gold sodium thiomalate - pharmacological aspects

Gold sodium thiomalate (GSTM) has been used in RA since the 1920s (Forestier 1935). GSTM is given as an intramuscular injection, initially once a week. After the injection the gold atom and thiomalate become separated and the gold atom binds to a serumprotein (Blocka et al. 1986) with an initial half-life of GSTM of about 5 days and excretion mostly by urine (Gerber et al. 1974). With repeated doses, the half-life is prolonged. Peak serum concentrations are estimated at 7 mg/L, and during maintenance therapy serum concentrations are around 1 mg/L (Smith et al. 1984). Gold is retained in the tissues, particularly in inflamed tissue such as synovial tissue (Vernon-Roberts et al. 1976), and is found in the liver and skin of patients many years after

cessation of treatment. There is no correlation between serum levels at steady-state and clinical effect (Gerber et al. 1972). There is an uptake of gold in the lysosomes of the synovial macrophages (Vernon-Roberts et al. 1976) with a formation of aurosomes (Oryschak et al. 1976) and a subsequent morphology change in monocytes (Ugai et al. 1979).

5.3.2 GSTM – biological effects

In vitro studies on the effect of GSTM have shown a downregulation of adhesion molecules (Heimburger et al. 1998) and neutrophil-dependent cytotoxicity in endothelial cells (Bratt et al. 2000) and a suppressed monocyte chemotaxis (Ho et al. 1978). The proliferation of lymphocytes (Sfikakis et al. 1993), fibroblasts (Matsubara et al. 1988) and DCs (Wang et al. 2002) is also suppressed. The antigen presentation and T cell activation mediated by DCs are also suppressed *in vitro* (Wang et al. 2002). Further, *in vitro* monocyte production of proinflammatory cytokines decreases; IFNγ (Lampa et al. 2002), IL-1 (Drakes et al. 1987), IL-2 (Wolf et al. 1988) and TNF as well as TNF mRNA expression (Mangalam et al. 2001). Concerning anti-inflammatory cytokines, an increase of IL-10 production from monocytes is reported (Lampa et al. 2002).

In vivo, GSTM treatment increases serum levels of IL-10 (Lacki et al. 1995) and decreases levels of IL-6 (Madhok et al. 1993; Lacki et al. 1995). In the synovial membrane, treatment with GSTM is reported to decrease adhesion molecules (Corkill et al. 1991) and CD68+ macrophages (Yanni et al. 1994), a biomarker correlated to disease activity (Haringman et al. 2005). A subsequent decrease in the expression of macrophage-derived proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNF is recorded (Yanni et al. 1994). The numbers of T and B cells remain unaltered, but the CD4+ T cells decrease in relation to the CD8+ T cells (Walters et al. 1987).

The effect of GSTM on pro-inflammatory cytokines has been suggested to be caused by a suppressive effect on NFκB translocation into the nucleus, through an effect of the thiol group of GSTM on the enzyme Inhibitory κB (Bratt et al. 2000; Jeon et al. 2000).

5.3.3 GSTM – clinical response and side effects

Response to GSTM takes several months, but remission is achieved more frequenctly than with methotrexate (Rau et al. 1997; Hamilton et al. 2001), while the effect on cartilage destruction is at least comparable to the effect of methotrexate (Rau et al. 2002). Adverse events are more frequent than for other DMARDs (Rau et al. 1997), in particular mucocutaneous side effects like skin rashes and mouth ulcers (Sambrook et al. 1982), sometimes preceded by eosinophilia. An association of the appearance of skin rashes and remission has been reported (Adams et al. 1950; Caspi et al. 1989).

GSTM treatment is now less used less than previously. Toxicity, the length of time before a perceptible effect appears and the initially frequent visits to the out-patient clinic are all reasons for other treatments being chosen for patients with high disease activity. The most important reason, however, is probably the introduction of new, less toxic therapies, i.e. biologics, with an almost immediate reduction of disease activity.

5.4 BIOLOGICS

5.4.1 Description of TNF-antagonists and biological effects

In the late 1990´s a new type of anti-rheumatic drugs appeared, biologics. The name refers to agents with a similarity to endogenous molecules, i.e. antibodies, soluble receptors and antagonistic cytokines. The most frequent used biologics are various TNF-antagonists. Biologics are more efficacious in RA than methotrexate (Klareskog et al. 2004), but when treatment is stopped the disease often flares. In today's daily practice, three TNF-antagonists are used in the treatment of RA. Infliximab is a chimeric IgG1kappa monoclonal antibody composed of a human constant attached to a variable region of a murine anti-human TNF antibody (Knight et al. 1993). Each infliximab molecule has the ability to bind to up to two TNF molecules in stable complexes with a high affinity. Up to three infliximab molecules can bind to one TNF molecule, thereby blocking all binding sites (Scallon et al. 2002). It binds to both soluble forms and monomers, dimers and trimers of transmembrane forms of TNF and subsequently complement activation or antibody-dependent cellular cytotoxicity induces lysis of these cells (Lugering et al. 2001). Etanercept is a dimeric fusion protein of the extracellular ligand-binding portion of the human soluble receptor 75kDa TNFR2, linked to the Fc portion of human IgG1 (Mohler et al. 1993). Etanercept binds one soluble TNF molecule as well as trimers of membrane-bound TNF (Scallon et al. 2002) and also has the ability to bind lymphotoxinα (Buch et al. 2004). Etanercept leaves one binding site of TNF free. The dissociation of TNF from etanercept is high and the dissociated TNF is bioactive, possibly through activation of TNFR1 (Scallon et al. 2002). It does not induce antibody-dependent cell lysis. Adalimumab is a recombinant human IgG1 monoclonal antibody specific for human TNF.

Concerning effects of TNF-antagonists on cells, monocytes decrease (Lorenz et al. 1996) whereas neutrophils and B cells are unchanged. Adhesion molecules decrease (Lorenz et al. 1996; Tak et al. 1996; Klimiuk et al. 2004). After treatment with infliximab the number of CD4+CD25+ T regulatory cells increased and their function is also restored with a normalised ability to suppress proinflammatory cytokine production from CD4+ T cells and monocytes in RA (Ehrenstein et al. 2004). An increased T cell reactivity has been reported previously with an increased ability to produce both IFNγ and IL-4 (Berg et al. 2001; Nissinen et al. 2004). Production of proinflammatory cytokines (TNF, IL-1β, IL-6 and IFNγ) from PBMC decreased during treatment with etanercept, whereas IL-10 production was unchanged (Schotte et al. 2004). Proinflammatory cytokines (IL-1β IL-1ra IL-6 IL-8 IL-18 MCP-1 VEGF) decrease (Elliott et al. 1993; Lorenz et al. 1996; Paleolog et al. 1998; Charles et al. 1999; Rooney et al. 2004).

