Immunological Mechanisms in Systemic Autoimmunity:
Autoantibodies and Chemokines in Systemic Lupus Erythematosus and During Treatment with TNF Inhibitors in Rheumatoid Arthritis

Catharina Eriksson
Till alla som önskat få en bok tillägnad sig
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ABSTRACT

Background. Rheumatoid Arthritis (RA) is an autoimmune inflammatory disease that, without powerful treatment, may lead to irreversible joint damage. During the past decade, anti-cytokine therapy has become available, e.g., infliximab, a chimeric antibody targeting the pro-inflammatory cytokine TNF that has a central role in the inflammatory process in RA patients. Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease that may affect all organs and is characterized by a massive antibody production. Chemokines, chemokine receptors and lipoprotein receptor-related protein 1 (CD91) are regulators of inflammation in autoimmune diseases and T-cell migration.

Objectives. The aim of this study was to get a deeper understanding how TNF blocking treatment influences inflammatory mechanisms and autoantibody formation in RA with special reference to similarities and differences with SLE.

Methods. In patients with RA treated with anti-TNF, and in SLE patients (ACR criteria) clinical evaluation was performed and blood samples analyzed. Autoantibodies were analyzed using indirect immunofluorescence, ELISA and multiplex flow cytometry in samples from anti-TNF treated RA patients (n=59) followed longitudinally for 54 weeks, in pre-diseased samples from SLE patients (n=38) and matched population-based controls (n=152). T-cell expression of chemokine receptors and CD91 was analyzed by flow cytometry, whilst serum levels of chemokines were determined using ELISA in anti-TNF treated RA-patients (n=24) followed longitudinally (30 weeks), and cross-sectionally in SLE-patients (n=23). Expression of mRNA for chemokines was analyzed in T-cells from SLE-patients (n=10) using PCR.

Results. After treatment with infliximab, RA patients produced ANA, anti-dsDNA and anti-nucleosome antibodies, but not anti-ENA antibodies. Although these antibodies are considered typical for SLE only one patient developed a transient lupus-syndrome. Antibodies against cell nuclear antigens, including ENA, were detected several years before the first clinical symptom of SLE; anti-SSA was the earliest detectable antibody.

In RA-patients before infliximab treatment, the T-cell expression of several chemokine receptors was elevated compared with healthy controls. In contrast, only one soluble chemokine, IP-10 was elevated. After treatment the levels of soluble MIP-1β, MCP-1 and IP-10, and the T-cell expression of CCR2 were decreased. In SLE-patients MIP-1β, MCP-1, SDF-1, IP-10 and RANTES in blood were elevated, whilst expression of CXCR5 and CCR6 on T-cells was lower than in healthy controls. T-cell expression of CXCR2 and CCR1 was elevated in active disease (measured as SLEDAI index), whereas the CXCR5 and CCR2 expression was lower in inactive SLE. In SLE patients with nephritis IP-10 was lower and T-cell expression of CXCR3 and CCR3
elevated compared with patients without nephritis. The expression of CD91 was higher on T-cells from patients not responsive to infliximab treatment compared with responders.

**Conclusion.** These findings indicate that anti-TNF (infliximab) treatment in RA-patients has a major impact on the production of autoantibodies and chemokines. The autoantibody profile in infliximab-treated patients was similar to that predating disease onset in SLE patients with the exception of anti-ENA being detectable in SLE, but the development of lupus-syndromes was rare. The expression of CD91 on T-cells may predict responsiveness to infliximab. The expression of chemokine receptors in SLE-patients seemed to be related to disease activity. Anti-nuclear antibodies were detectable years before clinical disease onset in patients who developed SLE suggesting a gradual pathogenic process.
Ledgångsreumatism (RA) är en livslång sjukdom som kan förstöra leder och ledernas funktion utan adekvat behandling. Skadorna på lederna orsakas av kronisk inflammation. De senaste åren har vi haft tillgång till s.k. biologisk behandling för RA-patienter. Ett av de vanligaste biologiska preparaten är infliximab, en antikropp som specifikt hämmar TNF, en molekyl som är central i inflammationsprocessen. I denna studie har vi undersökt den inverkan infliximab har på vissa specifika funktioner i immunsystemet.

Efter behandling med infliximab utvecklade många patienter antikroppar mot kroppsegna molekyler, s.k. autoantikroppar som är typiska för en annan reumatisk sjukdom, SLE. Endast en RA-patient utvecklade dock en övergående SLE-liknande sjukdom.

Kemokiner är en grupp molekyler som förmedlar signaler mellan celler inom immunsystemet. Kemokiner binds till specifika receptorer på cellytan av vita blodkroppar. De har en viktig roll vid inflammation i vävnader, då vita blodkroppar vandrar ut från blodbanan till inflammationshärden. Efter behandling med TNF-hämmare sjunker nivåerna av kemokiner i blodet vilket kan bidra till ett minskat utflode av vita blodkroppar till perifer vävnad och därmed minskad inflammation. För patienter med ledgångsreumatism leder minskad inflammation till att skador på lederna och därmed funktionsnedsättning kan reduceras.

CD91 är en molekyl som finns på ytan av vita blodkroppar ffa inom det ospecifika immunsförsvaret. Det är hittills inte beskrivet att denna molekyl uttrycks på T celler. Denna studie visade att CD91 finns på T celler, ffa på stora lymfocyter, d.v.s. aktiverade celler och att detta uttryck ökar hos de patienter som inte förbättrades av infliximab-behandling. CD91 skulle därför kunna vara en markör för patienter som inte förväntas svara på denna behandling, och som således bör få annan typ av behandling, men ytterligare studier behövs för att kunna fastställa detta förhållande.

SLE är en autoimmun sjukdom som kan drabba kroppens samtliga organsystem och leda till svåra organskador såsom exempelvis njursvikt. Det är viktigt att tidigt känna igen sjukdomen för att snabbt kunna sätta in behandling och förhindra permanenta organskador. Vi har i denna studie visat att de specifika antikroppar som förekommer vid SLE fanns i blodet flera år innan de första tecknen på sjukdom visar sig. Detta tyder på att sjukdomsutvecklingen börjar mycket tidigare än de kliniska sjukdomstecknen.
Vid SLE fann vi förhöjda nivåer av ett flertal kemokiner i blod jämfört med friska individer och att T-cells-uttrycket av kemokinreceptorer kan vara relaterat till sjukdomsaktiviteten.
ABBREVIATIONS

Ab  antibody
ACPA  anti-citrullinated-peptide antibody
ACR  American Colleague of Rheumatology
ANA  anti nuclear antibody
APC  antigen presenting cell
CD  cluster of differentiation
CI  confidence interval
CRP  C-reactive protein
CTLA  cytotoxic T-lymphocyte antigen
CTR  calreticulin
DAS-28  disease activity score (28-joints)
DC  dendritic cell
DMARD  disease modifying anti-rheumatic drug
ENA  extractable nuclear antigen
ESR  erythrocyte sedimentation rate
GM-CSF  granulocyte-macrophage colony stimulating factor
HAQ  health assessment questionnaire
HLA  human leucocyte antigen
HSP  heat shock protein
ICAM  inter-cellular adhesion molecule
IL  interleukin
IP  interferon-y inducible protein
LPS  lipopolysaccharide
MCP  monocyte chemotactic protein
MCTD  mixed connective tissue disease
MHC  major histocompatibility complex
MIP  macrophage inflammatory protein
MMP  metalloproteinases
NFκB  nuclear factor κ-light-chain-enhancer of activated B cells
PRR  pattern recognition receptors
PTPN  protein tyrosine phosphatase, non-receptor type
RA  rheumatoid arthritis
RANTES  regulated upon activation, normal T-cell expressed, and secreted
RF  rheumatoid factor
RNP  ribonucleoprotein
SD  standard deviation
SDF  stromal derived factor
SLE  systemic lupus erythematosus
SLEDAI  systemic lupus erythematosus disease activity index
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Sm</td>
<td>Smith antigen</td>
</tr>
<tr>
<td>SSA</td>
<td>Sjögren’s Syndrome A/Ro</td>
</tr>
<tr>
<td>SSB</td>
<td>Sjögren’s Syndrome B/La</td>
</tr>
<tr>
<td>sTNF</td>
<td>soluble TNF</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-alpha converting enzyme</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>tmTNF</td>
<td>transmembrane TNF</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNF-R</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T-cell</td>
</tr>
<tr>
<td>TSP</td>
<td>thrombospondin</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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</table>
LIST OF PUBLICATIONS

The thesis is based on the following papers that will be referred to by the appropriate Roman numeral:

I


II


III


IV


V

Eriksson C, Sundqvist KG, and Rantapää-Dahlqvist S. Changes in chemokines and their receptors after treatment with the TNF-inhibitor infliximab in patients with rheumatoid arthritis. (Manuscript)

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INTRODUCTION

1. The immune system

The main function of the human immune system is to protect the individual from pathogens such as bacteria, viruses and parasites and can be divided into an innate and an adaptive branch.

The innate immune system does not mediate immunological memory. It includes physical barriers, such as skin and mucosa, and a variety of cells and soluble molecules. This system reacts rapidly by receptors responding to molecular patterns on pathogens and damaged cells, and activates a wide range of inflammatory molecules. The innate immune system comprises phagocytic cells such as macrophages, granulocytes, dendritic cells (DC), and natural killer cells. DCs are the major antigen-presenting cells.

The adaptive immune system consists of T- and B-lymphocytes. Both develop in the bone marrow, the T-lymphocytes mature in the thymus and the B-cells in the bone marrow. The lymphocyte-mediated immune response can be either cell mediated via the T-cells or humoral mediated by antibodies from the B-cells. Activation through antigen specific receptors on the cell surface elicits clonal expansion of the lymphocytes. In T-cells, this activation generates a progression of the cells to CD8+ cytotoxic effector cells, CD4+ T-helper cells and long-lived memory cells, and in B-cells to antibodyproducing plasmacells.

