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**STUDIES ON THE THERAPEUTIC MODULATION OF
INFLAMMATION IN THE SYNOVIAL MEMBRANE OF
RHEUMATOID ARTHRITIS**

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To my family

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial membrane that can lead to joint deformity and physical disability. Despite recent progress in the therapeutic field of RA, the exact molecular mechanisms responsible for chronic joint inflammation are not yet completely understood. The overall aim of this thesis was to identify new molecular mechanisms responsible for inflammation in the rheumatoid joint and to understand how distinct anti rheumatic drugs act upon these mechanisms.

I first focused on validating arthroscopy as a research tool for better understanding of the molecular mechanisms of action of anti rheumatic drugs, demonstrating that rheumatologic arthroscopy is a safe method, with very few complications and allowing retrieval of representative tissue in clinical longitudinal studies. We also propose an easy to perform way to quantitate macroscopic joint changes based on photos acquired during arthroscopies.

Based on our validation study we then used this method to perform several mechanisms of action studies. We first investigated the effect of etanercept on synovial expression of lymphotoxin- α (LT- α) and tumor necrosis factor- α (TNF- α). As predicted from previous in vitro studies etanercept was able to decrease synovial expression of both LT- α and TNF- α . The effect was however limited to good clinical responders. We propose LT- α modulation as an additional but not essential mechanism to explain the clinical efficacy observed with this drug in clinical practice.

Defective apoptosis of lymphocytes is linked to pathogenesis of RA and glucocorticoids are good in vitro inducers of lymphocyte apoptosis. We therefore investigated the effect of intra articular glucocorticoids on synovial apoptosis demonstrating that in the complex milieu of rheumatoid joint glucocorticoids actually fail to induce lymphocyte apoptosis. We further demonstrate that monocytes are essential in rescuing synovial T cells from glucocorticoid-induced apoptosis through a soluble factor mediated mechanism, a feature that is specific for RA-derived synovial lymphocytes.

LL-37 is an anti microbial peptide belonging to the cathelicidin family with important functions in innate immune response but recently also implicated as a modulator of acquired immune responses. We therefore investigated a potential role for LL-37 in RA pathogenesis, demonstrating that the peptide is present at low levels in healthy synovium, but up regulated in the context of inflammation. We also identified synovial neutrophils and to a lesser extent macrophages as the main cell types expressing LL-37. Distinct modulation patterns of LL-37 by some but not all anti rheumatic drugs and correlation with local levels of inflammation suggest a potential direct contribution of LL-37 to synovial pathology in RA.

In conclusion, we demonstrated that arthroscopy is a safe and reliable research tool for studies on mechanisms of action of anti rheumatic drugs and pathogenic traits of the inflamed rheumatoid joint.

LIST OF PUBLICATIONS

- I. **Evaluation of arthroscopy and macroscopic scoring**
af Klint E, Catrina AI, Matt P, **Neregård P**, Lampa J, Ulfgren A-K, Klareskog L, Lindblad S. Arthritis Res Ther, 2009. 11(3): p. R81.
- II. **Etanercept decreases synovial expression of tumor necrosis factor and lymphotoxin- α in rheumatoid arthritis**
Neregård P, Krishnamurthy A, Revu S, Engström M, af Klint E, Catrina AI. Submitted.
- III. **Monocytes are essential for inhibition of synovial T-cell glucocorticoid-mediated apoptosis in RA**
Makrygiannakis D, Revu S, **Neregård P**, af Klint E, Snir O, Arthritis Res Ther, 2008. 10(6): p. R147.
- IV. **Identification of the antimicrobial peptide LL-37 as a potential mediator of synovial inflammation in rheumatoid arthritis**
Neregård P, Rekha RS, Engström M, Lindh M, af Klint E, Agerberth B, Catrina AI. Manuscript.

Related articles not included in the thesis

1. **The cathelicidins LL-37 and rCRAMP are associated with pathogenic events of arthritis in humans and rats**
Hoffmann MH, Bruns H, Bäckdahl L, **Neregård P**, Niederreiter B, Herrmann M, Catrina AI, Agerberth B, Holmdahl R. Ann Rheum Dis 2012;00: 1-10
2. **How to use synovial immunohistology as a tool for the better understanding of the clinical use of different antirheumatic treatments**
Neregård P, Catrina AI. The Journal of Clinical Rheumatology and Musculoskeletal Medicine 2011; 000:(000). Month 2011

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LIST OF ABBREVIATIONS

ACPA	Antibodies to citrullinated protein antigen
ACR	American College of Rheumatology
AMP	Antimicrobial peptide
APC	Antigen presenting cell
DMARD	Disease modifying anti rheumatic drug
EULAR	European League Against Rheumatism
FLS	Fibroblast-like synoviocytes
GC	Glucocorticoid
HLA	Human leukocyte antigen
HMGB1	High mobility group box protein 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTB4	Leukotriene B4
MAO	Mechanism of action
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MTX	Methotrexate
MΦ	Macrophage
NET	Neutrophil extracellular trap
NSAID	Non steroidal anti-inflammatory drug
OPG	Osteoprotegerin
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
ROS	Reactive oxygen species
SF	Synovial fluid
ST	Synovial tissue
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling
VEGF	Vascular endothelial growth factor

1 BACKGROUND

1.1 INNATE AND ADAPTIVE IMMUNITY

The immune system defends us against microorganisms that can cause infection. To protect the individual effectively the immune system must fulfil four main tasks. First **immunological recognition**, the presence of an infection must be detected. This task is carried out by the leukocytes in the innate immune system, which provide an immediate response, and by the lymphocytes of the adaptive immune system. The second task is to **contain** the infection and if possible **eliminate** it completely. To do that we need immune effector functions such as the complement system of blood proteins, antibodies and the destructive capacities of leukocytes among them lymphocytes. At the same time the immune response must be kept under control so that it does not do damage to itself. Immune **regulation**, the ability of the immune system to **self-regulate**, is an important function of the immune response and failure of such regulation can cause autoimmune diseases like RA. The fourth task is to protect the individual against recurring infections due to the same pathogen and therefore the adaptive immunity has evolved the capacity of generating an **immunological memory** [1].

Innate immunity serves as a first line of defense. It works as an early barrier to pathogens that acts immediately but does not generate a lasting protective immunity. Key components of the innate immunity are anatomical barriers such as the epithelia of the skin, the gastro-intestinal and respiratory tracts and professional phagocytes like neutrophils, macrophages and dendritic cells. Phagocytes can engulf microbes and destroy and eliminate them by degrading enzymes and cytotoxic mediators like antimicrobial peptides (AMPs) and reactive oxygen species (ROS). AMPs are naturally occurring peptides considered to be among the earliest developed molecular effectors of innate immunity [2, 3]. The immune system is triggered by detection of pathogen (non-self) associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) from invading organisms or injured structures. The macrophages, neutrophils and dendritic cells have receptors to recognize these patterns, so called pattern recognition receptors (PRRs). The dendritic cells work as a bridge between innate and adaptive immunity. They capture the microbial antigen, migrate to peripheral lymphoid organs and use their major histocompatibility complex (MHC)

class II molecules to display the antigen, in conjunction with co-stimulatory signals, to T cells. This initiates the early events in adaptive immunity where antigen specific T and B cells are activated [1]. After activation they undergo clonal proliferation and maturation. These features allow the adaptive immune response to be directed towards specific pathogens. Subpopulations of these cells later become memory cells that can quickly mount an immune response to the same microbe when reactivated.

Upon encounter with the antigen, clonal expansion of antigen-specific T and B cells occurs. The specificity of T and B cell receptors is acquired through gene translocation and mutations that allow an increase in affinity and specificity. Innate immune cells do recognize molecular motifs that are shared by various classes of microbes through genetically encoded PRRs, of which toll-like receptors (TLR) are a major family. TLRs are highly conserved across evolutionary time and serve to activate host defense through a signalling pathway that operates in most multicellular organisms. In vertebrates TLRs have a key role in enabling the initiation of adaptive immunity. TLR-4 for example detects the presence of Gram-negative bacteria through its association with the peripheral membrane protein CD14, which is a receptor for bacterial lipopolysaccharide (LPS). Other TLRs respond to other molecular patterns found on or in pathogens. TLRs activate the transcription factor $\text{NF}\kappa\beta$, which then induces the transcription of a variety of genes, including cytokines, chemokines and co-stimulatory molecules that have essential roles in directing the course of the adaptive immune response.

1.2 INFLAMMATION AND AUTOIMMUNITY

Inflammation is our body's response to injury and tissue damage. An acute inflammation as a response to an infection is a normal reaction that serves to remove the pathological agent and start healing and tissue repair. However the inflammation process itself may also contribute to the development of autoimmune diseases. The inflammatory pathway consists of inducers, sensors, mediators, and target tissues. Inducers initiate the inflammatory response and are detected by sensors. Sensors, such as TLRs, are expressed on cells present in the tissue like macrophages, dendritic cells and mast cells. They induce the production of mediators, including cytokines, chemokines, eicosanoids and products of proteolytic cascades. These inflammatory mediators act on various target tissues to develop changes in their functional status for optimizing adaptation to the infection or tissue injury, associated with the particular

inducers that elicited the inflammatory response [4]. Typical signs of inflammation are tumor (swelling), rubor (redness), calor (heat), dolor (pain), and functio laesa (decreased function).

A typical example where inflammation contributes to disease development is in autoimmune diseases such as RA. Autoimmunity results from the failure of self-tolerance like inability of lymphocytes to distinguish self from non-self, so that an immune response against the body's own cells and tissues leading to chronic inflammation will occur.

1.3 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovium, which outlines the inner cavity of synovial joints except for cartilage surfaces. It is a heterogeneous disease spanning several disease subsets with potential distinct pathogenic pathways [5-7]. Sir Alfred Garrod was in 1859 the first to define RA, even though the typical symptoms had been described far earlier [8]. Findings of population-based studies show that RA affects around 0.5–1.0% of adults in developed countries. The disease is two to three times more frequent in women than in men. The prevalence rises with age and is highest in women of 65 years and older [9]. The incidence ranges from 5 to 50 per 100 000 adults in developed countries and increases with age [10, 11]. The prevalence of RA varies geographically. The disease is more common in northern Europe and in the North of America compared with parts of the developing world, such as parts of the west of Africa [12-14]. Today it seems like the incidence of RA might be decreasing with onset later in life [15, 16].