5.4.2 Clinical effects and side effects of TNF-antagonists

All TNF-antagonists have a superior clinical effect to that of methotrexate in relieving symptoms, decreasing acute phase reactants and stopping joint destruction (Lipsky et al. 2000; Weinblatt et al. 2003; Klareskog et al. 2004). Clinical response is achieved for around 2/3 of patients during treatment with all TNF-antagonists, but 1/3 of patients are non-responders. Another 1/3 are good responders close to remission (Weinblatt et al. 1999; Lipsky et al. 2000; Weinblatt et al. 2003).

TNF-antagonists have been efficacious in, besides RA, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, uveitis, psoriasis and some orphan diseases like TNF receptor-associated relapsing syndrome and Mb Behçet (Keystone 2004).

TNF-antagonists are generally well tolerated, with 75% of the patients still on the drugs after 20 months (Geborek et al. 2002) and a low frequency of mild general side effects compared with DMARDs. The main serious side effects so far are infections (Day 2002). In particular, reactivation of tuberculosis has been reported (Keane et al. 2001; Wallis et al. 2004; Askling et al. 2005). These patients have had a high extent of extra-pulmonary tuberculosis and disseminated disease. Infliximab-treated patients have been at a higher risk of tuberculosis than etanercept treated patients and a shorter reactivation time is observed. The effect of TNF in maintaining stable tuberculosis granulomas is crucial (Kindler et al. 1989). Biological differences between the two TNF-antagonists, such as effects on transmembrane TNF and apoptosis probably explain this different incidence(Lugering et al. 2001).

A longer observation time is needed to assess the long-term effects of TNF-antagonists. Concerning cancer incidence, in particular an increased frequency of lymphomas has been feared. So far the incidence of lymphomas and other cancer diseases has not been altered in patients treated with TNF-antagonists compared with patients without TNF-antagonist treatment (Askling et al. 2005). A high frequency of autoantibodies, such as antinuclear antibodies, antibodies towards double-stranded DNA and anti cardiolipin antibodies are reported, in particular during infliximab treatment (Charles et al. 2000; Jonsdottir et al. 2004). There are few case reports of demyelinating disease and SLE-like syndromes during TNF-antagonist treatment (Moreland 2004), possibly related to changes in the immune response, such as an increased formation of autoantibodies. Recently we reported a case series of six RA patients older than 60 with RA and previous mild pulmonary fibrosis, who developed severe, and in several cases fatal, alveolitis during treatment with infliximab or etanercept (Tengstrand et al. 2005). The mechanism of this side effect may be a shift in the balance towards a Th2 immune response.

5.4.3 Other existing and evolving biologic therapies in RA

Many other cells and cytokines besides TNF have been identified as potential therapeutic targets in RA. Treatment with recombinant IL-1 receptor antagonist (IL-1ra) has been reported to be of value for some RA-patients, but has not been as successful as TNF-antagonists in achieving clinical response (Jiang et al. 2000). A combination of a TNF-antagonist and IL-1ra has been disappointing, with a high frequency of adverse events and no superior effect to etanercept alone (Genovese et al. 2004). Promising results in clinical trials in RA-patients have been reported for specific anti-cytokine therapies: an anti-IL-6 receptor antibody (Nishimoto et al. 2004) and an anti-IL-15 human monoclonal antibody (Baslund et al. 2005). Moreover, a beneficial effect in RA has been shown in terms of B cell depletion targeting the cell surface marker CD20, rituximab (Edwards et al. 2004), and T cell costimulation ligand depletion with fusion protein CTLA4Ig (Kremer et al. 2005), which binds CD80 and CD86 on antigen-presenting cells and blocks the binding with CD28 on T cells. Recombinant IL-10 has, however, not been efficacious in RA. This may be due to an upregulation of Fcγ-receptor expression in monocytes and macrophages during IL-10 treatment, potentiating an immune complex mediated proinflammatory response (van Roon et al. 2003).

6 AIMS

The general aim of this thesis was to investigate the clinical response of cytokine-modulating therapies in RA in relation to cytokine expression, gene polymorphisms and hormonal levels.

Specific aims:

To study if gold sodium thiomalate treatment in RA induces a Th2 shift in relation to clinical response and adverse events.

To study if the TNF-antagonist infliximab has any impact on the synovial expression of other proinflammatory cytokines, in particular IL-15, in relation to clinical response.

To study if cytokine gene polymorphisms have any impact on clinical response to the TNF-antagonist etanercept in RA.

To study if the TNF-antagonists etanercept and infliximab have any effect on the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonadal axis in RA in relation to clinical response.

7 PATIENTS AND METHODS

7.1 PATIENTS

All patients in the present studies met the ACR criteria of RA (Arnett et al. 1988) (Table 1). They were recruited at the out-patient clinic at Karolinska University Hospital in Huddinge (I, III, IV) and Solna (I, II, III). All patients gave their informed consent. The studies were approved by the local ethical committee.

7.2 DISEASE ACTIVITY

In papers I, II, III and IV, disease activity was assessed using the DAS28, a composite index including swollen joint count (SJC), tender joint count (TJC), erythrocyte sedimentation rate (ESR) in mm/h and the patient's assessment of general health (GH), measured on a visual analogue scale (VAS) (0-100 mm). The DAS28 is calculated as follows:
DAS28 = 0.56 * sqrt(TJC) + 0.28 * sqrt(SJC) + 0.70 * ln(ESR) + 0.014 * GH.

In paper IV, serum concentrations of high sensitive C-reactive protein (hsCRP) were determined by a high sensitive immunonefelometric assay using a commercial kit from Dade Behring GmbH, Marburg, Germany. Blood samples for CRP (II) and ESR (I-IV) were analysed immediately.

7.3 RESPONSE CRITERIA

In papers I and IV, EULAR response criteria were used. In EULAR response criteria, good responders are those with a DAS28 improvement from baseline of at least 1.2 and an end-point value of <3.2. Moderate responders are patients with 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 of <5.1. A patient is considered to be in remission if DAS28 is <2.6 (Fransen et al. 2004).
In paper II, ACR response criteria was used. ACR response criteria evaluate changes, calculated as a percentage from baseline. TJC and SJC must be improved separately, and in addition three of the following five variables must be improved: ESR or CRP, HAQ score, patient's assessment of disease activity, physician's assessment of disease activity and patient's assessment of pain. ACR 20%, 50% and 70% responses may then be calculated (Felson et al. 1995).

In paper III, a combined response criterion were used. The criterion for being a non-responder was to be a non-responder according to both ACR response criteria and EULAR response criteria. The group used for comparison consisted of all other patients with varying degree of response.