T-helper (Th) cells can be divided into distinct subpopulations [1]. T-helper-1 (Th1) cells which promote a T-cell mediated cytotoxic reaction, produce cytokines for opsonising and complement-fixing antibodies, antibody-dependent cell cytotoxicity, macrophage activation, and delayed-type hypersensitivity reactions. T-helper-2 (Th2) cells which promote B-lymphocyte differentiation to the plasmacells and production of antibodies. T-helper-17 (Th17) cells can elicit powerful immune reactions in tissues, probably meant to attack certain pathogens such as fungi, parasites and specific bacteria [2]. Regulatory T-cells (Treg) modulate the strength and duration of the immune reaction while follicular T-helper cells (Thf) interact with B-cells in the germinal centres of lymphoid tissues, thereby providing signals for long-lived antibody responses [3]. The T-helper subsets are shown in Figure 1. There is a degree of plasticity in these populations. Upon appropriate cytokine stimulation, especially during the early stages of differentiation, the T-helper cells can be reprogrammed into an alternative type of T-helper cell [4].
A normal immune function depends on the ability of immune cells to interact with each other, with other cell types and tissues in a proper manner. However, the immune system is not always perfect and defects in its functions may lead to different kinds of disease, for example, rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

### 1.1 Cytokines and their role in autoimmunity

Cytokines play an important role in the communication between cells of the immune system. As inter-cellular mediators, they regulate survival, growth, differentiation, and effector functions of cells. Cytokines are rapidly synthesized and secreted by different cell types in response to stimulatory signals. The main sources of cytokines are DC, Th cells, and macrophages. Cytokines often act in networks or cascades, and are classified on the basis of their biological effects. Only cytokines with great importance in autoimmunity are discussed here; TNF and chemokines are considered in separate chapters below.

IL-2, previously considered a main factor for T-cell proliferation upon antigen activation, has more recently been shown also to be essential for regulatory T-cell function [6]. Polymorphisms in the IL-2- and IL-2 receptor genes have been identified in several autoimmune diseases including ulcerous colitis, type I diabetes and SLE [7-9]. Low levels of IL-2 have been associated with several autoimmune diseases, suggesting a protective role for this cytokine in autoimmune disease [9-11].

IL-10 has an immunoregulatory function, mainly affecting antigen-presenting cells. It affects the Th1 response more than the Th2 response, and
suppresses the production of many pro-inflammatory cytokines and chemokines, and may even stimulate a Th2 response [12].

IL-12 and IL-23 are structurally related cytokines but act on different cell types. IL-12 promotes a Th1 response, and IL-23 promotes a Th17 response [13, 14]. IL-12 has been shown to be involved in collagen-induced arthritis in animal models and in human RA [15, 16].

IL-17 was first described as a transcription factor from T-cells that could promote the production of other cytokines and chemokines [17]. It could not be defined as a distinct Th1 or Th2 cytokine. The cytokines TGFβ together with IL-6 or IL-21 are found to be the differentiation factors for Th17 cells, and specific intra-cellular transcription factors have also been defined [18]. IL-17, IL-23, and the Th17 cells have, during recent years, been shown to be involved in the pathogenesis of several autoimmune diseases, including RA and SLE [19, 20].

Interferon (IFN) -α, -β, and -γ are produced during the early phase of an immune response against intracellular pathogens. IFN-α and IFN-β stimulate both innate and adaptive immune responses, and IFN-γ activates macrophages to secrete cytokines which support a T-cell mediated response [21, 22]. IFN-α is known to contribute to the pathogenesis of SLE [23]. Deletion of the IFN-α receptor in a mouse model leads to a delay in the development of disease and a milder form of SLE-like nephritis [24].

1.2 TNF and TNF-receptors

Tumor necrosis factor (TNF) was originally described as existing in two forms: TNF-α and TNF-β. However, it was subsequently decided to change the names to TNF and Lymphotoxin corresponding to TNF-α and TNF-β, respectively. Lymphotoxin, has many similarities to TNF, not only has its own receptor, but can also bind to the TNF receptors. This study focuses on TNF.

TNF is released not only from immune cells but also many others cell types as a response to alarm signals, e.g., from viruses and bacteria, cytokines as IL-17, granulocyte-macrophage colony stimulating factor (GM-CSF) and IFN-γ, tumour cells, irradiation, and ischaemia [25]. TNF is considered to be a major component of inflammatory processes and induces the production and secretion of many other pro-inflammatory cytokines. It is secreted in a soluble form (sTNF) after cleavage from the transmembrane form (tmTNF) by TNF-α-converting–enzyme (TACE). Both sTNF and tmTNF are functionally active and interact with the TNF-RI and TNF-RII receptors. The cell surface expression of these receptors differs with respect to cell types, affinities for the ligands and intra-cellular signalling
mechanisms [26]. The transmembrane form can act either as a ligand or as a receptor [27].

Both sTNF and tmTNF are able to bind to both variants of the receptors, but the soluble form preferentially binds to TNF-RI, and the membrane bound form to TNF-RII [28]. TNF binding to TNF-RI leads to activation of the transcription factor NF-κB, which activates cytokine- and other activation-product genes but also to apoptosis. Binding to TNF-RII leads to activation of NF-κB but not to apoptosis. TNF-RII can occur in a soluble form that can bind and neutralize sTNF thereby functioning as a natural TNF antagonist [29, 30]. TNF-RI can also occur in a soluble form but does so to a lesser extent.

TNF induces the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, which promote leucocyte extravasation [31]. It is involved in defence against bacterial infections, especially intra-cellular bacteria, e.g., mycobacteria. Control of intra-cellular infections includes the formation of granulomas that encapsulate the microorganisms. Intra-cellular killing of such bacteria in macrophages is also dependent on TNF, as illustrated by the observation that TNF-deficient mice are highly susceptible to tuberculosis infections [32].

TNF is also involved in the adaptive immune response by being linked to IL-2 induction and T-cell survival [33]. Other effects of TNF are the induction of several chemokines, and inhibition of the suppressive effect of regulatory T-cells [34]. TNF is, due to its central role in inflammatory processes, a key player in the pathogenesis of many autoimmune and chronic inflammatory diseases.

### 1.3 Chemokines and chemokine receptors

Chemokines are a group of small proteins acting as immunological signaling factors on mostly immune cells, but also on other cell types. They were first described as a group of cytokines with chemotactic properties. The chemokine receptors are seven transmembrane spanning domains coupled to G-proteins. Most chemokines and chemokine receptors have more than one ligand/receptor [35]. The chemokines are sub-divided into four groups depending on the first two cysteine residues in the amino acid sequence: CC, CXC, XCC and CX3C. The CXC-chemokine receptors have chemotactic effects on granulocytes and monocytes during acute inflammation, and participate mainly in the innate immune reactions. CCR chemokines and their receptors act more on the adaptive immune system and in chronic inflammatory processes (Table 1).
### Table 1.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Receptor</th>
<th>Responding cell type</th>
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<tbody>
<tr>
<td>CXCL1</td>
<td>GRO1</td>
<td>CXCR1, CXCR2</td>
</tr>
<tr>
<td>CXCL2</td>
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<td>CXCR2</td>
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<td>GRO3, MIP-2β</td>
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<td>RANTES</td>
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<td>CCL20</td>
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<td>XCL1</td>
<td>Lymphotactin</td>
<td>XCR1</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Fractalkine</td>
<td>CX3CR1</td>
</tr>
</tbody>
</table>

N=neutrophils, T=T-cells, B=B-cells, Mo=monocytes, Eo=eosinophils, NK=natural killer cells, DC=dendritic cells

Binding of a chemokine to its receptor leads to conformational changes and signaling to the nucleus via a G-protein pathway [36]. Chemokines participate in many cell functions of the immune system, such as cell trafficking, differentiation, angiogenesis, and have an important role in acute and chronic inflammation [37-43]. Mature lymphocytes recirculate between the blood, lymphatic organs and peripheral tissues and are in that process guided to the right place by locally expressed chemokines. Leucocyte migration from the blood vessels to inflamed tissue occurs in four steps; rolling, activation, adhesion and transendothelial migration, during which chemokines play an important role [44].

Some of the chemokines have been shown to have a role in autoimmune diseases such as RA [45-47]. Some of the chemokine receptors can be considered as markers for specific cells or activation states, e.g., CXCR3 and CCR5 as Th1 markers, and CCR3 and CCR4 as Th2 markers [48, 49]. CCR2 is often co-expressed on CXCR3/CCR5 expressing cells, typical of Th1 cells,
but can also be expressed on other cell types such as Treg cells [50]. Since chemokines play a key regulatory role in organ-specific leucocyte trafficking and cell activation by affecting integrin expression, chemotaxis, effector cell functions and cell survival they have been suggested as potential therapeutic targets for treatment of autoimmune or chronic inflammatory diseases. However so far this has not been successful, although several trials on both chemokine and receptor blocking in a variety of diseases are in progress [51, 52].

2. Autoimmunity

T-lymphocytes that react to autoantigens should normally be destroyed by negative selection in the thymus but this function is not absolute. Lymphocytes that have passed the central tolerance selection also have to maintain their tolerance in the peripheral tissues. This peripheral tolerance depends on multiple mechanisms. In some cases, a genetic defect is the reason for lost tolerance and the development of autoimmune disease.

Antibodies to self-molecules are present in all humans and can be found in healthy blood donors without any signs of disease and are even found in the cord blood from newborn babies [53, 54]. Autoreactive cells and antibodies can be beneficial for the individual, both in healing processes and maintenance of immunological homeostasis. Damaged cells and structures that are otherwise hidden, such as nucleosomes and apoptotic material that becomes available to the immune system can result in development of autoantibodies through immunization, whereas antibodies present in newborns are considered to be natural autoantibodies [55].

Disturbances in Th1, Th2 and Th17 pathways can be present in autoimmunity [56]. Normal function of Treg is considered important in preventing autoimmune diseases as shown in an animal model [57]. Cytotoxic T-lymphocyte–associated antigen-4 (CTLA4) downregulates the T-cell immune response and is important for the peripheral tolerance. CTLA-4 deficient animals show spontaneous T-cell lympho-proliferation and autoimmunity [58]. The cell surface receptor Programmed Cell death-1 (PD-1) and its ligand (PD-L1) are also important for both the induction and the maintenance of peripheral tolerance. Polymorphisms in the PD-1 gene have been associated with several autoimmune diseases, including SLE, type 1 diabetes, and RA [59-61].

Microorganisms are important environmental factors known to be involved in autoimmunity. The immune systems can cross-react with autoantigens in the response against microorganisms. Pattern recognition receptors (PRR) on APC’s recognize certain molecules on microorganisms.
Toll-like receptors (TLR’s) are an important group of PRR, and over-expression has been shown to promote systemic autoimmunity [62, 63].

2.1 Autoimmune diseases

Dysregulation in both the innate and adaptive immune systems can cause disease. When the innate immune system is activated without activation of the adaptive immunity the diseases are designated auto-inflammatory, and when the adaptive immune cells, T- or B-lymphocytes, are the main cause it is described as an autoimmune disease. Most immunological diseases are influenced by both of these two immune responses (Figure 2).

![Figure 2. The influence of innate and adaptive immune response in autoinflammatory and autoimmune diseases](image)

Autoimmune diseases are common with a prevalence of around 3% in the whole population, and are one of the most common causes of death in young and middle-aged women in the western world [64-66]. Autoantibodies, or autoreactive T-cells, are not sufficient for disease development [67, 68]. Genetic, environmental and immunological factors in combination are described as the etiology of autoimmune diseases (Figure 3) [69, 70].