RA is defined by a series of clinical criteria to ensure correct classification. A set of criteria, revised in 1987 by the American College of Rheumatology (ACR) (Table 1) was designed to help differentiate RA from other inflammatory arthritides [17]. These criteria were developed to define established disease and provide a standard for recruitment into clinical trials. Today we know more about the etiology and pathogenesis of RA and the importance of early diagnosis for the possibility of early aggressive treatment [5, 18]. To meet the need for earlier diagnosis and institution of effective disease-suppressing therapy to prevent or minimise the occurrence of the undesirable sequelae a new classification system was introduced, developed by the ACR and European League Against Rheumatism (EULAR) in 2010 [19] (*Table 1*). This new classification system redefines the current paradigm of RA by focusing on

features at earlier stages of disease that are associated with persistent and erosive disease, rather than defining the disease by its late-stage features like rheumatoid nodules and existent erosions.

<p>ACR 1987 criteria</p> <ol style="list-style-type: none"> 1. Morning stiffness (at least 1h) 2. Arthritis of three or more joint areas 3. Arthritis of hand joints (≥1 swollen joints) 4. Symmetrical arthritis 5. Rheumatoid nodules 6. Serum rheumatoid factor 7. Radiographic changes (erosions) <p><i>Four of these seven criteria must be present. Criteria 1-4 must have been present for at least 6 weeks</i></p>	<p>ACR/EULAR 2010 criteria</p> <ol style="list-style-type: none"> 1. Joint involvement (0-5) <ul style="list-style-type: none"> • One medium-to-large joint (0) • Two to ten medium-to-large joints (1) • One to three small joints (large joints not counted) (2) • Four to ten small joints (large joints not counted) (3) • More than ten joints (at least one small joint) (5) 2. Serology (0-3) <ul style="list-style-type: none"> • Negative RF and negative ACPA (0) • Low positive RF or low positive ACPA (2) • High positive RF or high positive ACPA (3) 3. Acute-phase reactants (0-1) <ul style="list-style-type: none"> • Normal CRP and normal ESR (0) • Abnormal CRP or abnormal ESR (1) 4. Duration of symptoms (0-1) <ul style="list-style-type: none"> • Less than 6 weeks (0) • 6 weeks or more (1) <p><i>Points are shown in parentheses. Cut-point for rheumatoid arthritis, 6 points or more. Patients can also be classified as having rheumatoid arthritis if they have: a. typical erosions; b. long-standing disease previously satisfying the classification criteria</i></p>
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Table 1. ACR 1987 criteria (left panel) were designed to classify established rheumatoid arthritis. 2010 ACR/EULAR criteria (right panel) are intended to classify both early and established disease.

RA is a heterogeneous disease that probably should be divided into at least two different subsets with different causes and severity. Traditionally the subsets have been divided based on presence or absence of autoantibodies [5]. Rheumatoid factor (RF) is the classic autoantibody in RA. IgM and IgA RF are key pathogenic markers directed against the Fc fragment of IgG. Today more specific antibodies, so called anti citrullinated-proteins antibodies (ACPAs) [20], are used for the identification of clinical subsets of disease, with more relevance in classification of RA according to shared features in genetic and environmental risk factors. Both RF and ACPA are clinically useful prognostic markers and predict a more aggressive, destructive disease [21].

RA is a complex genetic disease and the exact cause is still not known but it is considered to develop when genetically predisposed individuals are exposed to specific environmental risk factors [22, 23]. These genetic and environmental risks factors interact and result in molecular events that drive development of arthritis. Twin studies implicate genetic factors in RA with concordance rates of 15 to 30% among monozygotic twins and 5% among dizygotic twins [24]. Estimated contribution of genetic factors for RA is around 50% [24].

The strongest genetic risk factor for seropositive RA is linked to the MHC class II locus. The HLA-DRB1 alleles code for a common amino acid sequence, the *shared epitope*, located in the β chain of the HLA-DR molecule [25, 26]. This is a group of alleles that play an important role in antigen presentation by influencing the binding and presentation of arthritogenic peptides to autoreactive CD4⁺ T cells [27]. There are also other genes involved such as PTPN22 [28], coding for tyrosine phosphatase involved in T cell and B cell signalling [29]. At present over 30 non-MHC risk alleles for ACPA positive RA have been identified and validated through candidate gene studies and GWAS [30].

Smoking is the dominant environmental risk factor for developing RA [31], particularly in RF positive and ACPA positive patients. A gene-environment interaction between smoking and shared epitope provides a high risk of ACPA⁺ RA but not ACPA⁻ RA [32]. Other forms of bronchial stress, like silica exposure, increase the risk of RA among persons with susceptibility HLA DR4 alleles (reviewed in [33]). In a recent study overweight together with smoking was shown to be a risk factor for developing RA in a cohort of autoantibody positive individuals [34].

Several anti-rheumatic therapies are available today, either classic disease modifying anti rheumatic drugs (DMARD) with a broad non specific immune modulation effect or modern biologicals, partly native human substances produced by gene technology that target specific molecule in the immune system. Despite this it is still difficult to find the best treatment for each patient right from the beginning and still there are few complete remissions and limited effects in about 30% of the patients. This is partly due to limited knowledge on the exact mechanisms of action of each individual anti rheumatic drug in distinct clinical settings. Particularly in RA this type of research is partially restricted due to difficulties in investigating the site of active inflammation, i.e. the inflamed synovium. I focused therefore my thesis on validating arthroscopy as a research tool for better understanding of the molecular mechanisms of action of anti rheumatic drugs.

1.4 ARTHROSCOPY

Historically synovial membrane biopsies have been retrieved from end stage destructive joints at the time of arthroplasty. However this type of intervention limits the investigations to late chronic destructive stages of the joint inflammation not

relevant in the search of early biomarkers. Later on blind needle biopsy was introduced which could be used to retrieve synovial tissue in any stage of active disease. This is a safe, cheap method easy to perform but restricted to larger joints and not reliable in getting accurate samples [35, 36]. During the last years ultrasound guided biopsies are increasingly used, this method can be performed in both small and large joints under local anaesthesia [37]. It allows an indirect visual inspection and the best biopsy site is selected based on synovial proliferation and vascularisation. The biopsies taken have been shown to be reliable for histopathological assessment [38, 39]. Although this is an appealing technique it has some disadvantages, the bony surfaces might cause ultrasonic disturbances, which might limit the access area and it is also difficult to look into non-inflammatory joints. Further validation of this method is needed.

Arthroscopic biopsy have been used by rheumatologists since the late 1970's and is technically more complicated but provides better possibilities in getting good samples as one has direct vision of the synovial membrane in both small and large joints [36]. Since the introduction of local and regional anaesthetics this procedure can be performed safely in an office based setting [40]. The arthroscopic procedure is well tolerated and safe [41]. In a survey, in which information from 15682 arthroscopies performed by rheumatologists was collected, a complication rate of haemarthrosis was 0,9%, deep vein thrombosis 0,2% and wound and joint infection 0,1%. The total complication rate reported was 15.1/1000 arthroscopies, which is comparable to reports from orthopedic surgeons [40].

Several studies have addressed the issue of intra-articular variation of synovitis. Intra-articular variation has been confirmed macroscopically [42], microscopically for cells [43], protein expression [44] and gene expression [45]. In our studies we have developed a method where we take samples from maximum macroscopic active synovitis both distant and close to cartilage. When performing consecutive studies with repeated procedures we try to sample the biopsies close to the same sites as in the first procedure according to a specially arthroscopy map (study I). One study has shown that the inflammation in one inflamed joint is generally representative of that in other inflamed joints [46]. Therefore, it is possible to use serial samples from the same joint, selecting either large or small joints. Another study showed that biopsies taken from actively inflamed synovial tissues of patients with RA show expression of unique patterns of mRNA, provided that the biopsy has been taken in such a way that the

analysis is performed on cells from an inflamed site [47]. Both inter-individual and intra-individual variation must be taken into consideration when analysing gene expression in synovial tissue. Therefore sites of maximal inflammation and/or random sampling from multiple synovial sites should be considered.

For research purposes synovial tissue can be processed for histology, immunohistology (IHC), immunofluorescence, in situ hybridization, PCR, micro-array, proteomics and cell or tissue culture. Immunohistochemistry can be performed on formalin fixed, paraffin-embedded material or on samples that have been snap-frozen in liquid nitrogen.

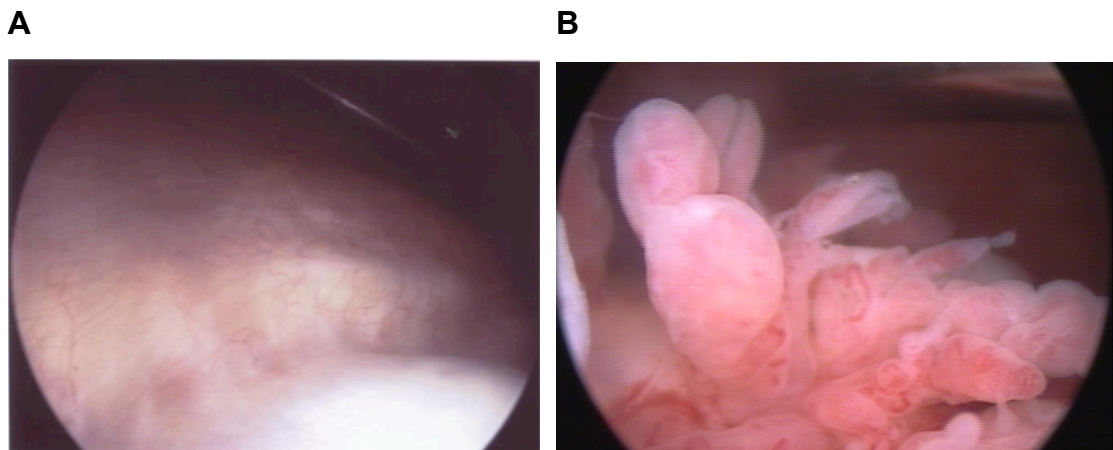


Figure 1

Arthroscopic images of a normal joint with thin transparent synovium (A) and a joint with on going inflammation (early RA) where we can see hypertrophied synovium with villi formation and increased vascularity (B).