7.4 FUNCTIONAL DISABILITY

In paper IV, functional disability was assessed using the Swedish version of the Stanford Health Assessment Questionnaire (HAQ) (Ekdahl et al. 1988). The HAQ score ranges from 0 to 3, where a higher score indicates a higher degree of disability.

7.5 TREATMENT

7.5.1 Gold sodium thiomalate (I)

The compound used for treatment was Myocrisin® (Aventis, Strasbourg, France), consisting of 20 mg gold sodium thiomalate (GSTM) and 20 μg phenyl mercury nitrate per mL. This preparation was given intramuscularly once a week starting with a dose of 10 mg and thereafter 20 mg/week for two weeks followed by 50 mg/week. After a total of 1000 mg the dose interval was prolonged. The research nurse who gave the injections also evaluated side effects. If side effects appeared, the dose was lowered or the injection interval prolonged.

7.5.2 TNF-antagonists (II, III and IV)

In paper II, three infusions of infliximab (Remicade®, Centocor, Leiden, The Netherlands) 3 mg/kg were given intravenously at 0, 2 and 6 weeks.

In paper III, etanercept (Enbrel®, Immunex, Seattle, WA, USA) doses of 25 mg were given subcutaneously twice a week during three months.

In paper IV, continuous treatment was given during two years with either etanercept (Enbrel®, Immunex, Seattle, WA, USA) 25 mg, given subcutaneously twice weekly or infusions of infliximab (Remicade®, Centocor, Leiden, The Netherlands) 3 mg/kg, given intravenously every eighth week after a three-dose induction phase with infusions at 0, 2, and 6 weeks.

7.6 ASSESSMENTS OF CYTOKINES

7.6.1 Enzyme-linked immunosorbent assay (ELISA) (I, IV)

Serum concentrations of IL-6 and IL-10 were determined with ultra-sensitive ELISA (R&D systems, St Paul, MN, USA) in papers I (IL-6 and IL-10) and IV (IL-6).

7.6.2 ELISPOT (I)

In paper I numbers of cytokine-producing cells (IFNγ, IL-6, IL-10) were analysed by ELISPOT. To prepare for the ELISPOT assessment, peripheral blood was collected in heparinized tubes. Peripheral blood mononuclear cells (PBMC) were then isolated using density gradient separation (Ficoll, Hypaque) and diluted to 1 x 10^6/mL in RPMI-1640 (Flow Laboratories, Irvine, Scotland) supplemented with glutamine, HEPES buffer, penicillin, streptomycin, and 10% of a defined batch of fetal calf serum (complete medium). Cell viability was assessed by trypan blue exclusion and always exceeded 95%. Cells were then used without further stimulation for the analysis of number of cytokine-producing cells, using the ELISPOT method.

The ELISPOT method allows detection of cytokine production at a single cell level (Czerkinsky et al. 1983; Sedgwick et al. 1983) and is 10-200 times more sensitive than ELISA (Tanguay et al. 1994). Conventional plastic ELISA plates were used instead of nitrocellulose plates as previously described (Ronnelid et al. 1997). The plates were coated with primary antibody (15 μg/mL), 50 μL/well overnight (16-20 h). Wells were washed and biotinylated secondary antibodies were added at 1 μg/mL overnight. Avidin-alkaline phosphatase (Dako, Glostrup,

Denmark) was added at a dilution of 1:250 and allowed to bind for 2 h. After washing BCIP 710-3 (Sigma, St.Louis, MO, USA) was added for 5 h to develop the spots. The number of cytokine-producing cells was counted using an inverted microscope. Antibodies used were 1-DIK (MabTech, Stockholm, Sweden) and biotinylated 7-B6-1 (MabTech) for IFNγ; 19F1 (Pharmingen, San Diego, CA, ISA) and biotinylated 12G8 (American Type Culture Collection, Manassas, VA, USA) for IL-10 and IL-6-I (MabTech) and biotinylated 39C3 (Pharmingen) for IL-6.

7.6.3 Incubation of PBMC with GSTM (I)

Before treatment, PBMCs from each patient were incubated with GSTM at 37°C for 16-20 h at concentrations of 0, 3, 12.5 or 40 μg/mL and analyzed for production of IL-10 with ELISPOT according to a previously used protocol (Lampa et al. 2002). Concentrations in this ranges have been used in previous in vitro studies and are described to be non-cytotoxic (Bratt et al. 2000).

7.6.4 Arthroscopic biopsies (II)

Samples from the synovial tissue in the knee joint were taken by arthroscopic biopsies. Biopsies were predominantly taken from areas of the synovial tissue with signs of maximal macroscopic inflammation. At the first arthroscopy the biopsy site was photo-documented and mapped to make it possible to take the biopsies at the second arthroscopy from the same area as the first biopsies. The biopsies were snap-frozen within 30 seconds in liquid isopentane, stored at -70°C and then embedded in OCT compound (TissueTek; Sakura Finetek, Zoeterwoude, The Netherlands) when sectioning was performed. Serial cryostat sections from the biopsy samples (7 μm) were placed on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and air dried for 30 minutes.

7.6.5 Immunohistochemistry (II)

Sections for cytokine staining were initially fixed for 20 minutes with freshly prepared 2% (volume/volume) formaldehyde (Merck, Darmstadt, Germany), dissolved in phosphate buffered saline (PBS; pH 7.4) at 4°C, washed in PBS, and then left to air-dry before storage at -70°C. Sections for phenotypic cellular characterization were fixed with acetone, then left to air-dry at room temperature before storage at -70°C (Ulfgren et al. 2000). The immunohistochemistry staining procedure, using EBSS / saponin 0.1% together with cytokine-specific monoclonal antibodies allows a positive staining of solely intra-cellularly expressed cytokine (Ulfgren et al. 1995). After fixation the sections were initially incubated with 1% hydrogenperoxidase and 2% sodium azide dissolved in EBSS-saponin in order to minimise background staining. The sections were incubated overnight with cytokine-specific monoclonal antibodies and subsequently a second antibody conjugated to biotin was added, followed by incubation with avidin-biotin peroxidase. The stainings were subsequently developed by incubation with diaminobenzidine. Negative controls using isotype matched IgG were included for each marker.

7.6.6 Computer-assisted image analysis (II)

A semi-quantitative analysis of the expression of cytokines and cell surface markers, considering the number of positive cells in the stained sections, was performed. Considering the cytokines IL-1α, IL-β and IL-15, stained synovial biopsy sections were evaluated by computerised image analysis, where the area of positive staining was expressed as a percentage of the total tissue area. This method has been evaluated and considered to be valuable in research studies (Haringman et al. 2005). In paper II, the analysis of an entire tissue section

typically involved 25–210 (median 70) microscopic fields, corresponding to an area of 0.7–9.1 (median 2.9) mm^2 at a magnification of x250.