![Figure 3. The etiology of autoimmune diseases is multifactorial.](image)
The frequency of autoimmune diseases increases with age, and most autoimmune diseases are more common in females [71-73]. Polymorphisms in genes coding for immunologically active molecules are associated with pathogenesis of autoimmune diseases. In RA there is a strong genetic influence as shown e.g. in twin studies, with the HLA-DR B1 shared epitope *0401 and *0404 being especially common [74-76]. In patients with SLE polymorphism in IRF5, STAT4, TNF genes, complement factors, type I IFNs and Fc receptors occur [77-83]. In disease characterized by Th1-polarisation, polymorphisms in the T-cell related molecule CTLA-4 is present [84, 85]. Polymorphism in the intra-cellular protein tyrosine phosphatase, PTPN22, is particularly related to autoantibody producing diseases, e.g., RA, SLE and Type I diabetes [86-88]. Epidemiological studies show differences in the prevalence of autoimmune disease among ethnic groups. In RA the frequency of certain HLA alleles, i.e., shared epitope *0401 and *0404, are higher in Caucasians than in Asian and Jewish populations [75].

Environmental influences are also important. Smoking has been suggested to be a risk factor for RA, SLE, MS and autoimmune thyroid disease [89-92]. Many medical drugs, such as hydralazine, procainamide, minocyclin, and TNF blocking agents, can induce or worsen SLE and lupus-like syndromes [93]. Infections are well known to trigger the onset of autoimmune inflammatory diseases, as was reviewed as early as 1975 [94]. Ultraviolet light can induce or suppress inflammation in the autoimmune diseases SLE and psoriasis, respectively [95, 96].

In the development of autoimmune diseases both cell mediated and antibody mediated immune processes are important. The humoral immune response, mediated by B-cells, is especially involved in systemic autoimmune reactions, in which autoantibodies have a pathogenetic role, such as in SLE [97, 98]. In RA and multiple sclerosis (MS), the Th1-response dominates, although there are also autoantibodies involved [99, 100].

### 2.2 Rheumatic diseases

The rheumatic diseases have a multi-factorial etiology. They are all more or less autoimmune, but unknown factors are also involved (Table 2). The immune response in rheumatic diseases can be dominated by a T- or a B-cell mediated response. In diseases where the B-cell mediated response is marked, the presence of autoantibodies detectable in blood and tissues is prominent.
Table 2. Rheumatic diseases

<table>
<thead>
<tr>
<th>Arthritis diseases</th>
<th>Systemic diseases</th>
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<tbody>
<tr>
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<td>ANA-associated diseases</td>
</tr>
<tr>
<td></td>
<td>Primary vasculitis</td>
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<tr>
<td>rheumatoid arthritis</td>
<td>systemic lupus</td>
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<td></td>
<td>erythematous</td>
</tr>
<tr>
<td>psoriasis arthritis</td>
<td>systemic sclerosis</td>
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<td></td>
<td>mixed connective</td>
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<td>juvenile idiopathic arthritis</td>
<td>tissue disease</td>
</tr>
<tr>
<td>spondylarthropathies</td>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>unspecified chronic arthritis</td>
<td>inflammatory myositis</td>
</tr>
<tr>
<td>reactive arthritis</td>
<td>undifferentiated</td>
</tr>
<tr>
<td></td>
<td>Kawasaki’s disease</td>
</tr>
<tr>
<td></td>
<td>Henoch-Schönlein’s purpura</td>
</tr>
</tbody>
</table>

Evidence of an autoimmune etiology has been shown in some animal models: in a model of RA mice injected with human serum from patients with RA developed a polyarthritis and mice injected with myeloperoxidase (MPO) abs developed glomerulonephritis [101-103]. In humans anti-ribonucleoprotein (RNP) abs was shown to induce an antigen driven autoimmunity [104]. In another experimental model, in which mice were immunized with human nuclear protein Ro/Sjögren-syndrom-A(SSA), mice initially developed autoimmunity specific for Ro/SSA, and subsequently to other, immunologically similar molecules. These findings suggest an epitope spreading as a theory for development of SLE [105]. In RA epitope spreading of citrullinated peptides has been shown before clinical symptoms [106]. Ankylosing spondylitis and psoriasis arthritis are considered autoimmune diseases although there is no strong evidence for a true autoimmune pathogenesis.

In many rheumatic diseases autoantibodies are important indicators used for diagnosis, and in a few cases also for monitoring disease activity. In SLE, the autoantibodies are preferentially directed towards intra-nuclear antigens whilst in RA they are against soluble intra- and extra-cellular proteins. Antibodies have been proposed to bind to molecules inside the cell, even though immunoglobulins are relatively large molecules (IgG molecular weight 150 kDa) [107]. They are able to pass through the cellular and nuclear membranes via certain receptors on the cell surface [108]. Rheumatic syndromes, and other autoimmune diseases, may have a long pre-clinical phase during which autoantibodies can be detected, which has been shown in insulin-dependent diabetes mellitus, RA, and SLE [109-113]. In patients diagnosed as undifferentiated connective tissue disease, anti-DNA abs was shown to be a marker for the development of SLE within the following five years [114]. Anti-cardiolipin antibodies have been found to precede thrombosis by a number of years [115].
RA is the most common of the arthritis diseases whilst SLE (and Sjögren’s syndrome) is the most common ANA-associated systemic inflammatory disease. This work is focused on RA and SLE.

3. Rheumatoid Arthritis (RA)

The incidence and prevalence of RA varies among different populations, in northern Europe and North America it is higher than in southern Europe and South America. In northern Europe the incidence is 24-36 cases per 100,000 individuals with a prevalence of 0.44-0.8% of the population [116]. In southern Sweden the overall prevalence of RA recorded during 2010 was 0.66% (women = 0.94%, men = 0.37%) and the incidence was estimated at 50/100,000 (women = 68/100,000, men = 32/100,000) [117]. Commonly the onset of RA occurs in middle age, but the disease can start at any age from childhood to very old age. RA most often has a progressive course with joint destruction and an increased mortality, particularly due to cardiovascular diseases [118]. Even though the main pathological process takes place in the synovium, RA is a systemic autoimmune disease.

Table 3. American College of Rheumatology (ACR) classification criteria of RA [119].

<table>
<thead>
<tr>
<th>4 of the 7 following criteria should be present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning stiffness lasting at least one hour</td>
</tr>
<tr>
<td>Arthritis in at least three joint areas</td>
</tr>
<tr>
<td>Arthritis in the hand joints</td>
</tr>
<tr>
<td>Symmetrical arthritis</td>
</tr>
<tr>
<td>Rheumatoid nodules</td>
</tr>
<tr>
<td>Presence of rheumatoid factor</td>
</tr>
<tr>
<td>Radiographic changes typical of rheumatoid arthritis</td>
</tr>
</tbody>
</table>

New classification criteria have been proposed in 2010 in which there is more focus on the early stage of disease [120]. The older classification criteria were based on a description of RA in the latter stages of disease [119].

RA is characterised by joint inflammation with an infiltration of T-lymphocytes, macrophages, antigen-presenting cells, and antibody producing plasma cells. Individuals carrying the known risk allele HLA-DRB1*0401/*0404 shared epitope (SE) develop the disease at a younger age, that is also the case for anti-CCP-positive individuals [121, 122]. Patients carrying these markers also have a higher risk of a more severe disease. Calreticulin has been shown to be a ligand for the shared epitope through
binding to the pattern recognition receptor CD91 [123]. A number of cytokines, both proinflammatory and downregulating, have been identified in inflamed joints. The inflammatory process in the joint is leading to destruction of cartilage and bone although in certain cases the inflammation is not prominent [124].

Evidence suggesting that RA is a T-lymphocyte-mediated autoimmune disease is increasing. T-cells are an abundant cell type in the rheumatoid synovium [125-127]. Furthermore the PTPN22 genes that help to set T-cell activation thresholds, and CTLA4, that down-regulates activated T-cells, have both been implicated in models of the etiology of RA [128, 129]. Individuals carrying the PTPN22 T allele show an increased relative risk of developing RA, whilst a combination of PTPN22 polymorphism and presence of anti-CCP antibodies very strongly predicts future onset of RA [130-133].

3.1 Autoantibodies in RA

RA may be difficult to diagnose in early stages of the disease, and laboratory markers are helpful. Rheumatoid factors (RF) have been used since many years as a diagnostic marker. They are antibodies directed against the Fc domain of IgG and may be of IgG, IgM, or IgA class. The most commonly detected in daily routine laboratories are of IgM class. RF was first detected by agglutination of human or sheep erythrocytes (Waaler-Rose), but can currently be analysed using many other laboratory methods [134]. Rheumatoid factors are produced not only in the joint in RA but also in the immune response to several infections, for example streptococcal infections, and in other inflammatory diseases such as Sjögren’s syndrome, SLE and mixed connective tissue disease (MCTD). Detection of RF was the only laboratory test included in diagnostic criteria for RA from 1988, although new criteria have been proposed 2010, which include the presence also of anti-CCP abs [119, 120].

Antibodies to citrullinated proteins were first described as anti-keratin or anti filaggrin abs during the 1970’s and 1980’s. During the 1990’s various studies showed that these antibodies were more specific than RF’s, and also predictive of radiological progression [135, 136]. During the early 2000th it was discovered that these antibodies was directed against citrullinated peptides, later referred to as anti-citrullinated peptides, anti-CCP abs or ACPA [137]. A second and a third generation of anti-CCP tests (CCP2 and CCP3) have been developed with an increased sensitivity and with the same high specificity as the initial test [136, 138]. RF’s and ACPA’s can be present
years before the onset of clinical disease, thereby facilitating prediction of RA, and high risk for joint destruction [111, 139].

3.2 Chemokines in RA

T-cells in the inflamed synovium of patients with RA have been shown to express the chemokine receptor CXCR3 [140]. The ligands of CXCR3 are induced by interferon-γ, a cytokine involved in the Th1 immune response. TNF in combination with interferon-γ has been shown to induce IP-10 [141]. Treatment with TNF inhibitors in patients with RA may alter lymphocyte trafficking as indicated by an accumulation of CXCR3 positive T-lymphocytes in the peripheral blood [142]. Several chemokine receptors have been reported to participate in the migration of B-cells into the synovium of RA-patients, [143]. SDF-1 and its receptor CXCR4, are important molecules in lymphocyte homing and are involved in inflammation and angiogenesis the expression of CXCR4 on synovial T-cells is highly elevated in RA [144].