1.5 BASIC ASPECTS OF THE SYNOVIAL MEMBRANE

The synovium is the central player in RA pathogenesis. A normal synovium (*figure 1A*) consists of two distinct layers, the lining or intimal layer and the sublining or subintimal layer. The lining layer is the superficial layer that is in contact with the intra-articular cavity and produces lubricious synovial fluid (SF). It is one to three cell-layers deep, containing macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. The sublining layer consists of scattered blood vessels, fat cells and fibroblasts residing in a matrix of fibrils and proteoglycans together with occasional mononuclear cells. When inflamed (*figure 1B*) the synovium undergoes profound changes, resulting in an increased volume and surface on macroscopic evaluation with accumulation of an inflammatory SF in the joint space. The lining layer becomes hyperplastic and forms an aggressive front termed “pannus” at the cartilage-bone junction, leading to the characteristic RA bone erosions. Important changes also occur in the sublining layer with massive mononuclear infiltration and blood vessel formation

[48] (*figure 2*). Increased joint influx and defective cell death (apoptosis) of resident cells are the mechanisms responsible for these cellular changes [49] and result in local accumulation of pro inflammatory cytokines (such as TNF). These cytokines further contribute to activation of synovial cells and perpetuation of chronic inflammation. The massive hyperplasia of the synovial membrane during inflammation does not only cause the majority of signs and symptoms of RA but also determines the outcome of the disease.

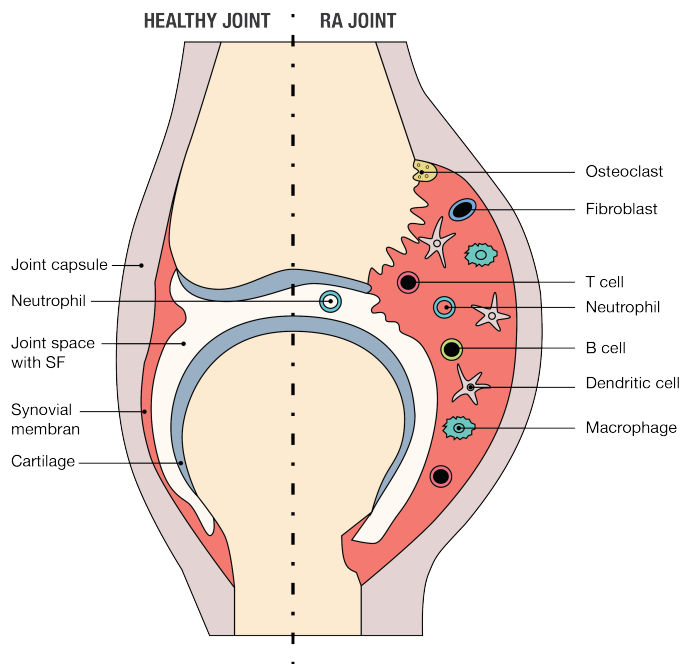


Figure 2

A schematic representation of a joint. A healthy joint to the left, with a thin synovial membrane, and an inflamed RA joint to the right, with a hypertrophied synovial membrane with massive influx of inflammatory cells.

1.6 CELL POPULATION IN THE INFLAMED RA JOINT

1.6.1 Monocytes/Macrophages

Macrophages ($M\Phi$) have phagocytic capacity and are central effectors of synovitis [50]. They are found both in the synovial tissue and SF. There are two types of macrophages in the RA synovial tissue, the macrophage-like type A synoviocytes in the lining and the sublining macrophages migrated as monocytes from the circulation and are diffusely distributed in the synovium. Both types have multiple functions like such as clearance of immune complexes, antigen presentation (MHC class II are overexpressed on $M\Phi$), mediation and regulation of local and systemic inflammation and tissue remodelling through release of different cytokines and growth factors (TNF-

α , IL-1, IL-6, IL-10, IL-13, IL-15, IL-18 and GM-CSF), mediation and regulation of monocyte migration and stimulation of angiogenesis by chemokines and chemoattractants, tissue degradation and post-injury tissue remodelling by matrix metalloproteinases (MMPs) [51]. They express several markers of the resident macrophage population including CD68, CD163 and CD14 [52]. In addition to the monocytes/MΦs central role in inflammation they are also involved in bone erosions due to their ability to differentiate into osteoclasts. Upon stimulation with TNF- α , IL-1, IL-6 and IL-17 synovial fibroblasts and activated T cells can upregulate RANKL expression on their surface which can engage its receptor RANK on the surface of monocytes and drive them into osteoclastogenesis [53]. Most of the macrophages in actively inflamed joints are localized in the synovial sublining rather than in the intimal lining layer [54]. Earlier studies have identified synovial sublining macrophage numbers as a potential biomarker for clinical efficacy in RA [55]. This was later confirmed in a study of synovial biopsies from 88 RA patients before and after treatment with different anti rheumatic drugs [50]. The validity of synovial tissue sublining CD 68 expression as a therapeutic biomarker has been confirmed across different academic centers [56].

1.6.2 Fibroblast-like synoviocytes (FLS)

FLS are non-phagocytic mesenchymal-derived cells. They can be found both in the lining and sublining layers. The FLSs found in the lining layer are highly activated and exhibit features with aggressive invasive properties. They are important in both initiation and perpetuation of RA and can contribute to maintenance of chronic inflammation through cell–cell contact and through elaboration of soluble products. In response to environmental stimuli and interactions with various cell types in the inflamed synovium FLS can secrete several mediators like cytokines, chemokines, growth factors and several other proinflammatory molecules like prostaglandins and leukotrienes. There are many ways to activate FLS, for example through activation of TLRs, exposure of cytokines and ligation of integrins by matrix molecules. The resistance to apoptosis characterizes FLSs and they have been linked to the progressive destruction of articular cartilage [52]. It has recently been shown in a SCID mouse model of arthritis that FLSs can migrate to a distant unaffected joint and invade and degrade the cartilage and thereby promote articular involvement [57]. In a very recent study, citrullinated fibronectin (cFn) was shown to inhibit apoptosis and increase pro-inflammatory cytokine secretion of RA FLSs [58]. This could be one possible

explanation for the increased number of FLSs that contribute to the hyperplasia in RA synovial membrane.

1.6.3 T cells

The T cells constitute around 30-50% of all cell types in the sublining and the majority are CD4⁺ with T helper (Th) 1 phenotype [52]. T cells are identified as CD3⁺ cells in the synovial tissue and are either CD4⁺ Th cells, CD8⁺ cytotoxic T cells or CD4⁺ regulatory T cells [59]. The cytokines present in the environment affect the differentiation of T cells towards a particular T cell lineage [60]. The Th1 subset mediates cellular immunity and is defined by IFN γ secretion. The Th2 is involved in humoral immunity and forms mainly IL-13 and IL-4, while Th17, the newest member of the T cell family is identified through its signature cytokine, IL-17. Th17 cells are important promoters of autoimmunity in RA [61]. Synovial-derived T cells have a phenotype that indicate chronic immune activation but express low levels of cytokines and show signs of anergy [60].

1.6.4 B cells

B cells and plasma cells are mainly found in the sublining layer of synovial membrane. Around 5% of sublining synovial cells are B cells. The pathogenic role of B cells in autoimmune disorders have historically been attributed to autoantibody production that would drive the inflammation locally either in soluble form or as immune complexes [62]. B cells contribute to RA through both antibody-dependent and antibody-independent mechanisms. Examples of antibody-independent functions are antigen presentation, T cell activation and polarization, organisation of other inflammatory cells and dendritic cell modulation. B cells display considerable phenotypic diversity [63].

1.6.5 Neutrophils

The phagocytic neutrophils are the most numerous and most important cells in innate immune responses, they take up a variety of microorganisms by phagocytosis and efficiently destroy them in intracellular vesicles using degradative enzymes and different antimicrobial substances, for example different antimicrobial peptides (AMPs) like LL-37, which I will describe later on in the text.

In the RA joint neutrophils are the first cells to be recruited at the sites of inflammation and accumulate mainly in the inflamed SF and to a lesser extent in synovial membrane

at the site of active destruction where they phagocytose immune complexes and release degrading proteases [64]. Resting peripheral blood neutrophils are relatively short lived, undergoing apoptosis within 12–18 h. Primed and activated neutrophils within tissues undergo molecular changes that extend their life span and alter their molecular properties, thereby allowing them to carry out many functions that have historically been attributed to macrophages. Delayed apoptosis, together with synthesis of inflammatory mediators like IL-8, TNF- α , IL-1, IL-6, IL-12, TGF- β and BLyS, and ability to present antigen to T cells via MHC II, makes tissue neutrophils capable of driving inflammatory processes. Many of the cytokines and chemokines implicated in RA are potent regulators of neutrophil activity (reviewed in [65]). Several recent reports have suggested a possible direct contribution of neutrophils in early RA pathophysiology and bone remodelling [66] by mediating Th17-responses [67], expressing PRRs [68-70], and mediating bone resorption via activating osteoclastogenesis [71]. To pass from the peripheral blood to the site of inflammation, the neutrophil adheres to the endothelial wall using selectins, integrins and adhesion molecules. Rolling arrest precedes transmigration through the endothelial lining of the blood vessel, and chemotaxis to sites of inflammation, for example the joint. (*Figure 3*)

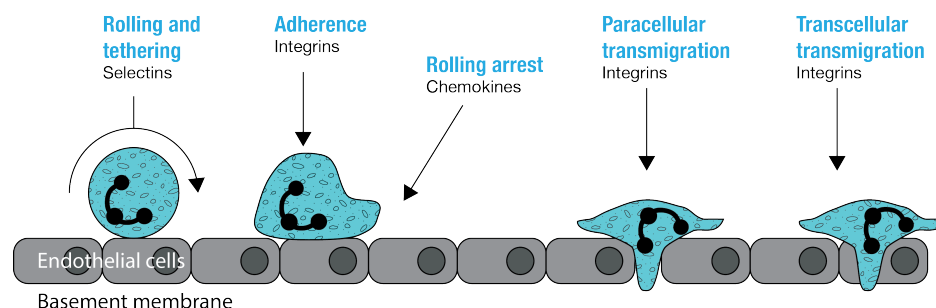


Figure 3

Diapedesis of neutrophils. How the neutrophils pass from the peripheral blood to the site of inflammation.