7.6.7 Cytokine specific monoclonal antibodies (II)

The following mouse IgG1 primary cytokine-specific monoclonal antibodies (mAb) were used for immunostaining: two anti- IL-15 mAbs were used, one neutralizing (B-E29) and one non-neutralizing (B-T15; both from Diaclone SAS, Besancon, France); a mixture of three anti-IL-1α mAb (1277-89-7, 1277-82-29, 1279-143-4; Immunokontakt, Bioggo, Switzerland); two anti-IL-1β mAb were combined (2D8 and 1437-96-15; Immunokontakt, Bioggo); two different TNF antibodies were used: TNF mab1/mab11 by pooling two anti-TNF mAb (both mouse IgG1, mAb 1 and mAb 11 from PharMingen, San Diego, CA, USA) and 2C8 (Biodesign, Saco, ME, USA); two anti-IFNγ antibodies Dic1 and 7B6 (Mabtech, Stockholm, Sweden); anti-CD3mAb mouse IgG1 (SK7, Becton-Dickinson, Mountain View, CA, USA); anti-CD68 mouse IgG1 (Ber-MAC3, Dako, Glostrup, Denmark) and anti-CD68 mouse IgG1 (PG-M1, Dako, Glostrup, Denmark). Matched IgG isotype controls were included for each marker.

7.7 GENOTYPING (III)

In paper III, DNA was extracted from EDTA blood by polymerase chain reaction (PCR) amplification (Mullis et al. 1986) with sequence-specific primers in a slightly modified method as described previously (Aldener-Cannava et al. 1996). Primers for amplification of the VNTR region in intron 2 of IL1RN gene were described in (Tarlow et al. 1993). The sequencies of other primers (–308 TNFG/A, –1087 IL-10G/A, codon 25 TGFB1) are described in (Padyukov et al. 2001).

HLA typing was performed by DR low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden), according to a previously described method (Olerup et al. 1992).

7.8 HORMONE ANALYSIS (IV)

In paper IV blood samples were drawn mainly between 8.00 am and 1.00 pm and serum and plasma were stored at -70°C. Serum concentrations of cortisol, testosterone, estradiol-17ß and luteinising hormone (LH) were determined by time resolved fluorescence immunoassay (TRFIA) using commercial kits obtained from Wallac OY, Turku, Finland ("Autodelfia®"). The values of LH are expressed as U/L of 1:st IRP LH 68/40 and of 2:nd IRP FSH 78/549 respectively. Plasma concentrations of adrenocorticotropic hormone (ACTH) and serum concentrations of dehydroepiandrosterone sulphate (DHEAS) were determined by chemiluminescence immunoassay using commercial kits from Nichols Products Corp. San Juan Capistrano CA ("Advantage®"). Serum albumin was determined by the clinical routine method of the Department of Clinical Chemistry, Karolinska University Hospital at Solna, Sweden.

7.9 STATISTICAL ANALYSES

For statistical analysis Statistica software, version 6.0, was used in papers II and IV. StatView software, version 5.0, was used in papers I and III.

In paper I, II and IV, the Mann–Whitney U test was used for the analysis of differences between groups and the Wilcoxon's signed rank test was used for the analysis of matched pairs.

In paper I, the Kruskal-Wallis test was performed to analyse the relation between cytokine production and clinical response.

In paper II and IV, the Spearman rank correlation test was used for correlations between variables.

In paper III, the Fischer's exact test was used to determine whether there was a random association between the observed alleles in the two studied populations or not.

In paper IV, Friedman's test, followed by posthoc analysis by Wilcoxon's signed rank test, was used for analysis of variables over time.

$p<0.05$ was considered statistically significant.

8 RESULTS AND DISCUSSION

8.1 SERUM CYTOKINE PRODUCTION DURING TREATMENT WITH GSTM (I).

Paper I presents a prospective study of 20 patients with RA starting treatment with GSTM. After 3 months the clinical response was evaluated using the EULAR response criteria. Side effects were continuously evaluated. Before treatment and after 4 and 12 weeks IFNγ, IL-6 and IL-10 production from monocytes and serum levels of IL-6 and IL-10 were measured.

After 3 months 6 patients were good responders according to EULAR response criteria, 8 were moderate responders and 3 were non-responders. A total of 10 patients developed skin reactions. 3 patients withdrew GSTM treatment due to severe skin reactions before the assessment at 3 months and 4 withdrew after the assessment, whereas 3 patients continued with a lowered dose and/or a prolonged interval between injections.

The numbers of IL-6-, IL-10- and IFNγ-producing cells measured with ELISPOT had significantly increased after 4 weeks treatment with GSTM. The number of IL-6 producing cells had also increased after 12 weeks. Furthermore, serum concentrations of IL-10 increased after 4 weeks, but serum concentrations of IL-6 did not change. There was no correlation between clinical response and cytokine production.

IL-10 production increased during GSTM treatment as previously reported (Lacki et al. 1995). IL-10 is an anti-inflammatory cytokine with immunoregulatory properties reviewed in (Akdis et al. 2001), produced by dendritic cells (Edwards et al. 2002), monocytes/macrophages (Fiorentino et al. 1991), T cells and B cells (Moore et al. 2001). IL-10 acts anti-inflammatory through inhibiting gene expression and synthesis of several proinflammatory cytokines, such as IFNγ, IL-1, IL-6 and TNF (Williams et al. 2004). IL-10 down-regulates both TNF and IL-1 in synovial cell lines (Katsikis et al. 1994) and synovial tissue (Chomarat et al. 1995). Maturation of dendritic cells is prevented by IL-10 (De Smedt et al. 1997; Steinbrink et al. 1997) and accordingly MHC class II expression is downregulated and antigen presentation is suppressed (de Waal Malefyt et al. 1991).

GSTM prevents DC maturation in vitro and has a suppressing effect on antigen presentation and T cell activation (Wang et al. 2002). Immature DCs produce IL-10 (Edwards et al. 2002) and activate T regulatory cells of different subsets (Bacchetta et al. 2005). Tr1 cell proliferation is stimulated by IL-10 (Groux et al. 1997), produced from immature DCs (Akbari et al. 2001; McGuirk et al. 2002). Tr1 cells produce IL-10, IL-5, IFNγ and TGFβ (Cottrez et al. 2000). Further, Tr1 cells regulate DCs to stay immature (Bacchetta et al. 2005), as in a positive feedback loop. Another subset of T regulatory cells, CD4+CD25+ cells, may suppress both Th1 and Th2 responses by a cell-to-cell contact (Stassen et al. 2004). In addition they can stimulate other Tr1-like cells to produce IL-10 (Stassen et al. 2004). The increased IL-10 production during GSTM treatment in our report may indicate an increased immunoregulation and tolerance to GSTM, mediated by immature DCs and T regulatory cells.