3.3 TNF in RA

TNF has been shown to be a key component not only in local inflammation but also in systemic inflammation through induction of IL-6 and other acute-phase reactants [145]. Production of TNF by cells in the synovium and macrophages is an early event in the inflammatory process resulting in secretion of a number of other pro-inflammatory cytokines including IL-1 and IFN-γ. In the acute phase of the disease TNF participates in pain, oedema, and other symptoms indicative of inflammation of the joint (arthritis). TNF and other proinflammatory cytokines induce production of MMPs known to participate in the tissue damage process of cartilage and bone in the joint [146]. Since cartilage damage is irreversible, it is important to initiate treatment at an early stage of the disease in order to avoid permanent disability. In the 1990’s several studies concluded inhibition of TNF to be an appropriate treatment for RA [147-149].

3.4 General aspects on treatment in RA

Vital for any treatment of RA, as well as the other rheumatic diseases, are depression of the inflammatory activity and the progression of the disease
leading to destruction of joints and other organs. Currently there is no treatment that can be described as a cure. Corticosteroids have dramatically beneficial effect. Many of the traditionally anti-rheumatic drugs, i.e., DMARDs (Disease Modified Anti Rheumatic Drugs), e.g., sulphasalazine, gold, and anti-malarials, have been used for a long time, but the mechanisms of action have not been clarified for all of them. Azathioprine and methotrexate are anti-metabolites that inhibit the activity of the immune system and have been used since the 1970’s.

It is important to evaluate the disease activity at a given moment, and to compare the activity state over time, and therefore a standardized index, Disease Activity Score (28-joints) (DAS28), based on the inflammatory status in 28 defined joints, the erythrocyte sedimentation rate and the patient’s global assessment on a visual analog scale, is used [150]. It is also important to evaluate the effect of any ongoing treatment and an index to achieve that, the so-called American college of rheumatology, 20% response (ACR20), is recorded [151]. In most studies of RA-treatments, patients with less than 20% improvement measured by ACR20 are considered to be non-responders. In this study the ACR20 response criteria was used, although there are also other instruments available for assessing the treatment response.

3.5 Treatment with TNF-inhibition in RA

Since 1999 the so-called biological drugs have been in use for the treatment of RA. These are antibodies or fusion proteins directed towards a specific molecule, a cytokine or a cell bound molecule, and inhibit the immune system in a very specific way. The most commonly used biological drugs applied to rheumatology are those that block the effect of TNF. As TNF is a cytokine produced at an early stage of the inflammatory cascade, blockage of this molecule is a powerful approach to the treatment of chronic inflammatory diseases, such as RA. Infliximab is a chimeric monoclonal antibody (30% mouse) against TNF [152]. It binds to both soluble and membrane bound TNF, and blocks the pro-inflammatory effects of this cytokine in a powerful way. Infliximab is administrated to patients by intravenous infusion under clinical supervision every 6-8 week and has a long half-life \textit{in vivo}. Etanercept is a TNF-Receptor II fusion protein that that acts primarily on soluble TNF, has a higher degree of dissociation from its target molecule and a shorter biological half-life time than antibody preparations [153]. This study is primarily focused on infliximab, which was
the most commonly used TNF blocking agent at the rheumatology clinic of Umeå University Hospital at the time when the patients were included.

At the molecular level, blocking of TNF has been shown to reduce the production of other pro-inflammatory cytokines such as IL-1 and GM-CSF by cultured synovial cells from patients with RA, and to decrease the production of acute-phase reactants like IL-6 and CRP [154, 155]. Furthermore, a reduction in tissue perfusion in the inflamed synovium has been shown by nuclear magnetic resonance, and in another study the characteristic histopathological findings associated with RA were modified [156, 157]. Induction of apoptosis in monocytes and T-cells has been shown both in an animal model and in man [158, 159]. Down-regulation of cytokine production by activated monocytes in humans but without signs of increased apoptosis was shown by Ringheanu [160]. Wijbrands et al showed no increase of apoptotic cells in the synovium after infliximab treatment and suggested that the mechanism of action of this drug is due to decreased migration of monocytes and other inflammatory cells into the joint [161].

From the clinical point of view blocking of TNF with infliximab over a period of two years induced a significantly lower DAS28, lower CRP and metalloproteases (MMP’s), less cartilage destruction measured by X-ray and Sharp score, and a decreased Health Assessment Questionnaire (HAQ)-score [162]. The most important side effects of TNF blocking therapy are severe infections, especially in skin and soft tissues, and an increased risk of tuberculosis [163, 164]. To date there is no indication of an increased risk of malignancies [165]. There is, however, a risk for autoantibody formation and development of other autoimmune conditions, which will be discussed further in the “Results and Discussion” section.

4. Systemic Lupus Erythematosus (SLE)

SLE is a systemic autoimmune disease that can affect most organ systems. Four of 11 criteria must be fulfilled for the diagnosis (Table 4) [166, 167].
Table 4. ACR classification criteria for SLE

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malar rash</td>
</tr>
<tr>
<td>Discoid rash</td>
</tr>
<tr>
<td>Photosensitivity</td>
</tr>
<tr>
<td>Oral ulcers</td>
</tr>
<tr>
<td>Arthritis</td>
</tr>
<tr>
<td>Serositis</td>
</tr>
<tr>
<td>Renal disorder</td>
</tr>
<tr>
<td>Neurological disorder</td>
</tr>
<tr>
<td>Hematological disorder</td>
</tr>
<tr>
<td>Immunological disorder</td>
</tr>
<tr>
<td>Anti-nuclear antibodies</td>
</tr>
</tbody>
</table>

The disease affects mostly females, and especially during their reproductive age. The course and prognosis is highly variable being dependent on the degree of inflammation in the various organs involved. Most patients have a disease course of “flares” with periods of remission. SLE is a disease with diverse clinical manifestations and variable severity between individuals and different populations [168, 169]. Individuals who develop SLE have also been found to gradually fulfil the number of clinical classification criteria before the diagnosis is defined which suggests a protracted pathogenesis [170]. The incidence is higher and disease development is faster in African-Americans than in Caucasians [171]. Anti-extractable nuclear antigen (ENA) antibodies are more common in Afro-Caribbean and African-American populations than in Caucasians [172-174].

As the producers of autoantibodies, B-cells have a central role in the pathogenesis of SLE. B-cell functions are broader than simply antibody production, and include activation of T-cells through the presentation of autoantigens, co-stimulation of T-cells, and production of pro-inflammatory or regulatory cytokines. B-cells can process and present autoantigens to naive T-cells resulting in autoreactive T-cells. Studies using animal models have demonstrated that SLE can develop in the absence of autoantibodies, but not in the absence of B-cells [175, 176]. Circulating B-cell activation factor (BAFF) is over-expressed in SLE-patients and lupus-prone mice, and seems to be of more import for the survival of autoreactive than of normal B-cells, and consequently may be a useful therapeutic target [177-179].

The introduction of modern therapy regimes (corticosteroids, anti-malarials, and cytotoxic drugs) during the past few decades has resulted in a considerably increased survival rate [180]. Taking a long-term perspective the morbidity from the disease, such as cardiovascular disease and osteoporosis, increases [181]. SLE is associated not only with characteristic autoantibodies, e.g., anti-nuclear antibodies, but also antibodies against
blood cells and cytoplasmatic proteins. An activity index, Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), in which the level of anti-double stranded DNA (anti-dsDNA) abs is included, and an index for permanent organ damages, Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) Damage Index are used to monitor disease progression [182, 183].

4.1 Autoantibodies in SLE

Detection of autoantibodies is very important for the diagnosis of SLE, and some of these antibodies are believed to be important in the pathogenesis and to reflect the inflammatory state of disease whilst others have unknown functions. A typical pathophysiological sign in SLE is the production of autoantibodies against nuclear antigens, which may precede the clinical manifestations. In a study by Arbuckle et al. in 2003 anti-nuclear abs were present in 78% of the patients before the clinical onset of disease, anti-(ds)DNA abs in 55%, and anti-SSA abs in 47% [112]. Anti-dsDNA abs has been shown to increase just before the diagnosis of SLE [184]. Development of a rheumatic disease in asymptomatic mothers expressing anti-SSA and/or anti-SSB abs identified by the birth of a child with congenital heart block, occurred in 48% of cases [185]. In another study, the detection of anti-SSB abs antedated clinical evidence of Sjögren’s syndrome by months and, in some cases, by years [186].

Acute-phase proteins and collectins are important molecules of the innate immune system. They recognize pathogens, activate complement, bind to apoptotic blebs, and stimulate uptake by phagocytes. These molecules also participate in the clearance of cellular and nuclear debris from apoptotic cells, and should be considered protective from developing autoimmunity. In SLE, autoantibodies directed against such protective molecules as complement factor C1q, CRP, serum amyloid P (SAP), mannose-binding lectin (MBL), and apolipoprotein A1 (APO A1) can be found [187, 188]. The function of the complement system is of great importance in SLE, and disturbances in the function as well as component deficiencies are described [189].

Antibodies against double stranded DNA are thought to be the most important antibody in the pathogenesis of SLE, and the most useful antibody to follow the inflammatory state, especially in patients with lupus nephritis [190, 191]. Anti-cardiolipin abs are associated with thrombosis [192]. ANA and anti-Smith (Sm)-abs are very important for the diagnosis of SLE but
have not been shown to be involved in engagement of specific organs [193, 194]. Likewise, anti-ENA abs are important disease markers, but as far as we know they don’t reflect disease activity and there is no need to follow them throughout the disease [195]. Touma et al showed that most of the autoantibodies in SLE were present within the first year of disease, and that anti-DNA abs was the only antibody that correlated with disease activity and organ damage after three and five years [196].

4.2 Chemokines in SLE

In recent years, several studies have reported up-regulation of chemokines, such as the B-cell attracting chemokine BCA-1 and IP-10, in blood and inflamed tissue in SLE [197-201]. Furthermore, chemokine receptor expression in RA and SLE on B-cells was reported to be different from each other, and related to disease activity and tissue damage [202-204]. CXCR4 and SDF-1 are thought to be important in the SLE pathogenesis by attracting T-helper and B-cells, hence for autoantibody formation, especially in lupus nephritis [205-207].