1.6.6 Dendritic cells

DCs play an essential role in the initiation and perpetuation of inflammatory arthritis by presentation of arthritogenic antigens to autoreactive T cells. Through their potent antigen-presentation ability they stimulate naïve T cells, direct effector cells function and polarize the T cell repertoire towards the Th1, Th2, or Th17 phenotypes. Myeloid DCs (mDCs) are considered especially important in promoting synovial inflammation. Plasmacytoid DCs (pDCs) are recruited in RA ST and comprise an antigen presenting cell (APC) population that might contribute to the local inflammatory environment,

particularly as a result of their capacity to produce cytokines in situ such as IFN- α , IFN- β , IL-15, IL-18 and IL-23p19 but also secondary to their potent function as stimulators of allogenic T cells. The number of synovial pDCs is especially increased in RA patients that are ACPA positive [72].

1.7 INFLAMMATORY BIOMARKERS, MEDIATORS

1.7.1 Cytokines

Cells can communicate and interact with each other either through direct cell-cell contact or via cytokines. Cytokines are proteins secreted by the cells of innate and adaptive immunity and they mediate many of the functions of the cells involved in immunity. Many cell types may produce the same cytokines and individual cytokines often act on diverse cell types. They are synthesized in response to inflammatory or antigenic stimuli and act locally by binding to high-affinity receptors on target cells. The cytokines that mediate innate immunity are mainly produced by activated macrophages (TNF, IL-1, IL-12, IL-18, IFN- γ , type I IFNs IL-10, IL-23, IL-27). Some of these macrophage-derived cytokines (IL-12, IL-18, IL-23, IL-27) also modify adaptive immune responses. However cytokines that mediate and regulate adaptive immune responses are produced mainly by antigen-stimulated T cells (IL-2, IL-4, IL-13, IFN- γ , TGF- β) [73]. A large number of cytokines are active in the joints of patients with RA and they play a major role in pathogenesis [74].

1.7.1.1 TNF- α

Based on synovial samples obtained from inflamed joints, tumor necrosis factor (TNF) was discovered as a key modulator molecule in RA for more than 20 years ago. Based on original studies which demonstrated elevated TNF concentrations at the sites of inflammation [75] it was proposed that this particular molecule drives disease pathology. It belongs to the TNF superfamily and consists of a protein expressed on the cell surface or present in a soluble form following cleavage by a protease called TNF- α -converting enzyme [76]. Both membrane bound and soluble forms are biologically active. Removal of excess TNF became a therapeutic goal [77-79]. Animal studies first demonstrated amelioration of chronic arthritis following TNF blockade [80-82] and a pilot study in RA patients, using a monoclonal anti-TNF antibody, showed impressive clinical results [83] and opened a new therapeutic era in RA [84]. TNF- α have several functions: it can activate leukocytes, endothelial cells and synovial fibroblasts, induce

production of cytokines, chemokines, adhesion molecules and matrix enzymes; suppress T reg function; activate osteoclasts and resorption of cartilage and bone; modulating neutrophil survival [85] (reviewed in [74]). TNF also has a dual effect on cell survival, being able either to promote cell death or to induce pro survival genes. While both TNF receptors promote early cell death, only TNFR1 can delay apoptosis via NF- κ B-controlled expression of pro-survival genes such as Bfl-1 and TRAF-1 [86].

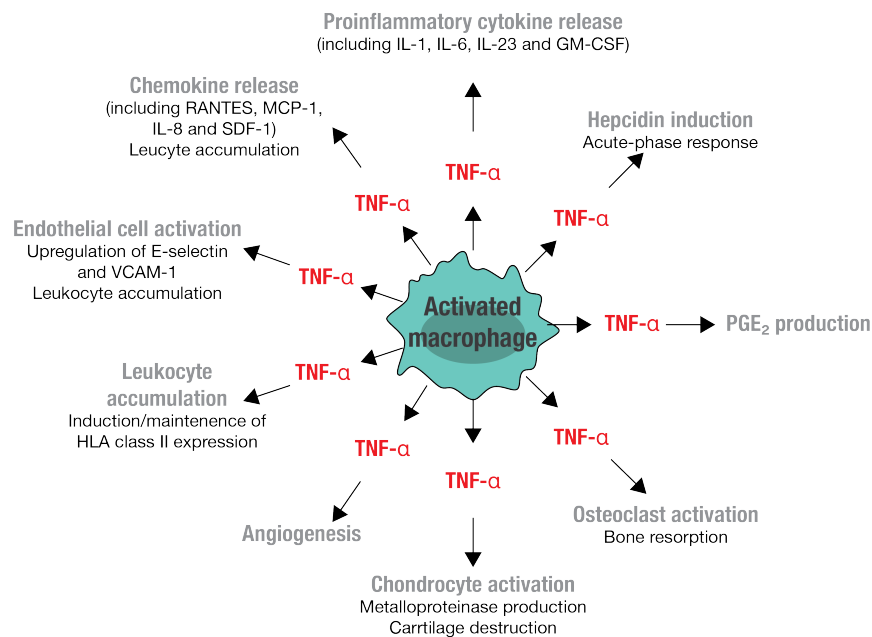


Figure 4
Simplified picture that outlines the typical TNF- α actions that are relevant to the pathogenesis of RA. Adapted from Brennan et al 2008 [74]

1.7.1.2 Lymphotoxin α (LT- α)

LT- α formerly known as TNF- β , was originally described in 1968 as a cytotoxic factor produced by T cells after antigenic or mitogenic stimulation [87]. In 1984, human LT- α was purified from a B-lymphoblastoid cell line and its structure was determined by classic protein-sequencing methods [88-90]. LT- α and TNF- α are 30% homologous in their primary amino acid sequence [91, 92]. LT- α is structurally similar to TNF- α . LT α is a soluble homotrimer composed of 17-kDa monomers and binds to and signals specifically through TNF receptors 1 and 2 (TNFR1 and TNFR2) to exert its biologic activities [93]. LT- α and TNF- α have many similarities but there are some distinct molecular and biological differences [94, 95]. The N-terminus of LT- α , unlike that of TNF- α , resembles a traditional signal peptide, making its conversion to a soluble form extremely efficient. LT- α is never found at the cell surface, a unique feature among the TNF superfamily members. LT- α is anchored to the cell membrane only in association

with membrane-bound LT- β , as LT- $\alpha\beta$ hetero-trimers. LT- $\alpha\beta$ is structurally distinct from LT- α and comprises two membrane-anchored heterotrimers, the predominant LT α 1 β 2 form and a minor LT α 2 β 1 form, both of which interact with the LT- β receptor (LT β R) [95].

LT- α is expressed by CD4⁺ Th 1 cells, CD8⁺ T-cells, NK cells, B-cells and macrophages [95]. LT- α has specific roles in the development and function of the immune system, mainly in lymphoid organ development, organization and maintenance of lymphoid microenvironments, host defense, and inflammation [96]. However, most of the evidence pointing to these roles came from genetically deficient mice and the relevance of LT- α in humans is less clear. In an animal model of collagen-induced arthritis, blocking of LT- α with a monoclonal antibody significantly improved the disease [97]. The main mechanism for improvement was attributed to the depletion of LT- α expressing Th1 and Th17 cells. Recently it has been demonstrated that LT- α can trigger activation of FLSs [98]. However LT- α was not detected in whole sera, plasma and synovial fluid of patients with RA, using commercially available ELISA kits.

1.7.1.3 IL-6

IL-6 is a cytokine produced by T cells, B cells, monocytes and fibroblasts, present at elevated levels in patients with RA. IL-6 signalling involves both a specific IL-6R and a ubiquitous signal-transducing protein, gp130 that is also utilized by other members of the IL-6 family. IL-6 signalling occurs by two mechanisms. Conventional signalling involves the binding of IL-6 to trans-membrane IL-6R on cells expressing this receptor. In contrast, trans-signalling involves binding between the complex of soluble IL-6R/IL-6 and membrane-bound gp130. Trans signalling allows IL-6 to affect cells that do not express IL-6R, including many synovial cells [99, 100]. The biological activities of IL-6 contribute to both systemic and local RA symptoms. IL-6 is a strong inducer of the acute-phase response, which can result in fever, secondary amyloidosis, anemia and elevations in acute phase proteins, such as C-reactive protein (CRP) [100]. The ability of IL-6 to induce B cell differentiation may lead to the formation of rheumatoid factor and other autoantibodies [101]. In joints, IL-6 promotes osteoclast activation and induces the release of MMP, thus contributing to joint damage [102, 103]. In patients with RA, IL-6 levels correlate with markers of disease activity and clinical symptoms. IL-6 signalling plays an important role in inflammatory cell migration by increasing the rate of cell adhesion and by inducing chemokine production in inflamed joints. [104].

1.7.2 Antimicrobial peptides

AMPs are naturally occurring peptides considered to be among the earliest developed molecular effectors of innate immunity [2, 3]. Two of the most extensively studied mammalian gene families of AMPs are the cathelicidins and the defensins [105]. The genes of mammalian cathelicidin consist of four exons, where exon four encodes the anti-microbial domain. CAMP (Cationic Anti-Microbial Peptide) is the single cathelicidin gene in humans. The human AMP LL-37 is a linear 37-residue peptide generated from the C-terminus of its precursor protein hCAP18 by a proteolytic cleavage [106].