The frequencies of skin rashes and subsequent discontinuations in some of the patients are in accordance with previous reports (Sambrook et al. 1982; Lockie et al. 1985; Rau et al. 1997). Patients without side effects had a higher in vitro IL-10 production from monocytes at baseline, before start of GSTM treatment, than patients with side effects such as skin rash. In nonallergic healthy individuals, the CD4+ T cells become anergic when exposed to nickel, another metal, as an effect of stronger regulation of CD4+CD25+ T regulatory cells, promoting tolerance to nickel (Cavani et al. 2003). On the contrary, in nickel allergic individuals CD4+CD25+ T

regulatory cells are unable to down-regulate the CD4+ response, tolerance is broken and an immune response is seen. In our study, the increased IL-10 production in individuals without skin rash may be a sign of a better ability in these individuals to regulate/tolerate the antigen presentation of GSTM. Immature DCs induce CD4+CD25+ T regulatory cells (Moseman et al. 2004) which down-regulate both a Th1 and Th2 response (Stassen et al. 2004). Tr1 cells producing IL-10 are also induced by immature DCs and by CD4+CD25+ cells (Stassen et al. 2004) .

Varying results were obtained concerning IL-6 production, with an increased production measured with ELISPOT, whereas serum levels measured with ELISA were unchanged. ELISPOT is at least 10 times more sensitive than ELISA (Tanguay et al. 1994) and detects only cytokine production from monocytes, whereas ELISA measures the net effect of production and consumption of cytokines. A relevant, but small increase in cytokine production, perceived by ELISPOT may be impossible to discern in an ELISA measurement.

We measured cytokine production several weeks before a clinical response to GSTM was to be expected, to be sure to record a possible early effect. In fact we saw an early increase in cytokine production of both IFNγ, a Th1 derived cytokine and IL-10, a Th2 derived cytokine. This may implicate that an early immunoregulatory effect is triggered by GSTM, whereas its clinical effects will only appear later. The effectuating cells for this immunoregulation may be Tr1, capable of producing both IL-10 and IFNγ (Cottrez et al. 2000). The delay in clinical effect mentioned above may explain why we did not find any correlation between the early cytokine changes and clinical response at that time. Time to effect may be several months (Rau et al. 1997), and when remission is obtained intermittent doses are required to avoid a disease flare. CD4+CD25+ T regulatory cells are reported to require continuous antigen exposure to remain active (O'Garra et al. 2004), which may explain why small intermittent doses of GSTM are enough to maintain a sustained remission in RA.

This study indicates an immunoregulatory rather than a strictly immunosuppressive effect of GSTM treatment in patients with RA. The increased IL-10 production during GSTM treatment may be a sign of as well as contribute to the immunoregulatory effects of GSTM in RA. It may also involve a shift from Th1 immune response typical of RA towards a Th2 immune response. One possible explanation is that factors of stronger immunoregulating influence than Th1 and Th2 cells, for example DCs and T regulatory cells are involved.

8.2 SYNOVIAL EXPRESSION OF IL-15 DURING TREATMENT WITH INFLIXIMAB (II).

In paper II, the effect of treatment with infliximab on synovial expression of IL-15, a pro-inflammatory cytokine, was studied with immunohistochemistry.

In nine patients with active RA and knee joint arthritis, an arthroscopy with multiple biopsies taken from the synovial tissue was performed before and after three infusions of infliximab. Synovial cellularity decreased and synovial expression of IL-15 decreased in 5 patients and increased in 4 patients after treatment with infliximab. ACR 70% response was achieved in 2 patients, ACR 50% response in 1 patient, ACR 20% response in 3 patients and 3 patients were non-responders. There was no correlation between synovial expression of IL-15 and response to therapy.

In RA, IL-15 is present in the synovial fluid and in the synovial membrane, predominantly expressed in the lining layer in macrophages, fibroblasts and endothelial cells (McInnes et al.

1996; McInnes et al. 1997; Thurkow et al. 1997; Oppenheimer-Marks et al. 1998). IL-15 shares one domain with IL-2, a T cell growth factor. T-cells, activated by IL-15, from both synovial tissue and peripheral blood from patients with RA induce TNF production from macrophages through cell-to-cell contact (McInnes et al. 1997). Further, IL-15 and IL-15 receptor α, a part of the IL-15 receptor form stable complexes on the cell surface of activated monocytes (Dubois et al. 2002), interacting with the rest of the receptor IL15Rβγ on CD8+ T memory cells. IL-15 is then recycled, leading to persistent appearance of surface-bound IL-15 (Dubois et al. 2002). In this way IL-15 stimulates the maintenance of CD8+ memory T cells (Zhang et al. 1998; Ku et al. 2000). In addition, activated T cells express TLR 2, a co-stimulatory receptor that acts together with IL-15 in maintaining T cell activation providing a link between the innate and adaptive immune system (Komai-Koma et al. 2004). Altogether, conditions for sustained T cell activation are obtained by IL-15, something that is suggested as being central in the pathogenesis of RA. We performed this study to elucidate the effect on IL-15 in relation to cell surface markers and other cytokines in the synovial tissue when another central cytokine, TNF, is blocked by infliximab.

Synovial expression of IL-15 was demonstrated in all patients at baseline. There was no consistent change in the synovial expression of IL-15 after three infusions of infliximab, in 5 patients the expression decreased whereas in 4 an increase was seen, but IL-15 was still present in all but one biopsy. The synovial tissue of that patient was taken at an arthroplasty and showed none or little expression of other cytokines or CD-markers, as previously reported without explanation (Catrina et al. 2002). There was no correlation between expression of IL-15 after treatment and clinical response to infliximab, measured as ACR response. The remaining expression of IL-15 in the synovial tissue after treatment with infliximab may implicate that despite an ameliorated synovial inflammation, conditions for a sustained T cell activation still may be present.

The observation of an overall decreased synovial cellularity after TNF antagonist treatment found in this paper is consistent with previous studies (Tak et al. 1996; Ulfgren et al. 2000; Smeets et al. 2003; Catrina et al. 2005). Our finding that CD68+ synovial mononuclear cells decreased significantly after treatment with infliximab 3 mg/kg, whereas T cells only showed a decreasing trend has also been reported before (Smeets et al. 2003). A decrease in the expression of CD68+ cells has also been suggested to be a synovial tissue biomarker of good response to therapy (Haringman et al. 2005).