5. Lipoprotein receptor-related protein 1/ CD91

CD91, also called lipoprotein receptor-related protein 1, is a pattern-recognition receptor (PRR) which binds α2-macroglobulin, lipoproteins and heat shock proteins (HSP) on APC’s where it promotes endocytosis [208]. It is also a receptor for thrombospondin-1 (TSP-1) and calreticulin (CTR), and is expressed by T-cells following stimulation of the T-cell antigen receptor (TCR) [209]. The expression of CD91 on T-cells seems to regulate migration and is important for TCR/CD3 induced IFN-γ production [210, 211]. CD91 expression is induced by stimulation of the CD3 complex [209]. The chemokines SDF-1 and RANTES have been shown to induce expression of CD91 and its ligand (TSP-1) on T-cells [210]. The HLA-DRB1*0401/*0404 shared epitope is common in RA and has been reported to trigger signalling through CTR and CD91 indicating that CD91 and its ligands have an important role in the pathogenesis of RA. HSP’s are formed in the tissues by stress signals such as trauma, ischemia, and inflammation. Soluble HSP’s may promote, and regulate, the immune responses in autoimmune inflammation [212].
AIMS

This study was performed to get a deeper understanding how TNF blocking treatment influences inflammatory mechanisms and autoantibody formation in RA with special reference to similarities and differences with SLE.

We examined RA-patients on TNF-blocking treatment in with respect to
- induction of autoantibodies
- chemokines and chemokine receptor expression on blood T-cells
- expression of CD-91 on blood T-cells

We also studied patients with SLE with respect to
- chemokine levels in blood and chemokine receptor expression on blood T-cells
- autoantibodies predating onset of symptoms of disease
METHODOLOGY

6. STUDY POPULATIONS AND CONTROLS

6.1 Paper I

Twenty-three patients with SLE (ACR criteria) [166, 167] were consecutively recruited into the study. The patients were evaluated clinically and scored according to the SLE Disease Activity Index, SLEDAI [182]. Blood samples were collected from these SLE patients, from a reference group suffering another inflammatory disease seven female patients with RA [119], and from 15 healthy controls. The demographic data for the SLE-patients, stratified for active (SLEDAI ≥6) and inactive (SLEDAI < 6) disease, are presented in Table 5. Ten patients had nephritis, 16 joint manifestations and 20 skin manifestations. The mean age of the seven patients with RA was 54.3 years (range 25–72 years), the mean disease duration 20.1 years (range 0.3–40 years) and their mean ESR 24 mm/hr (range 1–46 mm/hr).

<table>
<thead>
<tr>
<th>Table 5. Basic data for the patients with SLE included in paper I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active SLE (n=11)</strong></td>
</tr>
<tr>
<td>Age at onset, mean±SD, years</td>
</tr>
<tr>
<td>Disease duration, mean±SD, years</td>
</tr>
<tr>
<td>SLEDAI, median</td>
</tr>
<tr>
<td>Q1-Q3</td>
</tr>
<tr>
<td>SLICC, median</td>
</tr>
<tr>
<td>Q1-Q3</td>
</tr>
<tr>
<td>Anti-DNA positive, n (%)</td>
</tr>
<tr>
<td>Prednisolone, n (%)</td>
</tr>
<tr>
<td>Cytotoxic drugs and/or anti-malarials, n (%)</td>
</tr>
</tbody>
</table>

6.2 Paper II

Fifty-nine patients (48 female and 11 male) with RA according to the ACR criteria [119] were consecutively recruited into the study. Fifty-three patients were treated with infliximab (3 mg/kg body weight) at inclusion, after two and eight weeks, and subsequently at intervals of eight weeks. The other six
patients were treated with etanercept (25 mg) twice weekly. Blood samples were collected from all of the patients before treatment and after 14, 30, and 54 weeks. The sera were stored at -80 °C until analysis. Demographic data on these patients are presented in Table 6.

**Table 6.** Demographic data of the patients in paper II. The figures refer to the number of patients. The age and disease duration are presented as mean values.

<table>
<thead>
<tr>
<th></th>
<th>Infliximab (n=53)</th>
<th>Etanercept (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F/M</strong></td>
<td>43/10</td>
<td>5/1</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td>54.8 (26-79)</td>
<td>48.3 (35-65)</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Disease duration, years</strong></td>
<td>13.8 (2-37)</td>
<td>9.1 (1.3-15)</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Corticosteroids, median dose</strong></td>
<td>30(57%)</td>
<td>3(50%)</td>
</tr>
<tr>
<td>7.5mg/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methotrexate (5-20mg/week)</strong></td>
<td>43(81%)</td>
<td>2(33%)</td>
</tr>
<tr>
<td><strong>Other DMARD’s</strong></td>
<td>19(36)</td>
<td>2(33%)</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DAS28 at onset</strong></td>
<td>5.9(±0.14)</td>
<td>6.6(±0.52)</td>
</tr>
<tr>
<td>(mean±SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DAS28 at 54 weeks</strong></td>
<td>3.7 ± 0.2</td>
<td>3.2 ± 0.33</td>
</tr>
<tr>
<td>(mean±SEM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 anti-malarials (3/1), minocycline (1/1), cyclosporin A (4), azathioprine (8), sulphasalazine (2), leflunomide (2) and injectable gold (1)

### 6.3 Paper III and Paper V

In these two reports, the study population consisted of 24 patients fulfilling the classification criteria for RA [119]. Twelve had established RA with disease duration of 1-36 years and 12 had early RA (eRA) with duration less than one year. In Paper V there was only complete data from the analysis in nine patients with early RA, and the statistics of laboratory values are based on nine individuals in that work. There were also nine healthy controls and a small group of three patients with SLE as a control group for another inflammatory disease. The patients with established RA were treated with infliximab and the early RA-patients with traditional DMARDs. The infliximab dosage for all patients was 3 mg/kg body weight. All patients receiving infliximab were also treated with DMARDs. Five of the patients
with eRA and four of the infliximab treated patients were receiving corticosteroids (<10 mg prednisolone daily). All patients were assessed clinically, the DAS28 [150] score calculated, and blood samples collected at baseline and after 14 and 30 weeks treatment. Blood samples were also taken from the infliximab treated group at week two and six. The sera were stored at -80°C until analysis. The ACR20 response to treatment was calculated after 14 and 30 weeks [151]. In nine healthy controls and three patients with SLE blood samples were taken on one occasion. Basic data on patients and controls are summarized in Table 7.

**Table 7. Basic data on patients and controls included in Paper III and Paper V**

<table>
<thead>
<tr>
<th></th>
<th>Anti-TNF-treated RA</th>
<th>Early RA</th>
<th>SLE</th>
<th>Healthy Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>paper III</td>
<td>paper V</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>F:M</td>
<td>8:4</td>
<td>8:4</td>
<td>3:0</td>
<td>6:3</td>
</tr>
<tr>
<td>Age (years, median (range))</td>
<td>55.7 (43-69)</td>
<td>54.2 (19-66)</td>
<td>53.2 (19-66)</td>
<td>55.3 (42-62)</td>
</tr>
<tr>
<td>Disease duration (years, median (range))</td>
<td>12.9 (1-36)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>19.5 (0.5-45)</td>
</tr>
<tr>
<td>DAS28 baseline (median (range))</td>
<td>5.86 (3.7-7.0)</td>
<td>3.21 (1.2-5.3)</td>
<td>2.95 (1.2-5.3)</td>
<td></td>
</tr>
</tbody>
</table>

### 6.4 Paper IV

Thirty-eight patients (three male and 35 female) fulfilling the ACR criteria for SLE [166, 167] were identified as donators of blood samples to a biobank before onset of symptoms of the disease. The registry of patients with SLE at the rheumatology clinics in northern Sweden, with the date of onset of symptoms given, the registry of the Medical Biobank and the registry of the Maternity cohort of northern Sweden were co-analyzed. The Medical Biobank consists of three cohorts – the Västerbotten Intervention Project, the Northern Sweden part of World Health Organization (WHO) study for Monitoring of Trends and Determinants in Cardiovascular Disease (including Västerbotten and Norrbotten), and the Mammary Screening Project in Västerbotten. The Maternity cohort of Northern Sweden comprises samples from pregnant women from the four northernmost
counties of Sweden who have undergone screening for rubella since 1975. Nineteen of the samples were from the Medical Biobank and 19 from the Maternity cohort. A nested case-control study, designed 1:4 was performed with the identified individuals, who later developed SLE, referred to as “pre-symptomatic individuals” and controls from the same population based cohorts matched for sex and age at the time of blood sampling, and area of residence (n=152). The samples from three pre-symptomatic individuals and six controls from the Maternity cohort were insufficient for analysis. The mean age (range) at blood donation of those individuals who developed SLE was 36.9 (16.8 – 60.2) years and of the matched controls 36.7 (17.8 – 62.3) years. The mean age at onset of symptoms of SLE was 44.5 years (range 19-65) and at the diagnosis was 47.4 years (range 22-68). The basic data on the patients and controls are presented, together with basic results from the study in Table 9 (page 44).

7. LABORATORY METHODS

7.1 Cells

In paper I the peripheral blood mononuclear cells (PBMC) were isolated and stored over night before the staining procedure. Venous blood was collected into EDTA-coated tubes and density gradient centrifuged within two hours. The cells were stored in culture medium overnight. In papers III and V the cells were stained in the whole blood from EDTA-tubes within 4 hours and the erythrocytes lysed with a lysis buffer.

7.2 Flow cytometry

A two-colour staining analysis was performed. In paper I mouse-anti-human phycoerythrine (RPE)-conjugated anti-CD3 and unconjugated mouse anti-human CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CCR1, CCR2, CCR3, CCR5, and CCR6 were used. Secondary antibody (Fluorescein isothiocyanate (FITC)-conjugated Goat anti-mouse) was added to each sample. The antibodies used in paper III and V were mouse-anti-human FITC-conjugated anti-CD3 and anti-CD14, RPE-conjugated antibodies against CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR4, CCR5 and unconjugated mouse-anti-human against CXCR4, CXCR5, CCR3, and CCR6. To detect CD91, RPE-conjugated anti-CD91 was used. TSP-1, CRT and CD47 were
detected with unconjugated mouse-anti-human monoclonal antibodies and a polyclonal RPE-secondary goat-anti-mouse.
Ten thousand living cells/sample were analysed. Flow cytometry was performed on a FACScan (Becton Dickinson) and using LYSIS II Software.

7.3 Quantitative immunocytochemistry.

Lymphocytes from two healthy individuals were incubated in medium in the presence or absence of infliximab, thereafter fixed with paraformaldehyde, and was subsequently allowed to adhere to glass slides. Mouse anti-human antibodies to TSP-1 and CD91 were added, followed by detection of the primary antibody using biotin/avidin/HRP. Photomicrographs were taken of each slide and the mean staining intensity (arbitrary units) was determined. (Paper III)

7.4 Analysis of chemokines and cytokines in blood

Plasma and serum samples were stored at -80°C prior to analysis. The chemokines MCP-1, MIP-1α, MIP-1β, IL-8, IP-10, SDF-1α, the cytokines TNF and IL-10 were measured in plasma (paper I) and in serum (paper V) using enzyme linked immunosorbent assay (ELISA). Soluble dipeptidylpeptidase IV/CD26 and RANTES was only analysed in serum.