1.7.2.1 LL-37

LL-37 is expressed in many cell-types and stored as the precursor hCAP-18 in large quantities in the specific granules of neutrophils [107]. LL-37 has a direct antimicrobial action but also diverse immune-modulatory effects such as increased chemotaxis of neutrophils, monocytes and T-cells [105], activation and maturation of B-cells and dendritic cells [105, 107, 108] and differentiation of macrophages towards macrophages with a pro-inflammatory signature [109]. It is known that cathelicidins and other AMPs influence adaptive immunity by acting on antigen presenting cells (APCs). Cathelicidins are secreted and taken up by macrophages, B cells and DCs and their effects on these cells lead to selective immune activation [105, 110, 111].

Immature monocyte derived DCs transport LL-37 into the cytoplasm and nucleus where LL-37 acts to up regulate CD68 and HLA-DR expression [112], these markers are associated with activation of the adaptive immune response.

LL-37 is produced by keratinocytes and neutrophils in wounded skin [113] and appears to be involved in the pathogenic events by binding locally released self-DNA and self-RNA in psoriasis, thus forming large aggregates that are resistant to nuclease-attack and transport and retain the nucleic acids in the early endosomes of pDCs, thereby leading to extended activation of TLR-7 and TLR-9 [114, 115] and production of type I IFN while inhibiting TLR-3 responses [116]. Patients with SLE [117, 118] but also a subtype of RA patients [119] display a type I IFN signature as measured by peripheral blood mononuclear cells (PBMC) gene expression. Given the importance of AMPs for development of SLE and psoriasis, there is a possibility that AMPs could also be mediators in TLR-stimulated pathways leading to induction of other autoimmune

diseases that are characterized by reactivity to nucleic acids, such as for example arthritis. The CAMP gene is constitutively expressed in the healthy state but can be increased in certain conditions, for example in psoriasis or SLE [120, 121].

LL-37 has also been detected as a component of the so-called NETs (neutrophil extracellular traps) structures that are formed following dying neutrophils. NETs contain self-DNA, AMPs and histones and they can serve as immunogens in SLE and additional autoimmune conditions by activating pDCs to produce type I IFN via TLR to induce a type I IFN signature [114, 122-124]. We have recently shown that LL-37 plays a pathogenic role in mediating synovial inflammation in an animal model of arthritis [125].

1.8 APOPTOSIS IN RA

Apoptosis, programmed cell death is an evolutionary conserved, multi-step process by which a cell dies, quietly. In contrast to necrosis the contents of the apoptotic cell are not released, the cell is cleared by phagocytosis and does not result in an inflammatory response. Apoptosis can be initiated by a variety of stimuli through different intracellular pathways, death receptor (DR) pathways or mitochondria-dependent pathways, the common link being activation of the caspases [126]. A hallmark of apoptosis is the degradation of DNA by cleavage between nucleosomes. To detect DNA strand breaks in tissues the TUNEL method, which labels DNA fragments, is commonly used [127]. Electron microscopy is another method frequently used; it identifies changes in nuclear morphology that define apoptosis [126].

The extrinsic pathway, the death receptor pathway is initiated through ligation of specific death receptors, FAS, by their ligands, FasL, TNF-related apoptosis-inducing ligand (TRAIL) receptors-1 and -2 and TNF receptors-1 and -2. Engagement of a death receptor with its ligand (e.g. FasL, TRAIL and TNF- α) induces apoptosis via activation of caspase-8 [128]. Fas-FasL interaction result in the recruitment of Fas associated death domain (FADD) and pro-caspase 8, which is cleaved to activate caspase 8. Activated caspase 8 either directly cleaves caspase 3 or activates BH3 interacting domain death antagonist (Bid), which disrupts mitochondrial integrity through the action of the pro-apoptotic molecules Bcl-2 associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK). In some cells, especially M Φ , growth factor can activate the PI3K/PKB pathway. The phosphorylation and activation of protein kinase B (PKB) by PI3K (phosphatidylinositol 3-kinase) regulates the anti-apoptotic protein

myeloid cell leukaemia-1 (Mcl-1) and A1 expression. NF- κ B activated by TNF- α has the same effect. Mcl-1 and AP1 (activator protein 1) together with Bcl-2 maintain mitochondrial integrity by inhibiting the damage promoted by aggregation of BAX and BAK. Following the loss of mitochondrial integrity cytochrome c is released and together with Apaf-1 (apoptosis protease activating factor 1) and pro-caspase-9 the apoptosome is formed activating caspase 9 and subsequently caspase 3. Caspase-3 promotes the characteristic features of apoptosis, including DNA fragmentation and cell death. Mitochondrial apoptosis might be directly induced by cytotoxic chemotherapy, UV irradiation, growth factor withdrawal or induction of p53 [129]. (Figure 5).

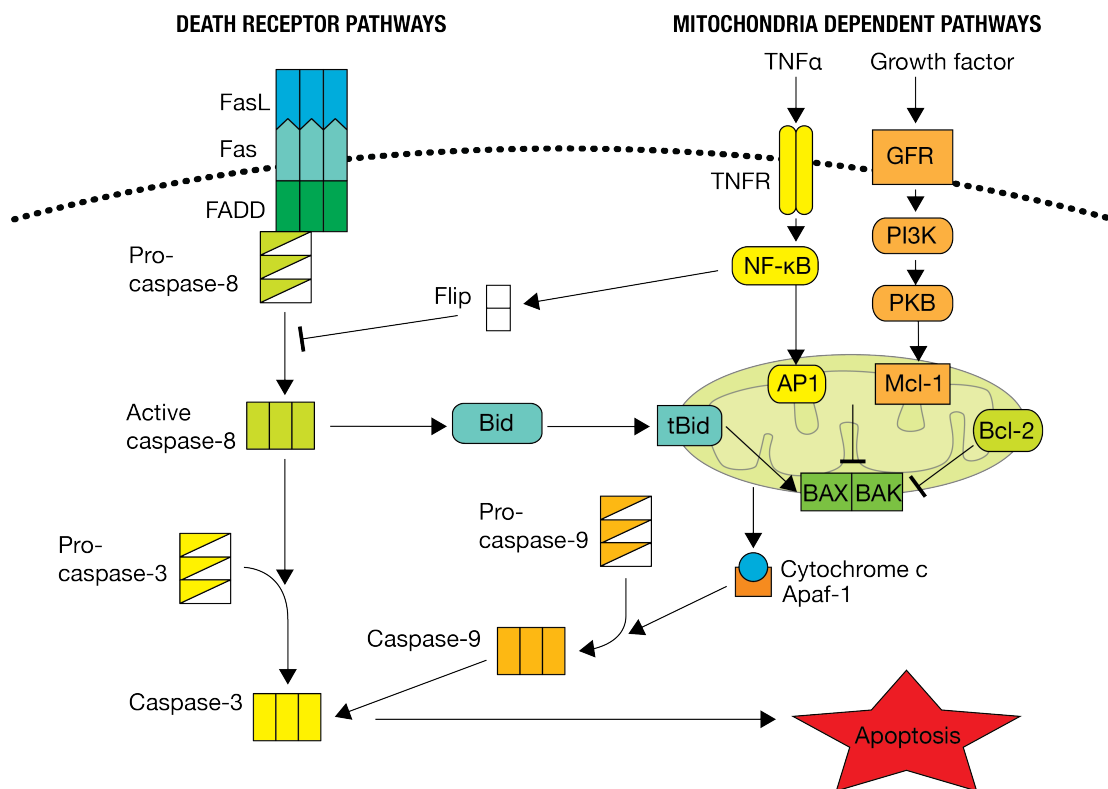


Figure 5

Schematic representation of apoptosis induction and suppression in RA synovial cells. (FADD: Fas associated death domain, TNFR: TNF receptor, NF- κ B: nuclear factor κ B, GFR: growth factor receptor, PI3K: phosphatidylinositol 3-kinase, PKB: protein kinase B, FLIP: Fas-associated death domain-like IL-1 β -converting enzyme inhibitory protein, Bid: BH3 interacting domain death antagonist, tBid: truncated Bid, Mcl-1: myeloid cell leukaemia-1, AP1: activator protein 1, BAX: Bcl-2 associated X protein, BAK: Bcl-2 homologous antagonist killer, Apaf-1: apoptosis protease activating factor 1)

The massive hyperplasia of the synovial membrane in RA is partly due to a defective apoptosis of infiltrating and resident synovial cells [52]. There are several potential mechanisms that can explain the resistance to apoptosis in RA. Histological studies have demonstrated low levels of apoptosis in the RA synovial tissue [127, 130], between 1% and 3% of synovial cells, despite the presence of both cell death receptors (Fas and TNFR) and cell death ligands (Fas-ligand and TNF) in the inflamed synovium

[131, 132]. Neutrophils that have migrated into RA joints display a delay in apoptosis, and thus have an enhanced potential to mediate host tissue damage because of their extended life span [133, 134]. It has been suggested that this delayed apoptosis in neutrophils could be partially due to the hypoxic conditions in the joints [135]. The anti-apoptotic Mcl-1 of the Bcl-2 family is involved in the pathophysiology of inflammatory disorders, and has been shown to be elevated in synovial fibroblasts [136], macrophages [137] and lymphocytes, as well as neutrophils [135] from inflammatory arthritis patients. Dysregulation of the intrinsic apoptotic pathway in RA has been reported to occur via increased expression of BAX and Bcl-xL in synoviocytes [138] and B cells [139], and Bcl-2 in CD4⁺CD28-T-cell clones [140] and RA synovial tissue [141]. This may be explained by high expression of Fas-associated death domain-like IL-1 β -converting enzyme inhibitory protein (FLIP) in synovial tissue [142]. FLIP inhibits caspase-8 activation by blocking its engagement with intracellular domains on death receptors, and expression of FLIP is under the control of Nuclear Factor (NF)- κ B, a transcription factor that is activated by TNF- α [143]. RA-FLSs are relatively resistant to apoptosis and through both cell-cell interactions and the secretion of soluble factors they also prevent infiltrating B and T cells from undergoing apoptosis [144].