TNF-producing cells, detected by TNF-neutralising antibodies mab1 and mab11, were exclusively seen in three patients with a subsequent good response to infliximab, fulfilling at least ACR50% response. Although the number of patients in this respect was small, the results are in concordance with those of a previous study of shorter duration (Ulfgren et al. 2000). Also in terms of the effects of synovial cellularity and expression of TNF mAb1 and mAb11, IL-1α and IL-1β, our results are in agreement with previously published studies (Ulfgren et al. 2000; Smeets et al. 2003). The results of staining with the new TNF-binding antibody 2C8 differed from those obtained with mAb1 and mAb11 in the sense that 2C8 shows rather abundant binding to extracellular material adjacent to cells that are intracellularly stained with this antibody. In addition, more cells are stained with 2C8 than with mAb1 and mAb11, indicating that staining with 2C8 is more sensitive than staining with mAb1 and mAb11. Nevertheless, the specificity of the 2C8 antibody for TNF seems high, because the positive staining was blocked totally by a recombinant TNF. The two sets of anti-TNF antibodies display different sensitivity and possibly also differences in binding to intracellular TNF only (mAb1 and mAb11), or to both intracellular and extracellular TNF (2C8).

This open label study was performed without a placebo group, with the patients as their own controls. This study design may be considered relevant as previous studies concerning serial synovial biopsies from patients receiving placebo or ineffective therapies showed no changes in the biopsy findings (Ruderman et al. 1995; Smeets et al. 1999; Cunnane et al. 2001). Biopsies at the first arthroscopy were taken from areas with maximal inflammation. If sufficient material was provided at the first arthroscopy, a second arthroscopy was performed with biopsies taken from the same area. This minimises the risk of a bias at the second arthroscopy. The differences in time when biopsies are taken in relation to the first (o-21 days) and the last infusion of infliximab (2-5 weeks) was not considered a major problem as only minor detectable decreases in serum levels of infliximab were detected 4 weeks after infusion (St Clair et al. 2002).

In our study we have chosen to study IL-15 detected by a neutralising antibody that detects IL-15-producing cells and a non-neutralising antibody that in addition also detects IL-15 bound in the tissue. We considered that the important part in the regulation in the synovial tissue is the expressed IL-15 protein. The micro-array technique may provide additive information on mRNA expression in the synovial tissue, to complement data on the expressed protein measured with immunohistochemistry.

In conclusion, we consider IL-15 to be independent of TNF-antagonist treatment. Thus, IL-15 may be a remaining therapeutic target in patients without effect of TNF-antagonists or in addition to TNF-antagonists in partial responders. Recently a 12-week double-blind phase I/II randomised controlled trial showed promising results of an IL-15 monoclonal antibody in 30 patients with RA (Baslund et al. 2005), indicating a possible future role in RA treatment.

8.3 GENETIC FACTORS PREDICTING RESPONSE TO TNF-ANTAGONISTS (III).

In paper III we investigated if genetic factors, such as HLADRB1/SE or some variations in cytokine genes, relevant to RA, have any impact on clinical response to etanercept. The clinical response rate was evaluated after 3 months in 123 patients with RA.

We did not find any correlation between the presence of HLADRB1/SE and clinical response to etanercept in our material. This lack of association between HLADRB1/SE and clinical response has been reported for treatment with infliximab and conventional DMARDs (Mottonen et al. 2002; Gossec et al. 2004; Marotte et al. 2005). In another study of 457 Caucasian patients the presence of two SE copies correlated with a higher ACR50% response rate to etanercept than in patients with none or one copy (Criswell et al. 2004), indicating a gene dose response association. In that study also patients with presence of HLADRB1/SE together with TNF–308G responded better, indicating perhaps the influence of a linkage disequilibrium with other genes important for immune responses.

Further, we investigated if single nucleotide polymorphisms (SNP) in the promoter region for–308 TNFG/A, –1087 IL-10G/A, in the intron 2 of IL-1 receptor antagonist gene (IL1RN) and in first exon of transforming growth factor beta 1 gene (TGFB) had any impact on clinical response to etanercept. There was no correlation between response and any SNP alone. A combination of the genotype TNF –308GG (=TNF1/TNF1) and IL-10 –1087GG (n=23) was correlated with a higher frequency of responders to etanercept, 95%, compared with 77% in patients with all other combinations (n=100), p=0.031 (Fisher's exact test).

Eight SNPs have been described within the TNF promoter at positions –1031T/C, –863C/A, –875C/T, –575G/A, –376G/A, –308G/A, –244G/A and –238G/A (reviewed in (Bayley et al. 2004). In our study the selection of genotypes that may have an influence on the phenotype was

performed taking into account that the less frequent allele –308TNFAA (TNF2) has been described to be functionally relevant, resulting in increased transcription of TNF (Kroeger et al. 1997; Wilson et al. 1997) and increased production in vitro (Bouma et al. 1996; Braun et al. 1996; Louis et al. 1998), though the contrary has also been claimed (Brinkman et al. 1995). The IL-10 promoter SNP studied has been linked to low IL-10 production in vitro (Turner et al. 1997; Rieth et al. 2004). The same combination of polymorphisms in the promoter region of TNF and IL-10 that we studied is described to be functionally relevant in rejection to transplants (Turner et al. 1997). Also theoretically the combination of polymorphisms used in this study, of –308TNFGG and –1087GG may implicate a low immune response, as it may be considered to correlate with a phenotype with presumed low serum levels of TNF and high serum levels of IL-10.

We report in addition that a combination of A2 allele for intron 2 IL1RN and rare C allele in codon 25 of TGFβ1 gene (n=4) was correlated with a higher frequency of non-responders (n=3) to etanercept, compared with non-responders (n=21) among patients with all other combinations (n=119), p=0.023 (Fisher's exact test). Also these polymorphisms have been suggested to be of functional importance (Awad et al. 1998; Perrier et al. 1998).

There are other studies of genetic predictors for treatment response to TNF-antagonists. Concerning the –308TNFG/A polymorphism, both an association (Mugnier et al. 2003) and a lack of association (Martinez et al. 2004) with response to infliximab have been reported. Another TNF gene polymorphism, the –857 TNF, has been reported to be associated with response (Kang et al. 2005). Concerning the IL-10 gene, a microsatellite allele with association to low IL-10 production (R3), has been suggested to be associated with good response to TNF-antagonists (Schotte et al. 2005). Other gene polymorphisms that have been suggested to be of importance for the response to TNF-antagonists are TNF receptor II 196 T/G with contradictory results (Fabris et al. 2002; van der Helm-van Mil et al. 2004) and Fcγ receptor IIIA polymorphism (Tutuncu et al. 2005).

Multiple comparisons are always a drawback in studies like this one. A high number of comparisons was carefully avoided through the conscious choice of a few functionally relevant SNPs and combinations of SNPs. Also concerning clinical response criteria we performed a dichotomous division in responders and non-responders to keep the number of comparisons low. We considered it important in this study to identify patients without the slightest tendency to be responders to etanercept. Consequently, a non-responder was defined as a patient failing to fulfil either ACR 20% response or EULAR moderate response. The responder group used for comparison was all other patients with varying degree of response. Taking an approach like this, all patients in the non-responder group may be considered as true non-responders implicating a high relevance to clinical practice.