7.5 Analyses of autoantibodies

The autoantibodies analysed in paper II were RF by Waaler-Rose (haemagglutination, in house protocol), ANA (screening dilution 1:100), anti-smooth muscle abs and anti-mitochondrial abs by IIF on rat tissue by indirect immunofluorescence (IIF, in-house protocol), anti-histone abs, anti-extractable nuclear antigens (ENA: anti-Sm, RNP, SSA, SSB, Scl-70, and Jo-1) anti-proteinase-3 abs, anti-myeloperoxidase abs, and anti-cardiolipin (aCL, IgG) abs by ELISA. Antibodies against double stranded DNA (ds-DNA) were analysed by IIF on Crithidia luciliae coated slides for both IgG (heavy and light chain conjugate) and IgM, by ELISA, and by Farr assay. Anti-nucleosome abs (IgG) were analysed by ELISA.

In paper IV a multiplex detection analyses was performed using the AtheNA Multi-Lyte ANA-II Plus Test kit on a Bio-Plex Array Reader (Luminex, Labmap™ system). The antibodies included in this kit are anti-SSA (52+60kDa), -SSB, -dsDNA, -RNP, -Sm, -Jo-1, -Scl-70, -Centromere B and –
histone abs. The cut-off levels for each antibody recommended by the manufacturer were used. ANA were performed with IIF on HEp-2 microscope slides at a screening dilution of 1:100 and evaluated using a UV microscope at 20 x magnification. Analyses of autoantibodies from the patients after diagnosis were performed with AtheNA Multi-Lyte ANA-II Plus Test and at the immunological routine laboratory at the hospital (ANA with indirect immunofluorescence HEp-2 or rat tissue, anti-dsDNA on *Critidiae Luciliae* IIF slides and for the other antibodies by different ELISA’s or immunoblot.

7.6 RNA extraction and polymerase chain reaction (PCR)

Total RNA was isolated from PBMC, RNase inhibitor was added to the RNA and the sample stored at -80°C until used. cDNA was prepared from RNA using random hexamers and murine leukaemia virus reverse transcriptase. Reverse transcription (RT) was performed.

The following primers were used:

SDF-1α: 5’-TAGCCCGGCTGAAGAACAACAACA-3’
5’-CCCAGGGAGCAGAGGAGATG-3’
RANTES: 5’-TGCTCTGTCATCTCATATTCTG-3’
5’-GAGTTGATG TACTCCCAACGAC-3’
MIP-1α: 5’-CCCTCGTGTCCTCTTGCA-3’
5’-CACCAGCTCTTAGGTCGCTG -3’
MIP-1β: 5’-TGCTCTCTCTCATGCTAGTA-3’
5’-GTACTCCGGCCAGGAGGT-3’
β-actin 5’-GGGGCGCCCCAGGACGAC-3’
5’-CTCTTAATGTACGCGACAGTTTC-3’

In all, the cDNA reaction mixture and the specific primers were used in a standard protocol to amplify the 511 bp (SDF-1α), 235 bp (RANTES), 254 bp (MIP-1α), 233 bp (MIP-1β) and 540 bp (β-actin) fragments. PCR products were analysed on agarose gels, stained with ethidium bromide and analysed under UV light (Paper I).

7.7 Statistics

All statistical analyses in these papers were performed using the SPSS package (SPSS for Windows 14.0 or higher, SPSS Inc., Chicago, IL, USA). Non-parametric testing was applied for statistical comparison due to small numbers and the not exactly normal distribution of all measurements in all studies. The non-parametric Kruskal–Wallis one-way analysis of variance by ranks was used when three or more groups were included in the analyses. If
there was a significant difference between the groups in the test, or when two groups were compared, the groups were further compared two by two using the Mann–Whitney U test. In Paper II the Friedman two way analysis of variance by ranks for related samples test was used and a Chi-square test for trends used for testing categorical data. In Paper IV continuous data were compared by Wilcoxon’s signed rank test for matched pairs (pre-symptomatic individuals versus SLE-patients) and conditional logistic regression analyses (pre-symptomatic individuals versus control subjects). Relationships between categorical data were compared using Chi-square analyses or Fisher’s exact test. Corrections for multiple comparisons have been performed in the preparation of Paper IV. Wilcoxon signed rank test was used for paired samples and Spearman non-parametric analyse for correlation tests. All p-values are two sided and a p value ≤ 0.05 was considered statistically significant.

### 7.8 Ethical aspects

All participating individuals gave their informed consent, and all studies were approved by the Regional Ethics Committee of the University Hospital, Umeå, Sweden.

- Paper I: dnr 00-196
- Paper II, III, V: Um dnr 00-404
- Paper IV: dnr 07-066M
RESULTS AND DISCUSSION

8.1 Chemokines and chemokine receptor expression on T-cells (paper I and paper V)

In RA: The IP-10 level in serum was significantly elevated in RA-patients compared with healthy controls, and decreased significantly after the first infusion of infliximab. The serum level of MCP-1, MIP-1β and RANTES was slightly raised in RA-patients than in controls, and the levels of MCP-1 and MIP-1β levels decreased significantly during treatment with infliximab whereas RANTES was not affected. The concentration of MCP-1 in serum was higher at baseline in the RA-patients who did not respond to infliximab treatment as compared with responders, however the difference did not reach statistical significance (p=0.07) (paper V).

The T-cell expression of CXCR2, CCR1 and CCR2 was elevated in patients with RA compared with healthy controls in paper I. The T-cell expression of CXCR1, CXCR2, CXCR3, CXCR5, CCR2, CCR4 and CCR5 was significantly higher in RA patients than in healthy controls and CCR1 showed a tendency to be elevated in paper V. Two weeks after the first infliximab infusion a significant decrease of the expression of CCR2, an increase of CXCR5 expression and a non-significant decrease in CXCR1 was noted.

In SLE: The blood levels of IP-10, SDF-1, MCP-1, MIP-1β, and RANTES were significantly higher than in controls, but there were no significant differences between patients with inactive and active disease. The PCR analysis of peripheral blood mononuclear cells from SLE-patients showed high expression of MIP-1β RNA in active disease whilst other chemokines did not differ between patients and healthy controls. The expression of chemokine receptors CXCR5 and CCR6 on T-cells was lower in SLE-patients than in healthy controls. It was in the inactive (SLEDAI <6) patients that the significance was defined for both. The expression of CXCR2 was elevated in active SLE (SLEDAI ≥6) and lowered in inactive disease compared with controls. In active SLE, the T-cell expression of CCR1 was close to significantly higher than in inactive disease and healthy controls, and in inactive disease the CCR2 expression was depressed both compared with patients with active disease and healthy controls.

The expression of CXCR3 and CCR3 on T-cells was elevated and the CXCR3-ligand IP-10 was depressed in SLE patients with nephritis compared with patients without this manifestation.
The ratio between Th1 and Th2 cells, measured by CCR5/CCR3 quote was elevated in RA-patients compared with healthy controls and with SLE-patients, but there was no such difference between SLE and controls. The main results from paper I and paper V are summarized in Table 8, being presented as the p-values. The actual values are not presented in this table due to different expression levels in the two papers.

### Table 8. Main results from paper I and paper V on soluble chemokines and chemokine receptor expression on T-cells in blood, presented as p-values.

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>SLE (paper I)</th>
<th>RA (paper V) Baseline</th>
<th>RA, baseline versus 2 weeks infliximab treatment (paper V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10/IP-10</td>
<td>0.0005</td>
<td>0.012</td>
<td>0.005 ↓</td>
</tr>
<tr>
<td>CXCL12/SDF-1</td>
<td>0.038</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CCL2/MCP-1</td>
<td>0.017</td>
<td>ns (0.085)</td>
<td>0.037 ↓</td>
</tr>
<tr>
<td>CCL4/MIP-1β</td>
<td>0.0001</td>
<td>ns (0.10)</td>
<td>0.028 ↓</td>
</tr>
<tr>
<td>CCL5/RANTES</td>
<td>0.0012</td>
<td>ns (0.10)</td>
<td>ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemokine receptors</th>
<th>SLE (paper I)</th>
<th>RA (paper V) Baseline</th>
<th>RA, baseline versus 2 weeks infliximab treatment (paper V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1</td>
<td>ns</td>
<td>0.018</td>
<td>ns (0.062) ↓</td>
</tr>
<tr>
<td>CXCR2</td>
<td>ns</td>
<td>0.013</td>
<td>ns</td>
</tr>
<tr>
<td>CXCR3</td>
<td>ns</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>CXCR4</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CXCR5</td>
<td>0.051*</td>
<td>0.04</td>
<td>0.028 ↑</td>
</tr>
<tr>
<td>CCR1</td>
<td>ns</td>
<td>ns (0.077)</td>
<td>ns</td>
</tr>
<tr>
<td>CCR2</td>
<td>ns</td>
<td>0.03</td>
<td>0.016 ↓</td>
</tr>
<tr>
<td>CCR3</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CCR4</td>
<td>na</td>
<td>0.003</td>
<td>ns</td>
</tr>
<tr>
<td>CCR5</td>
<td>ns</td>
<td>0.0001</td>
<td>ns</td>
</tr>
<tr>
<td>CCR6</td>
<td>0.002*</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Lower than controls (other values were higher than controls).
ns=not significant, na=not analyzed, ↑= increased value versus baseline, ↓= decreased value versus baseline

Some important factors may influence the results of these studies. All patients, both those with established RA who started infliximab treatment and the early RA-patients, had ongoing treatment with DMARD’s and some also with corticosteroids. All but two patients with SLE, both with inactive disease, were receiving pharmacological treatment. However, the low
numbers of patients was the main limiting factor in these studies. The number of patients receiving TNF-blocking drugs was limited in the area of Västerbotten with a population of approximately 250,000 inhabitants. We wanted to study just one kind of anti-TNF medication, due to partly different mechanisms of action between the various drugs, and consequently infliximab, that was the most commonly used product at the rheumatology department of Umeå University Hospital, was selected. Additionally, when the RA-patients were stratified into responders and non-responders the two groups consisted of few individuals. With small study groups it is more difficult to obtain good statistical power. It is difficult to undertake studies on patients who do not respond to the treatment because continuing a non-effective treatment is not ethical. Some of the studied patients were non-responders to infliximab after 6 months, i.e., they had some effect from the medication but did not achieve the 20% improvement required according to the ACR20 criteria. Due to the long interval between the infusions after the first infliximab dose, and possible also an network effect between the chemokines and their receptors, it was decided to concentrate on processing the results from the first two weeks of treatment; the observable changes being most obvious at that time (paper V). Differences in the results from the two studies concerning the expression of chemokine receptors on T-cells are probably due to the different staining procedures. In paper I, the mononuclear cells were isolated before the staining procedure in preparation for analysis by flow cytometry, whereas in paper V the cells were stained without initial isolation to minimize affection of inflammatory molecules during the isolation procedure. In all experimental protocol steps there is a loss of cells, and this is especially the case for the lymphocytes from SLE-patients that are very vulnerable in vitro.