1.9 TREATMENT

1.9.1 Classic anti rheumatic drugs

While more modern therapies, such as biologicals, have been developed based on pre existing knowledge about disease pathogenic mechanisms, classical anti rheumatic drugs, such as glucocorticoids and methotrexate, have been empirically used in RA based on the assumption that these drugs will exert their effect through mechanisms described in disease states others than RA. It is therefore important to characterize the disease specific mechanisms of action of these drugs for a more rationale use in clinical practice. However surprisingly few studies are available.

1.9.1.1 Methotrexate (MTX)

MTX is the most frequently used disease-modifying anti-rheumatic drug (DMARD) and it suppresses disease activity and reduces joint damage [145]. It is usually the first DMARD administered to patients with RA. It should be initiated when the disease is first diagnosed. The dose used and escalation of dosing has increased in recent years.

Folic acid is given to limit toxic effects. Findings of observational studies show that many patients remain on methotrexate and it achieves good outcomes [146]. MTX is a folate antagonist but the precise mechanism of action in the treatment of RA is unclear, it is thought that MTX prevents de novo pyrimidine and purine syntheses, required for DNA and RNA syntheses, and consequently inhibits cellular proliferation of lymphocytes involved in the inflammation process. At the cellular level, MTX and/or MTX-polyglutamates directly inhibit dihydrofolate reductase (DHFR), thymidylate synthase (TS) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase. Other folate enzymes such as methylenetetrahydrofolate reductase (MTHFR) may be influenced indirectly. MTX enters the cell via the reduced folate carrier (RFC1), whereas several multi-resistance proteins (MRPs) and P-glycoprotein (P-gp) probably facilitate cellular efflux [147].

Methotrexate has been shown to down regulate RA synovial inflammation with a decrease in the number of synovial macrophages probably due to deactivation of the endothelium with reduced expression of cytokines [148] and adhesion molecules [149]. A potential beneficial effect on cartilage has been suggested by the significant down modulation of synovial MMPs [149, 150] and our yet unpublished data suggest also an effect on bone metabolism through specific decrease of synovial RANKL expression. Only few studies are available in other diseases than RA, namely in psoriasis arthritis, where methotrexate decreased inflammatory synovial infiltrates and MMP expression without any effect on hypervascularity, a prominent feature of this type of arthritis [151, 152].

We have recently shown that MTX directly affects the RANKL/RANK/OPG system and inhibits osteoclast formation, a mechanism that might explain the bone sparing effect observed for MTX in clinical practice (Revu, S et al, Manuscript).

1.9.1.2 Glucocorticoids

Glucocorticoids (GCs) are potent anti-inflammatory agents that modulate apoptosis of immune cells. Most studies on mechanism of action (MAO) of GCs have addressed the effect of systemic administration. In this respect systemic GCs decrease macrophage number and tends to decrease the numbers of T cells, B cells and FLSs, probably through down regulation of the expression of synovial chemotactic factors [153] and adhesion molecules [154]. As a more specific synovial consequence of systemic GC, up regulation of the anti inflammatory S100A8 protein has been reported [155]. Real-

time quantitative PCR (Q-PCR) identified synovial expression of IL-8 and MMP-1 as biomarkers correlating with clinical response in a placebo controlled study of systemic GCs [156]. GC activities can be divided in genomic effects mediated through cytosolic glucocorticoid receptors (GRs) that need hours to become evident at the cellular and tissue levels and non genomic effects mediated through membrane-bound GR or nonspecific physicochemical interaction with the cell membrane which might explain some of the immediate effects observed with GC administration in vivo [157]. There are several synthetic GCs, such as triamcinolone hexacetonide (for local intra-articular administration) and methylprednisolone (for both local and systemic administration), used in clinical practice today. Differences in the mechanisms of action of these two compounds have been previously reported [158].

Few reports on the MAO of local administrated GC are available. We have previously demonstrated that ia GCs decrease the number of synovial T cells, whereas the number of macrophages remained unchanged [159]. We could also show that overall synovial protein expression of TNF- α , IL-1 β , extranuclear HMGB-1, VEGF, and ICAM-1 was reduced at follow up tissue sampling, while no significant effects were observed regarding vascularity following ia GCs. In contrast, expression of IL-1 α , VEGF, and cytoplasmic HMGB-1 protein in vascular endothelial cells was not affected. GC therapy down regulated levels of messenger RNA (mRNA) encoding IL-1 α and IL-1 β , but not TNF or HMGB-1. Synovial cell infiltration and pro-inflammatory cytokine expression were affected in a multifaceted manner by ia GC treatment. Marked reduction of synovial T lymphocytes, TNF, IL-1 β , extranuclear HMGB-1, ICAM-1, and VEGF occurred in association with beneficial clinical effects [160]. We further demonstrated that GCs could change the pro bone destructive synovial phenotype through specific down regulation of RANKL expression [159] and modulate the synovial prostaglandin pathway [161]. Our group has focused on the studies of mechanisms of action of ia GC, an adjuvant therapy largely used in different clinical settings for RA treatment.

1.9.2 Biologicals

1.9.2.1 *TNF antagonists*

TNF was discovered as a key modulator molecule in RA for more than two decades ago. In the search for understanding the pathogenesis of RA and cytokine biology TNF and interleukin-1 (IL-1) emerged as key factors in inflammation. Originally studies on animal models of RA showed good effect of TNF blockade [80-82]. A pilot study was performed in RA patients using a neutralizing chimeric monoclonal anti-TNF antibody, cA2, now called infliximab also gave very positive results [83]. There was strong symptomatic response, with relief of tiredness, lethargy and morning stiffness, reduction of swelling and tenderness. This study was followed by a larger multicenter study, a double-blind placebo-controlled trial with the same antibody, performed in four European centres, which showed the same good results [84]. Re-treatment of eight patients from the first open study was also successful [162]. Today there are five TNF-antagonists available: Infliximab, a chimeric monoclonal antibody; Etanercept, a recombinant TNF receptor-Fc fusion protein; Adalimumab, a fully human monoclonal antibody; Certolizumab, a recombinant humanized antibody Fab' fragment; Golimumab, a fully human monoclonal antibody. The mechanisms of action of TNF antagonists have been intensively studied but still there are many questions unsolved. A class effect of different TNF antagonists is the decrease in synovial cellularity observed by immunohistochemistry for cell surface markers as early as 48 hours and maintained up to 2 months after treatment initiation [163, 164]. The decrease concerns mainly intimal and sublining macrophages and is less pronounced for plasma and T cells, suggesting that TNF antagonists mainly target the macrophage synovial compartment. Further histological examination of arthroscopic obtained synovial biopsies provided even insights into the mechanisms behind this change in cellularity consisting in both decrease expression of adhesion molecules with consecutive reduction of cell recruitment [165] and increased apoptosis with consecutive increase in the clearance of resident cells [166]. Using similar methodology, the direct and selective modulation of the receptor activator of the NF- κ B ligand (RANKL) pathway has been proposed to be responsible for the bone protective effect observed with these drugs in clinical studies [167], while MMP modulation is thought to be important for the cartilage protective effects. TNF antagonists also down regulate expression of synovial and serum cytokine levels [168-171]. Most of the studies on the effect of TNF antagonists have been conducted with Infliximab-treated patients with RA.

1.9.2.2 Rituximab

Rituximab is a chimeric monoclonal antibody directed against CD20 antigen expressed by B cells. Treatment of chimeric mice with anti-CD20 mAb inhibited the production of IFN- γ and IL-1 β , indicating that APCs other than B cells could not substitute in maintaining T cell activation. Different studies have shown that treatment with rituximab causes a rapid and specific decrease in numbers of B cells in the synovium after 4 weeks. The early synovial tissue response varied between patients but in the peripheral blood there was a marked B cell depletion in nearly all patients [172, 173]. Prolonged follow-up showed good clinical response and a more pronounced decrease of B cells after 16 weeks but also a significant decrease in sublining macrophages, T cells and plasma cells supporting the concept that B cells have a big role in synovial inflammation [56, 174-176].

1.9.2.3 Abatacept

Abatacept, a CTLA4-Ig fusion protein, attenuates T cell activation as it regulates the activation of T cells by inhibiting the CD80/86:CD28 co-stimulatory pathway that is required for the proper T cell activation. Abatacept had minimal effect on synovial cell composition but significantly down modulated IFN γ gene expression in the inflamed RA synovium [177].

1.9.2.4 Tocilizumab

Tocilizumab is a humanized anti-interleukin-6 (IL-6) receptor monoclonal antibody, which binds to circulating soluble IL-6 receptor and membrane-expressed IL-6 receptor, inhibiting IL-6 by binding to both forms of IL-6 receptor [178].

2 AIMS OF THIS THESIS

2.1 GENERAL AIM

The overall aim of this project was to identify new molecular mechanisms responsible for inflammation in the rheumatoid joint and to understand how distinct anti rheumatic drugs interact with these mechanisms.

2.2 SPECIFIC AIMS

1. To develop and validate a standardized procedure to obtain synovial biopsies for further molecular studies (study I)
2. To characterize synovial expression of LT α and TNF- α in therapy resistant RA and their modulation by anti TNF treatment (study II)
1. To characterize synovial T cell apoptosis and its modulation by intra articular glucocorticoids in active RA (study III).
3. To investigate a potential new role for LL-37 as a pro-inflammatory molecule in RA and to study its modulation by distinct anti rheumatic drugs (study IV)

3 RESULTS AND DISCUSSION

The main focus of my research has been to look at the synovial membrane in RA patients. Arthroscopy with consecutive synovial immunohistology is a major research tool to dissect molecular mechanisms at the site of inflammation, without further in vitro manipulation, needed for fine characterization of both new and more classic anti rheumatic drugs. This will eventually contribute to a better use of different combination therapies in clinical practice and a better understanding of differences between therapies in terms of both effectiveness and adverse reactions. The ultimate goals of this type of research are identification of both new therapeutic targets and synovial biomarkers for predicting disease course and therapy response.