Another drawback is a low number of patients included and particularly, a low number of non-responders. In a pharmacogenetic study a considerable higher number of patients are needed to be able to draw conclusions about possible predicting factors for clinical response.

There has been a debate about the relevance of single nucleotide polymorphisms (Bayley et al. 2004), in particular with data on a relevant linkage disequilibrium in the region of HLA class II and III (1999), where also the TNF gene is situated. It has been claimed that the importance of TNF-promoter polymorphisms for development of RA may be hard to interpret, as this region represents very high linkage disequilibrium with neighbouring HLA DRB1 gene (Newton et al. 2003) and one effect can mimic another.

Since this study was performed there has been considerable progress in sampling data on gene polymorphisms with the micro-array technique, which allows scanning for hundred thousands of gene polymorphisms at the same time. A whole genome case-control linkage disequilibrium mapping is recently reported with data on RA-associated polymorphisms in two loci (Yamamoto et al. 2005). A scanning of thousands of genes also introduce major concerns about multiple comparisons and requires development of statistical methods to make useful interpretations. A selected study on relevant SNPs of cytokines or cytokine signalling pathways, known to be important in the pathogenesis of RA, may be an equally good approach and the two methods may complement each other.

Another approach in pharmacogenetic studies may be identification of genes with altered gene expression after drug exposure, in order to obtain information about drug specificity, efficacy and toxicity (Ospelt et al. 2005). Further, information of still upregulated genes during treatment may give clues to understand pathogenesis and analysis of differences between responders and non-responders may be a first step to develop new therapies. There are not yet any reports of correlations between gene expression signatures and disease outcome or response to therapy in RA. This type of approach is already frequent in cancer research (van de Vijver et al. 2002). In rheumatic diseases upregulated IFN genes in SLE-patients correlated to disease activity (Bennett et al. 2003), whereas in arthritis comparisons between juvenile RA and healthy controls (Jarvis et al. 2004) as well as between early RA and late RA (Olsen et al. 2004) have been performed.

In conclusion, our study may support a significant role of cytokine gene polymorphisms, associated with a presumed phenotype with low inflammatory response, in prediction of treatment response to etanercept in the individual patient.

8.4 HPA- AND HPG-AXES DURING TNF-ANTAGONIST TREATMENT (IV).

In the fourth and last paper, 81 patients with long-standing established RA were investigated concerning the impact of either infliximab (n=56) or etanercept (n=25) on hormone levels of the HPA (ACTH, cortisol, DHEAS) and HPG (LH, estradiol, testosterone) axes.

Disease activity, measured as DAS28, and inflammatory markers (hsCRP, ESR, IL-6) were profoundly reduced in most patients during two years treatment with TNF-antagonists. There was no correlation between hormone levels of neither the HPA nor the HPG axis and inflammatory mediators, as reported previously during 12-16 weeks' short-term therapy (Straub et al. 2003; Straub et al. 2005). Further, we found no correlation between changes in hormone levels and clinical response according to EULAR response criteria. We did not either find any differences in hormone levels between patients in remission and non-responders.

At baseline low hormone levels of the peripheral HPA axis were seen in female subgroups. Low sex- and age-adjusted DHEAS levels were thus recorded in 4 patients without glucocorticoid (GC) treatment, and cortisol levels <150 nmol/L were recorded in 8 patients, all but one with concomitant low dose GC. In parallel low baseline testosterone levels were recorded in 9 of 18 male patients, which is consistent with previous reports (Gordon et al. 1988; Tengstrand et al. 2002). Low baseline levels of the HPA and the HPG axes were not correlated with disease activity or inflammatory markers and did not significantly change during two years of treatment with TNF-antagonists.

Instead, both in patients with low hormone levels at baseline and in patients with normal levels according to the reference levels, a considerable longitudinal stability in individual hormone

levels was found between baseline and two years: DHEAS (r_s=0.93, p<0.0001), cortisol (r_s=0.32, p<0.01), LH (r_s=0.75, p<0.001), testosterone (r_s=0.70, p<0.01) and estradiol (r_s=0.74, p<0.001). There was no effect whatsoever of the massive reduction in inflammation on this stability. In RA, longitudinal studies on hormone levels are lacking, but in healthy individuals such a longitudinal stability is reported regarding DHEAS and testosterone in women (Yildiz et al. 2004) and regarding testosterone (Maes et al. 1997) and estradiol (Gennari et al. 2003) in men. Twin studies have also suggested a hereditary contribution to cortisol and DHEAS levels (Meikle et al. 1997; Bartels et al. 2003). However, in one study of postmenopausal female RA-discordant HLA-identical twins, sex hormone levels were concordant concerning testosterone and estradiol, but DHEAS levels were lower in RA-affected siblings (Deighton et al. 1992).

DHEAS levels increased significantly from baseline to one and two years in female patients without GC treatment, but the individual stability mentioned above was still present. An improved physical function, measured with HAQ, was correlated with DHEAS levels after one (r_s=-0.44, p=0.008) and two years (r_s=-0.46, p=0.008). DHEAS is an androgen that has been correlated to overall well-being (Baulieu 1996) and in female Sjögren patients low levels have been associated with both diminished mental wellbeing and quality of sexual life (Valtysdottir et al. 2003). The increased DHEAS levels may be an effect of increased physical activity, as reported in healthy individuals (Kraemer et al. 2001; Tissandier et al. 2001), but in patients with RA strength and endurance training during 12 weeks had no effect on DHEAS levels (Hakkinen et al. 2005). Increased DHEAS levels may also be an effect of the TNF-antagonist treatment, but in a report of adalimumab treatment in patients with RA during 12 weeks no effect was recorded (Straub et al. 2005). The lack of correlation between DHEAS levels and inflammatory markers or disease activity indicates that the increased DHEAS level correlated with HAQ score is rather an effect of increased ability to be more physically active. This may be a more general effect, relevant to any efficective anti-rheumatic treatment rather than a specific effect of TNF-antagonists.