SDF-1 levels in serum were elevated in SLE-patients independent of disease activity. Elevated chemokine levels in serum have also been reported by others [213, 214]. T-cell expression of CCR2 was depressed in patients with low disease activity although the CCR2-ligand MCP-1 was elevated both in inactive and active SLE. Another study reported decreased CCR2 expression during disease flare [215]. Circulating T-cells in patients with kidney involvement showed elevated CXCR3 and CCR3 expression. Others have shown up-regulated CXCR3 and CCR3 in skin and kidney of SLE-patients [216, 217]. The level of the CXCR3-ligand, IP-10 in peripheral blood was lower in SLE-patients with nephritis than in those without nephritis suggesting a relationship to kidney function. Lacotte et al made an interesting interpretation that patients with SLE nephritis could have a lower sensitivity to secreted inflammatory chemokines [218]. Disease activity seems to correlate better with chemokine receptor expression than with the corresponding chemokine levels in blood. The different expression of
chemokine receptors on blood T-cells may reflect a redistribution of cells from tissues. The low T-cell expression of CXCR2, CXCR5, CCR2 and CCR6 in SLE may be a consequence of receptor internalization or immune-modulating therapy [219].

The reduced level of IP-10 was significant after the first infliximab infusion in RA but thereafter the level was reduction was lower. As TNF can induce IP-10 this could reflect a high TNF level in active RA and a decrease of TNF after infliximab treatment [141]. Consistent with earlier reports MCP-1, MIP-1β, and RANTES were almost significantly elevated levels before anti-TNF treatment compared with controls, whilst MCP-1 and MIP-1β were decreased after treatment [220-222]. The T-cell expression of CXCR5 was elevated compared with controls at baseline, and after treatment with infliximab the expression increased significantly, however all values were very low and the relevance is difficult to interpret. The levels of CXCR3 expressing T-cells in the blood during infliximab treatment remained high. The number of CXCR3 bearing T-cells in blood after anti-TNF treatment has been reported to increase, interpreted as a pooling of Th1 cells in the blood due to decreased trafficking [142]. Reduced trafficking of leucocytes into synovial tissue could be one mechanism of action of anti-TNF treatment. CCR4 on T-cells was significantly higher in RA-patients than in healthy controls as has been shown earlier [140]. This expression did not change after infliximab treatment, possibly reflecting a lower impact on Th2 than on Th1 response by infliximab.

RA is considered more of a Th1 mediated disease than SLE that has a more even distribution between the Th1 and Th2 responses, as we could see in our studies. The chemokines with their main action in the Th1 response, i.e., IP10 and MIP-1β, decreased during treatment with infliximab whilst their corresponding receptors on T-cells, CXCR3 and CCR5, were less affected by the treatment. This could possibly be a consequence of a change in the activity level of the Th1 cells during TNF blockade. The typical Th2 chemokine receptors, CCR3 and CCR4, and the CCR3-ligand RANTES were unaffected during the infliximab therapy. The changes after the first infliximab infusion were more pronounced in terms of the soluble chemokines than in the corresponding receptors and possibly result in at least a transient change of the Th1/Th2 balance.

Soluble CD26/dipeptidyl peptidase IV was analysed as a molecule related to several chemokines (paper V). CD26 is a multifunction molecule that may regulate the function of the chemokines RANTES, SDF-1, and IP-10. CD26 can be considered an inhibitor for development of autoimmune diseases, since it cleaves these chemokines and consequently may alter their functions
In RA-patients the CD26 level was close to being significantly lower (p=0.054) than in healthy controls. The SLE-patients had the lowest values. An inverse correlation was seen between RANTES and soluble CD26. Low levels of soluble CD26 have been reported to correlate with disease activity in patients with SLE [224]. In RA-patients an inverse correlation between CD26 and cell-infiltrating into the joint, CRP values and SDF-1 levels has been reported [225]. In our study there was a tendency towards increased CD26 levels during anti-TNF treatment of the ACR20-responding patients, however the increase was not significant (p=0.10), whereas the non-responders did not change their values at all. The results in our study are consistent with the studies cited above and indicate that CD26 is an important factor in autoimmune processes (paper V).

8.2 CD91 in RA (paper III)

All studied groups of individuals were found to express CD91 on large lymphocytes (probably activated T-cells); a finding that has probably not previously been reported. In contrast, the expression on small (inactive) T-cells was low in all individuals. The CD91 expression on T-cells was almost significantly higher in RA-patients at baseline compared with healthy individuals whilst the small group of SLE-patients did not differ from the controls. The CD91 expression in those RA-patients responsive to infliximab treatment was close to healthy controls before and after treatment, whereas in the patients who did not respond to treatment the levels were much higher after treatment. However, before treatment the expression was not significantly higher in this group. One possible hypothesis could be that the high expression of CD91 on T-cells in the non-responding patients binds a higher amount of soluble HSP’s that can act as immunoregulators. Another possible explanation may be that high T-cell expression of CD91 simply reflects up-regulation by antigens or chemokines.

CD91 is also a receptor for lipoproteins. On phagocytic cells CD91 binds lipoproteins and form the foam cells that are involved in the pathogenesis of atherosclerotic plaques and cardiovascular disease. Patients with RA are known to have an increased risk of cardiovascular disease although it is not known whether CD91 on T-cells is implicated in that context.

The ligands of CD91, calreticulin, thrombospondin-1, and CD47 were expressed at the same level on T-cells from control subjects and from RA-patients, and did not change significantly during infliximab treatment. CD91 and its ligands was also analysed on monocytes and the expression was similar to that in healthy controls and was not affected by the treatment.
8.3 Autoantibodies in RA during anti-TNF treatment (paper II)

Many of the patients with RA treated with infliximab developed autoantibodies with new specificities, but none of the etanercept-treated did so. There were more patients with adverse side effects of the treatment with infliximab than etanercept, but only one individual developed a transient lupus syndrome.

RF: 90% of the RA patients were RF-positive, in the Waaler–Rose test with a titre of 80 as the cut-off level before the anti-TNF-treatment and 82% after one year. Before treatment, the median titre was 1280, and then decreased gradually to 560 after 54 weeks. Others have also reported decreased RF levels during anti-TNF treatment [226, 227, 228]. There are conflicting results concerning correlation between RF levels and treatment response. Although this was not calculated, it is reasonable to believe that the treatment response was related to RF values. In the patients who received infliximab the RF levels were reduced more than in those receiving etanercept.

Anti-Cardiolipin abs: Two patients had this antibody at baseline and one thereafter. Formation of anti-cardiolipin abs has been reported in patients who developed drug-induced lupus after anti-TNF treatment [229].

Anti-ENA abs: Only one patient had SSB abs after 54 weeks treatment. Other studies have reported similar results, i.e., that anti-ENA is not induced by anti-TNF treatment [230-233].

Anti-Histone abs: One patient who developed a lupus syndrome became positive for anti-histone abs.

Anti-Nucleosome abs: Ten % of the RA-patients had anti-nucleosome abs at baseline and the number increased significantly during the study. The concordance between anti-nucleosome abs and anti-DNA abs was at least 78% for both IgG and IgM. Benucci et al. have reported similar finding [234, 232].

ANA: At baseline, 24% of the patients were ANA positive. During infliximab treatment the number of positive patients increased until 30 weeks (77%), and the mean titres increased until 54 weeks. Many other studies have reported similar results [230, 235, 236]. Almost all patients with positive ANA had a homogenous pattern, a few mixed homogenous and speckled pattern and a few speckled. All of the ANA samples that had just a speckled pattern were negative for anti-DNA abs. In the etanercept-treated group, three patients (50%) were positive from start and their titres did not change during therapy.

Anti-DNA abs: At inclusion, only one patient had IgG abs to DNA and one had IgM, both with low titres, as analysed with IIF on Crithidia luciliae. At 14 weeks, 45% were IgG positive and 59% had IgM abs; after 30 weeks 66%
and 85%, respectively, and; after 54 weeks 45% and 70%, respectively. The median titres were highest at week 30 for IgM isotype and thereafter falling to 10 after 54 weeks. The IgG median titre was highest week 30 and 54, although the mean value at week 54 was slightly higher than week 30.

A number of patients were followed-up further during the routine health care. After 76 weeks, 50% (n=14) and after 94 weeks 29% (n=17) were still anti-DNA positive in terms of the IgG isotype. In the samples identified at week 94, the median titre was back to zero as before treatment, but there were still a few patients with high titres (Figure 4). Follow-up at 94 weeks for ANA showed that the number of ANA positive patients were still high, 60% at 76 weeks (n=15), and 57% after 94 weeks (n=21). Similar findings, that ANA was longstanding whereas anti-DNA was a transient phenomenon, have been reported in at least two other studies [230, 236].

![Figure 4](image-url)

**Figure 4.** The median titre of anti-DNA of IgM and IgG isotypes after treatment with infliximab (n=27 week 0-30, n=23 week 54, n=14 week 78, n=17 week 94).

Analysis of anti-DNA abs by ELISA after 30 weeks of treatment showed only one positive individual. Using the radioimmunoassay (FARR) no patients were positive at baseline, whilst after 14 weeks 40% of the patients were positive after 30 weeks 25% were positive. In the etanercept treated patients, one patient was positive after 30 weeks with low titre for IgG isotype in the IIF test but was negative after 54 weeks.

The main limitations in paper II were that there were no disease or healthy control groups, and no decisions were made based on treatment response,
such as ACR20. For that reason, it was not possible to analyse for any correlation between antibody formations and treatment response. All patients in this study were also treated with traditional anti-rheumatic medication (DMARD’s) and many with low dose corticosteroids. None of these drugs are known to give rise to autoantibodies, with the possible exception of gold, but only one person was treated with this drug. There may also be a small risk of lupus syndromes after treatment with sulphasalazine. However, TNF inhibitors were added after the patients had failed to respond to their treatment regimes. It is rather likely that the autoantibody formation after TNF inhibitor treatment is depressed by the combination with DMARD’s, especially by methotrexate. There were a number of dropouts because of side effects or lack of efficacy, consequently the number of samples analysed at the later time points was lower than at the beginning of treatment. The frequency of IgG anti-DNA positive patients was very high, possibly explained by the fact that an IgG conjugate to both heavy and light chains that was used. This can interfere with IgM and generate both IgG and IGM-binding antibodies.