3.1 PAPER I - EVALUATION OF ARTHROSCOPY AND MACROSCOPIC SCORING

Safety of and yield of adequate synovial samples by arthroscopy

We have established an arthroscopy research unit at our department since 1998 with 408 procedures until 2005. We have validated the safety of the procedure and its usefulness as a research instrument. In our hands rheumatologic arthroscopy was a safe method with very few complications (two major and one minor complication; two haemarthrosis and one wound infection, respectively). Importantly, other major complications, such as septic arthritis or deep vein thrombosis, did not occur. In our study no arthroscopy procedure lasted more than one hour, and we kept the irrigation volume at a minimum, which might contribute to lowering the risk of infection as this has before been described as a risk factor for infection [40]. Despite the use of local anaesthesia, pain might still be a problem. In our study the physician responsible for the procedure notified occurrence of pain in each case, but it was not captured in a formal protocol. We had to prematurely terminate 12 procedures (3% of the cases) due to pain. In one patient the pain induced at the time of the arthroscopy lasted for two weeks. The large majority of the patients who were asked to do a second follow up arthroscopy consented to the procedure indicating that the experience of pain was low.

Yield of biopsies adequate for histology was 83% over all, 94% for knee joints and 34% for smaller joints. The low rate of adequate histology from small joints might be an obstacle that is possible to overcome with the right training and the use of ultrasound guidance [37] as other groups have reported better success rate [179]. In our case we

decided to restrict the studies mainly to knees, ankles and wrists if repeated biopsy sampling of the same joint was required.

Macroscopic scoring

There is an obvious need to correlate macroscopic findings and microscopic/molecular analysis of inflamed joints. Several different macroscopic scales have been used [41, 42, 180] and have been found to correlate with molecular SM features [180, 181]. Only a few studies have been published on intra and inter-variation of different raters in macroscopic scoring. We created a method for macroscopic scoring using printed photographs of synovitis obtained at arthroscopy. Fifty images were scored by seven raters twice, regarding hypertrophy, vascularity and synovitis using a five-point scale (0-4). Intra-individual scoring variation was low; at the second scoring 99% of all scores were within one point of the first scores using a five-point scale. Further, a perfect match between first and second scoring sessions was reached in 70% of scores, and no single parameter had a substantially greater intrinsic variability. We also showed low inter-rater variation: 1036 of 1050 individual scores (98.7%) were within one point from the median score. The range was two points or less in 139 of 150 (92.7%) image scores.

Some of the images and scores from the first set were used to create a reliable and easy to use macroscopic synovial scoring system for arthroscopy (Macro-score). These written instructions were tested on the same set of 50 photographs by five control-raters with no previous experience of arthroscopy. Without any other instructions they scored well and the time to understand the score and perform the score was about 2- 2.5 hours.

3.2 PAPER II - ETANERCEPT DECREASES SYNOVIAL EXPRESSION OF TUMOR NECROSIS FACTOR AND LYMPHOTOXIN- α IN RHEUMATOID ARTHRITIS

TNF- α is a key modulator of chronic inflammation in RA synovium, but less is known about LT- α , another pro inflammatory member of the same cytokine family. Few previous reports have investigated the expression in the synovium. Etanercept is the only TNF antagonist acting as a soluble receptor and able not only to block TNF- α but also LT- α , at least in vitro. High levels of expression of synovial LT- α have therefore been proposed as a possible mechanism to explain why certain patients might respond to etanercept while being resistant to treatment with TNF antagonists that exclusively block TNF- α such as infliximab [182]. However this original speculation has been

dismissed in a subsequent study demonstrating no significant differences in synovial baseline expression of LT- α in responders and compared to non-responders to infliximab. Buch et al looked at a subgroup of patients (n=5) demonstrating total failure to respond to infliximab but measurable clinical response to etanercept. In these patients there was no significant difference in expression of LT- α before treatment with etanercept compared with infliximab good responders (ACR 50/70) [183]. We further confirm this by demonstrating similar baseline expression of synovial LT- α in both responders and non-responders to etanercept treatment. In another study from the same group they could see that baseline synovial TNF- α and IL-1 expression did not predict infliximab response but synovial TNF- α level expression was reduced in all patients after infliximab treatment except for the worst non-responders [184]. Earlier it has been shown that TNF- α synthesis was reduced 2 weeks after infliximab treatment and that high level of synovial TNF- α production prior to treatment may predict responsiveness to therapy [168]. Another group has also showed the same results in a larger study with 143 patients. Here they could confirm that the baseline level of TNF- α expression was a significant predictor of response to infliximab [185]. In a study that was originally designed to examine the effectiveness and safety of etanercept in RA patients that had failed infliximab they could show good effect of etanercept but an exploratory analysis of serum LT- α and anti-infliximab antibodies was also performed. That analysis did not show any evidence of relationship between LT- α levels and response to etanercept [186].

Etanercept down regulates synovial LT- α and TNF- α expression in good clinical responders

To our knowledge this is the first study to investigate the effect of etanercept on synovial cytokine expression in relation to clinical response to therapy in RA patients. We demonstrate that etanercept decreases synovial expression of both TNF- α and LT- α and that this effect is restricted to good clinical responders. Clinical response to etanercept could not be predicted by differential synovial expression of these cytokines at baseline. We demonstrated that LT- α was present in a majority of the investigated RA biopsies (n=46) with large variations between different patients. LT- α was detected in both lining and sublining layers in a majority of patients, with high inter individual variations in a similar manner with other synovial cytokines [44]. Infliximab treatment had no effect on synovial expression of LT- α . These findings taken together suggest the existence of a specific and TNF-independent regulation of synovial LT- α by etanercept.

Another possible explanation is that the low number (etanercept n=12, infliximab n=11) of the patients might prevent identification of meaningful changes specifically in the infliximab group. However, similar numbers of patients allowed detection of significant changes in the etanercept group and in previous studies on infliximab [44, 166, 167]. We observed higher baseline levels of synovial TNF- α expression in responders as compared to non-responders to etanercept, but the difference was not statistically significant. No such difference was however observed for LT- α , suggesting that LT- α is rather a bystander than a major driving cytokine of the rheumatoid synovial inflammation.

3.3 PAPER III – MONOCYTES ARE ESSENTIAL FOR INHIBITION OF SYNOVIAL T CELL GLUCOCORTICOID-MEDIATED APOPTOSIS IN RHEUMATOID ARTHRITIS

Defective synovial apoptosis is one important mechanism contributing to local cell accumulation and perpetuation of inflammation in RA. RA synovial T cells express a phenotype suggesting chronic immune activation but have been found resistant to apoptosis [187, 188]. GCs are known inducers of T cell apoptosis, mainly through the mitochondrial pathway [189]. Our group have previously shown a decrease in the number of synovial tissue T cells after treatment with ia glucocorticoids in a wide range of arthritis types and suggested that this finding might be the consequence of reduced cell trafficking to the joints [160]. However apoptosis induction by GCs might be an additional mechanism and the aim of this study was to investigate if this was true.

All patients included in the study were clinical responders as evaluated by physician assessment during arthroscopies. The clinical response was paralleled by a significant decrease in the number of ST T cells as evaluated by CD3 staining, without changes in the number of ST macrophages, as evaluated by both CD68 and CD163 staining. We confirmed low levels of apoptosis in synovial tissues obtained from active arthritis that were unchanged following local administration of ia injection of GCs. The synovial apoptosis was evaluated using TUNEL technique and staining for active caspase-3 and confirmed with dual-immunofluorescence CD3/TUNEL. Similar, RA SF T cells were resistant to GC-induced apoptosis when cultured *in vitro* in the presence of monocytes. However upon SF isolation and separate culture of T cells, apoptosis induction was readily detected after exposure to GCs (dexamethasone, triamcinolone and methylprednisolone, had all the same effect). Transwell co-culture of monocytes

and T cells demonstrated that soluble factor(s) and not cellular contact are essential for T cell resistance to GC-mediated apoptosis. It has earlier been demonstrated that monocytes isolated from RA SF express IL-15 [190], a cytokine able to up regulate Bcl-2 expression [187] and to render activated T cells resistant to glucocorticoid-mediated apoptosis [191]. This feature appears to be RA-specific as T cell apoptosis induction was observed in co-cultures of cells, obtained from psoriatic arthritis patients, in the presence of dexamethasone at similar doses. In conclusion, we demonstrate that monocytes rescue synovial T cells from glucocorticoid-induced apoptosis, a feature that seem to be specific for RA.

3.4 PAPER IV – IDENTIFICATION OF THE ANTIMICROBIAL PEPTIDE LL-37 AS A POTENTIAL MEDIATOR OF SYNOVIAL INFLAMMATION IN RHEUMATOID ARTHRITIS

LL-37 is originally described as an anti microbial peptide belonging to the cathelicidin family with important functions in innate immune response but recently also implicated as a modulator of acquired immune responses [105, 192-194]. We have recently reported that rCRAMP, the rat homologue of human LL-37, plays a pathogenic role as a local and systemic mediator of inflammation in an animal model of arthritis. This suggests that LL-37 may contribute to RA pathogenesis [125]. To extend these findings and add new knowledge on the role of LL-37 in human RA we investigated the expression of LL-37 in synovial membrane and its relationship to local inflammation. We started with a screening of LL-37 expression performing immunohistochemistry on different tissues from a patient with RA where we looked at bone marrow, lymph node and synovial biopsies. We also looked at lung biopsies from patients with RA (*Figure 6*) and synovia from other arthritis like psoriatic arthritis and we could in all of the different tissues identify LL-37 expression.