In our study, the subgroups of female patients with low levels of peripheral adrenal hormones mentioned above had a significantly lower age at disease onset than patients with normal levels. In female patients without concomitant prednisolone with a low age- and sex-adjusted DHEAS level at baseline the median age at onset of RA was 27 (range 21-30) compared with 47 (21–65) in patients with normal levels, p=0.0028. Similarly, in female patients with very low level of cortisol <150 nmol/L, the median age at onset of RA was 30.5 (23–53) compared with 42 (21–74) in patients with cortisol >150 nmol/L, p=0.040. DHEAS levels in relation to age at disease onset have been investigated by Deighton et al (Deighton et al. 1992), reporting numerically but not statistically significantly, lower DHEAS levels in postmenopausal women with disease onset before menopause compared with women with a postmenopausal disease onset. However, no age-adjustment of DHEAS levels was performed, so comparison is impossible. In young women with RA, low levels of DHEAS (Feher et al. 1984; Masi et al. 1999; Imrich et al. 2005) and cortisol (Masi et al. 1999), are reported and low levels of DHEAS have been described to precede disease onset in premenopausal women (Masi et al. 1999). The female subgroup we present could plausibly correspond to this subgroup earlier described, but prospective longitudinal studies are needed to confirm this finding.

The low levels of adrenal androgens and the inadequately low levels of cortisol in RA have been suggested to be the consequence of long-term inflammation (da Silva 2002). In this subset of young female patients low levels of peripheral adrenal hormones may instead provide the prerequisite for an early disease onset. A self-limiting immune response may not be inhibited strongly enough if the HPA-axis is down-regulated and it makes it impossible to resolve arthritis spontaneously. It can be speculated whether these stable low levels also precede the disease onset and whether the hypoactive HPA axis may contribute to the chronicity of the

disease. If so, a constitutional risk factor of a hypoactive HPA axis may define this subgroup of young female patients with RA.

In conclusion, treatment with TNF-antagonists in patients with RA during two years has no major impact on either the HPA axis or the HPG axis, regardless of response to therapy. The increased DHEAS levels may be associated with an improved physical function, rather than being a direct effect of decreased inflammation. The individual stability in hormone levels during the two years suggests a stable hormonal homeostasis, regardless of RA in a clinically active or controlled phase. An individual stability in the subgroup of female patients with signs of a hypoactive HPA axis and an early disease onset may indicate that the low levels of cortisol and DHEAS precede and contribute to the disease onset.

9 GENERAL DISCUSSION

This thesis focused on treatments of rheumatoid arthritis with drugs that may modulate cytokine interactions. The hypothesized immunoregulatory mechanism of action of GSTM treatment in RA was supported by an increase in IL-10, IL-6 and IFNγ-production. Synovial expression of IL-15, important in T cell activation and a therapeutic target present in the synovial tissue, was independent of infliximab treatment. This finding may indicate that IL-15 is a remaining target for therapy in RA. Further, a combination of cytokine polymorphisms, –308TNFGG and –1087GG, theoretically consistent with a low ability of immune response, may be relevant in predicting clinical response to etanercept. Finally, hormone levels of the HPA and HPG axes were independent of TNF-antagonist treatment, and, instead, an individually highly stable hormone homeostasis seemed to be of importance. A subset of women with low adrenal hormone levels had a disease onset at a young age. Presuming a stable individual hormonal homeostasis, these low adrenal hormone levels may even precede disease onset.

There are some more general aspects in the presented studies that need to be addressed.

RA is a heterogeneous disease in many ways. The clinical presentation ranges from mild to severe disease. There is evidence of different disease outcome for patients depending on their sex, menopausal status, smoking status, presence of HLADRB1/SE, anti-CCP and RF, as well as to erosive disease and disease activity. A variability in the inflamed synovial tissue is also described in immunohistochemistry studies (Klimiuk et al. 1997; Tak et al. 1997; Ulfgren et al. 2000), and is further supported by gene expression microarray data from the synovial tissue (van der Pouw Kraan et al. 2003). Satisfactory results for divergent treatment strategies, such as depletion of B or T cells or inhibiting different proinflammatory cytokines, indicate that different pathogenetic mechanisms contribute to disease in RA. Further clinical response and therapy side effects vary between patients (Elliott et al. 1993; Weinblatt et al. 1999). This heterogeneity may have an impact on the studies in this thesis. When a small number of patients are analysed, interpretation of data may be harder.

On the other hand, results from even a small number of patients with deviating side effects (paper I) or a lack of response (paper I and II) may provide data that can generate further hypothesis. Identification of subsets of patients with RA may give clues to a better understanding of different mechanisms in the pathogenesis.

Further, changes in synovial expression of cytokines, cells producing different cytokines or hormonal axes are in the respective study put in relation to clinical response criteria. Concerning RA, there is a lack of consensus regarding response criteria. Validation between the EULAR response criteria and the ACR response criteria on a group level has been successful (Verhoeven et al. 2000), but for an individual patient there may be a considerable difference between the two sets of criteria. Particularly concerning good response, concordance between the different criteria may be low (Gulfe et al. 2005). When a small number of patients are included in a study, this implies that interpretation of data in relation to clinical response must be done with some caution.

Genetic predictors are attractive, as they are present already at disease onset, even before patients fulfil the disease criteria. Effective treatment may then be introduced early, as a long time to initiation of DMARDs is a strong risk factor for worse long-term outcome (Möttönen et al. 2002). In a review by Scott (Scott 2003), the clinical utility of genotypes and phenotypes in predicting clinical response in RA was considered uncertain. Cytokine gene promoter

polymorphisms may provide valuable information in studies, but results have been diverging in RA and not consistent enough for use as predictors (Scott 2003). Concerning HLADRB1/SE, associated with worse prognosis, we found no correlation between HLADRB1/SE and clinical response to etanercept in established RA (III). In early RA however, a better prognosis in HLADRB1/SE+ patients is reported if combination therapies are used compared with methotrexate in single therapy, indicating a disease modifying effect of this genotype (Lard et al. 2002; Criswell et al. 2004; De Vries-Bouwstra 2005). As HLADRB1/SE is reported to be inexistent in anti-CCP antibody negative patients with RA (Huizinga et al. 2005), anti-CCP status may provide a selection criteria for combination therapy. Identification of different subsets of patients with divergent clinical response may be a way to develop individualised treatment startegies.

Hormone levels of the HPA and HPG axes seem unaffected of TNF-antagonist treatment (IV). Instead very stable individual levels are reported, both in patients with low levels from start and patients with normal levels. This suggests a stable hormonal homeostasis, regardless of RA in clinically active or controlled phase. We found that women with low levels of cortisol and DHEAS had a significantly lower age at disease onset compared to women with normal levels. Provided that longitudinally stable hormonal levels exist, these women may constitute another subgroup of patients with RA. A confirming prospective study of the frequency and characterisation of this phenotype in relation to other women with RA and healthy controls may provide valuable information.

In conclusion, studies on the effect of therapeutic interventions, like GSTM and TNF-antagonists, in RA may provide information on mechanisms of action, RA pathogenesis and remaining targets. The identification of subsets of patients with distinct phenotypes or genotypes may provide further insight into potentially different pathogenetic mechanisms and predictors for response to therapy. In a future perspective patients with RA may benefit of a more individualised treatment.

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