Induction of nuclear autoantibodies after TNF specific antibody treatment is an established phenomenon [230, 235, 236]. Patients also develop antibodies towards infliximab and adalimumab (that are antibodies against TNF), but to a lesser extent to etanercept (a synthetic TNF receptor). Formation of anti-nuclear antibodies and these drug-specific antibodies does not seem to correlate [236]. There are several possible mechanisms for autoantibody formation after anti-TNF treatment. First, anti-TNF therapy may decrease the Th1 immune response more than the Th2 response, thereby turning the immune response towards a humoral response with the B-cell stimulation and autoantibody formation [237]. Infliximab binds membrane bound TNF that may result in increased apoptosis and lysis of cells, in turn leading to more free extra-cellular apoptotic blebs, free nucleosomes and DNA [158, 238, 239]. Lowering the acute phase reacting proteins may influence the elimination of circulating immune complexes, and thus enhance autoantibody formation to nuclear antigens. Priori et al have shown that circulating free nucleosomes rise in the blood already two hours after infliximab infusion and that circulating nucleosomes are strongly associated with formation of anti-nuclear antibodies. Patients without circulating nucleosomes did not produce ANA, and ANA production correlated with a reduction of CRP [235]. Another factor is that TNF itself increases the level of CD44 and CD8 positive cells that participate in the elimination of apoptotic blebs, and blocking of TNF decreases these cell types, and further to reduced elimination of apoptotic materials [240]. In our, as well as other studies, the anti-DNA abs that develop during anti-TNF antibody treatment are detected by IIF on Crithidia luciliae. However, these
DNA abs are not detectable with other laboratory methods using human DNA, suggesting that these antibodies may be directed specifically against the *Crithidia*. It has also been hypothesised that these autoantibodies may be heterophilic, directed against species other than humans [241].

Studies comparing classical drug-induced lupus and anti-TNF induced lupus have been performed showing that anti-TNF-induced lupus has more similarities with idiopathic SLE than lupus syndromes induced by other drugs [242-244]. In the classical form of drug-induced lupus there is no association with antibodies to dsDNA or complement consumption, whereas this occurs in the anti-TNF treated patients. Nearly all patients with the classical form of drug-induced lupus are positive for anti-histone antibodies, whilst these only occur in approximately half of patients with anti-TNF-induced lupus. Clinically anti-TNF-induced lupus patients have more cytopenia and skin manifestations than other drug-induced lupus syndromes. However, in both groups the lupus manifestations recover after withdrawal of the treatment. A group of patients fulfils the criteria for both RA and SLE and is called “rhupus”. In individual cases, this could be one possible explanation in RA patients after receiving anti-TNF, however, this cannot be the case in other inflammatory diseases treated with anti-TNF.

### 8.4 Autoantibodies before clinical disease in SLE (paper IV)

Twenty-two of 35 pre-symptomatic individuals (63%) had detectable autoantibodies in their blood before onset of symptoms, *i.e.*, predating disease by 5.6 years (± 4.7; mean ± SD) before the onset of symptoms and 8.7 (± 5.6) years before SLE diagnosis. Ten of these patients had one autoantibody, whilst 12 were positive in between two and seven autoantibody-tests. From the maternal cohort six of 16 individuals (37.5%) were positive for at least one autoantibody before symptom onset, and the corresponding number in samples from the Medical Biobank was 13 of 16 women (81%) and all of the three men in the cohort (100%).
Table 9. Basic data on patients and controls, and basic results from paper IV presented as the mean value and range.

<table>
<thead>
<tr>
<th></th>
<th>All Presymptomatic Individuals</th>
<th>Antibody positive Presymptomatic Individuals</th>
<th>Antibody negative Presymptomatic Individuals</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>35</td>
<td>22</td>
<td>13</td>
<td>152</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td>(62.9%)</td>
<td>(37.1)</td>
<td></td>
</tr>
<tr>
<td>F/M</td>
<td>32/3</td>
<td>19/3</td>
<td>13/0</td>
<td>140/12</td>
</tr>
<tr>
<td>age, blood sampling</td>
<td>36.9 (17 - 61)</td>
<td>43.22 (17 - 61)</td>
<td>28.3 (19 - 50)</td>
<td>36.7 (18 – 62)</td>
</tr>
<tr>
<td>age, symptom onset</td>
<td>44.5 (19 - 65)</td>
<td>49.2 (19 - 65)</td>
<td>36.0 (23 - 58)</td>
<td></td>
</tr>
<tr>
<td>age, SLE diagnosis</td>
<td>47.4 (22 - 68)</td>
<td>51.5 (26 - 68)</td>
<td>40.3 (22 - 58)</td>
<td></td>
</tr>
<tr>
<td>number of abs*/individual, pre-dating symptoms</td>
<td>1.4 (0-7)</td>
<td>2.2 (1-7)</td>
<td>0 (0-2)</td>
<td>0.16</td>
</tr>
<tr>
<td>number of abs*/patient after SLE diagnosis</td>
<td>3.1 (0-4)</td>
<td>3.3 (0-4)</td>
<td>2.8 (0-4)</td>
<td></td>
</tr>
</tbody>
</table>

*number of positive antibody-tests

ANA was the most common antibody, being found in 16 individuals (45.7%) whilst anti-SSA abs was the first to appear at 6.6 (± 2.5) years before onset of symptoms. Anti-Sm abs and anti-centromere abs appeared closest to onset of disease, but there was only one patient each with this antibody. The presymptomatic individuals from the rubella screening biobank were younger than those from the Medical Biobank at the time of blood sampling, symptom onset and diagnosis of SLE, and these samples contained fewer antibodies. The most common onset symptom was arthritis. Those who had serositis as their first symptom of SLE had the highest number (2.5) of antibodies per individual followed by arthritis (1.6).

In the control group ten individuals (6.7%), all female, were ANA positive, two with a borderline titre (1:100). In total 21 controls were positive in any antibody test, four individuals in two tests (SSA+RNP, ANA+SSA, and two ANA+Jo-1). Two men in the control group had antibodies; one had SSA and RNP, and one SSA. None of the controls was positive for SSB or Sm. The
three pre-symptomatic individuals with SSB abs had all SSA abs and two had a speckled ANA. There was a tendency for relationship between higher age and anti-SSA and anti-SSB positivity (p=0.09 and p=0.10, respectively) (Figure 5) and a significantly positive relationship between older age and number of autoantibodies. The number of antibody-positive individuals increased most during the year before the onset of symptoms, particularly ANA and anti-DNA abs, as shown in Figure 6. There were no relationships between any specific antibody and the initial symptom in the pre-symptomatic samples.

Figure 5. Age at blood sampling, first symptom, and SLE diagnosis in relation to autoantibody positivity.

Figure 6. The number of individuals with antibodies present before disease onset, presented as the accumulated number of antibody-positive individuals and years before onset (the year 0 = disease onset).
Anti-DNA abs and ANA had the highest odds ratio (OR) and likelihood ratio (LR) for developing SLE, but there were too few values to estimate the ratios for SSB abs and Sm abs. The most common ANA pattern was speckled in both pre-symptomatic individuals and controls, but a homogeneous pattern dominated in the SLE-patients after diagnosis (not shown in the paper). The speckled ANA pattern seems to be most unspecific and is the most common among healthy people with a positive ANA [245].

The current study encompasses some important limitations. SLE is not a common disease, and the number of SLE-patients who had donated blood to one of the blood banks in Västerbotten is low. Since the number of patients is low it is difficult to obtain statistical differences, especially when the individuals were stratified into subgroups. Additionally the amount of serum or plasma was a limiting factor. For that reason a multiplex method, in which many analytes could be detected in a small amount of sample, was chosen. In the multiplex test kit we used it was not possible to differentiate between the SSA antigens Ro-60kDa and Ro-52kDa, since these are mixed in this test. Antibodies against Ro-60 are more specific for SLE than Ro-52. It would have been interesting to separate these two antigens both in the pre-symptomatic individuals and in the controls. Anti-Scl-70 and anti-Jo-1 that are unusual antibodies may be more sensitive for detection with our analyse kit than with other methods [246, 247]. Autoantibodies against nucleosomes are though to be an early event in the lupus pathogenesis, but were not included in the multiplex analysis kit. As in many other studies on SLE very few of the patients were male, and consequently few age matched male controls. Furthermore, all of the men in this study were older than 50 years at the time of blood donation. Swedish law concerning personal integrity limited the possibility to gather facts about the control individuals except date of birth, gender and residential area at the time of donating blood.

The findings in paper IV are consistent with other published studies, although in those studies several ethnic groups were included. Both the incidence of SLE and presence of autoantibodies may differ between ethnic groups [112, 173, 184, 248]. This study is the first to include only individuals from northern Europe. A link between the number of antibodies and onset symptom was revealed that probably has not been reported previously. Furthermore, the findings is consistent with the hypothesis of a disease progression due to epitope spreading as has been described both in animal models and in SLE-patients [102, 249, 250].
In conclusion this work has shown that anti-TNF treatment affects chemokines levels in blood which may influence the leucocyte trafficking and be a part of the mechanism of acting in this kind of treatment. An increase in T-cell expression of CD91 in RA-patients who did not respond to anti-TNF-treatment could indicate CD91 to be a possible marker for response to this treatment. After treatment with anti-TNF (infliximab) many RA-patients developed new autoantibodies against chromatin-related nuclear antigens, but very few developed new autoimmune diseases. In SLE-patients the blood levels of chemokines and T-cell expression of chemokine receptors were altered and suggest an association with disease activity and kidney engagement. Autoantibodies were detected in the blood several years before symptoms of disease in SLE-patients. The findings of antibodies towards nuclear antibodies in pre-symptomatic individuals who later develop SLE (paper IV), and in infliximab-treated RA-patients (paper II) were similar with respect to chromatin-related structures, but differed concerning anti-ENA abs.
CONCLUDING REMARKS

- TNF-blocking treatment with infliximab in patients with RA induced formation of autoantibodies typically found in SLE-patients, but lupus-like syndromes was rarely induced.

- TNF-blocking treatment affected the levels of chemokines in blood in RA-patients. The influence on chemokines and their receptors may be part of the mechanism of action for anti-TNF medications.

- The CD91 expression on T-cells was high in RA patients who did not respond to anti-TNF treatment, and may be a marker of unresponsiveness.

- The levels of chemokines and the expression of their receptors on T-cells were altered and suggest a relation to disease activity and kidney engagement in patients with SLE.

- Autoantibodies were detected in blood several years before the onset of symptoms of disease in individuals who developed SLE later in life suggesting a prolonged pathogenetic process.

- The autoantibody formation in RA patients after TNF-blocking treatment (infliximab) was similar to that in individuals who subsequently developed SLE with regard to antibodies against chromatin structures, but differed with respect to anti-ENA antibodies.
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