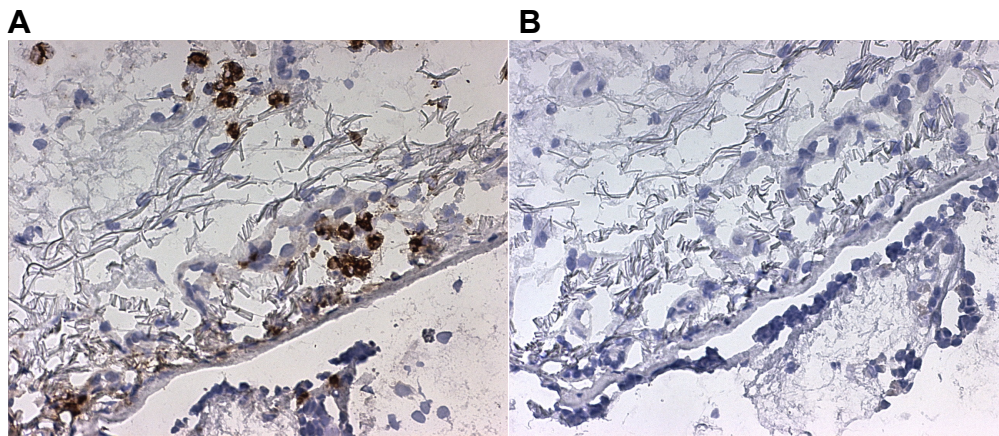


Figure 6

Immunohistochemical analysis of LL-37 expression in lung tissue from a patient with RA. LL-37 expression in cells with neutrophil morphology (A), negative control (B). Magnification x25

LL-37 was expressed in a majority of the 37 pre-treatment RA synovial biopsies, both in the lining and sublining layers. The peptide was also detected by western blot in the inflamed SF, while only low levels of LL-37 were detected in the healthy synovium. Treatment with adalimumab and ia GCs but not MTX resulted in a significant down-regulation of synovial neutrophils and LL-37 expression. Using serial and double-fluorescent immunostaining we could confirm that neutrophils and to a lesser extent macrophages were the main cell-types expressing LL-37. We also found LL-37 expression in the endothelium in some of the biopsies from healthy individuals and following intra-articular glucocorticoids, which is intriguing and its relevance for the synovial biology needs further investigation.

Previous studies have demonstrated that a broad spectrum of AMPs are expressed to different extent in normal, inflamed and pyogenic synovial membranes at both RNA and protein level [195]. However, synovial distribution of LL-37 has only been investigated at the RNA level showing that LL-37 mRNA is present in the synovial membranes of RA and osteoarthritis but absent in healthy and pyogenic arthritis [195-197]. In accordance with this we here demonstrate very low levels of LL-37 expression on peptide level in healthy synovium and with distinct up-regulation in active RA that could be reverted by anti-rheumatic treatment. Similar to our current study we have previously shown over-expression of the rat cathelicidin rCRAMP during pristane-induced arthritis (PIA). The induced expression was most pronounced at the very early phase of disease but sustained until the late, chronic stage (*figure 7*).

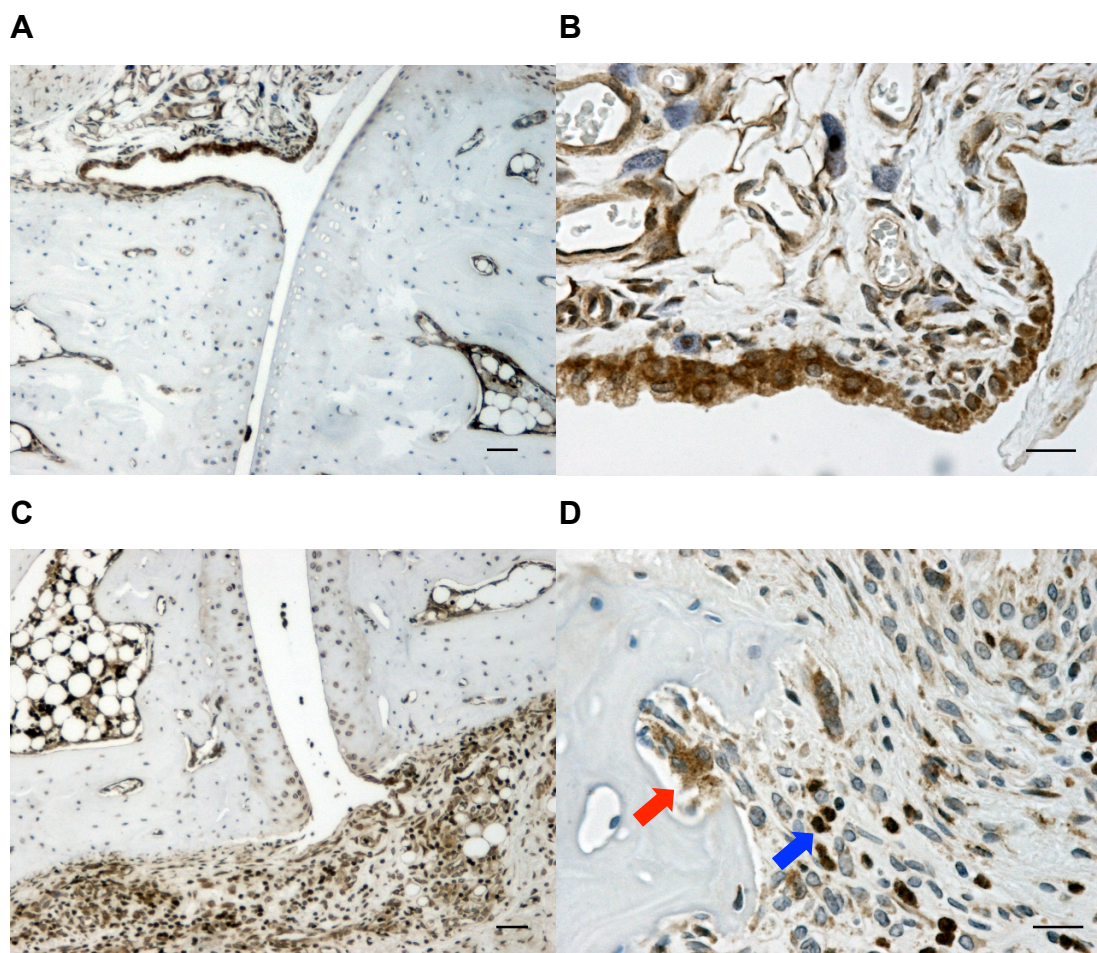


Figure 7

Immunohistochemical analysis of rCRAMP expression in hind paws of Dark Agouti rats at day 0 and 23 after pristane injection. In naïve animals (A and B) rCRAMP is expressed in synoviocytes (especially in the synovial lining (B with magnification x40)), part of the chondrocytes and in osteoblast-like cells of bone marrow. During pristane-induced arthritis (PIA) (C and D), rCRAMP is also strongly expressed in inflammatory cells of the infiltrating pannus tissue. Magnification x40 (D) shows strongest expression in cells with neutrophil morphology (blue arrow) and intermediate expression in macrophage-like cells and multinucleated osteoclasts (red arrow).

Low level of LL-37 expression in the normal synovium and septic arthritis with up-regulation in the context of sterile inflammation suggests that LL-37 mainly contributes as mediator of the local adaptive immune response in RA, and not acting as an antimicrobial defensive peptide.

LL-37 is capable of attracting various cell-types such as phagocytic leukocytes, immature dendritic cells, and lymphocytes, together with its capacity to stimulate IL-8 production and mast cell degranulation offers some insights in its potential pro-inflammatory role in arthritis [107, 198]. Recently a novel mechanism has emerged by which LL-37 can contribute to the generation of autoimmune diseases. It has been shown that in psoriatic lesions excessive LL-37 accumulates with subsequent binding of self-DNA and -RNA, forming large aggregates. These aggregates are resistant to

degradation and retained in the endosomes of pDCs, thereby leading to detrimental production of type I IFNs [114, 123, 124, 199]. Furthermore, in the sera of patients with systemic lupus erythematosus (SLE), immune complexes of AMPs and DNA forming neutrophil extracellular traps (NETs) trigger activation of dendritic cells. NETs can serve as auto-antigens to trigger B cell activation, as demonstrated by antibodies against AMPs [122, 123, 200]. A vast majority of the cells expressing LL-37 were identified as neutrophils, which are part of innate immunity and crucial for the pathogenic defense. These cells also have an important role in the modulation of several immune functions. They are the first cells to arrive at sites of inflammation [201] and are able to release cytotoxic components such as ROS, nitrogen species, AMPs, proteases and other inflammatory mediators. Neutrophils are mainly found in the SF of RA patients but may also be detected in the ST [65, 202]. Recently, neutrophils have been implicated as important mediators of the synovial inflammation in early stages of the disease, in a similar way as we observed in the pristane-induced model of arthritis in rats [125, 203]. However in our current study neutrophils and LL-37 were up regulated in both early and long-standing RA seemingly more dependent on inflammation than of disease duration. Treatment with adalimumab and intra-articular GCs decreased both the number of neutrophils and LL-37 expressing cells, while no such effect was observed for methotrexate. It is tempting to speculate that this difference is due to different stages of the disease, with adalimumab and glucocorticoid treated patients both having longstanding RA while methotrexate treated patients are newly diagnosed with RA. However no differences in the level of expression of either neutrophils or LL-37 were observed among the three treatment groups and both early and longstanding RA had higher levels of expression as compared to healthy individuals. As far as changes in LL-37 expression paralleled changes in the number of synovial neutrophils we believe that the most obvious explanation is a decrease in the local synovial recruitment of neutrophils induced by adalimumab and intra-articular glucocorticoids. Despite previous reports suggesting that methotrexate also influence neutrophils functions at least *in vitro* we were not able to see any significant down-regulation of synovial neutrophils following methotrexate treatment *in vivo* despite good clinical results in a majority of the patients. One additional possibility is a direct effect of adalimumab and intra-articular glucocorticoids on LL-37 expressing cells and lack of such effect for methotrexate, as suggested by our pilot screening *in vitro* in the LL-37 expressing cell line.

4 CONCLUSION

In this thesis we have demonstrated that:

- In our hands rheumatologic arthroscopy is a safe method with very few complications. For knee joints it is a reliable method to retrieve representative tissue in clinical longitudinal studies. Our results are similar to other few centers performing rheumatologic arthroscopies and are part of a continuous effort to develop international standards in this area. (Paper I)
- We have also created an easy to use macroscopic score, that needs to be validated against other methodologies, which we hope will be of value in further developing international standards in this area (Paper I)
- Etanercept treatment modulates synovial expression of both TNF- α and LT- α in vivo, an additional mechanism that explains the clinical efficacy observed with this drug in clinical practice (Paper II)
- Monocytes are essential in rescuing synovial T-cells from glucocorticoid-induced apoptosis through a soluble factor mediated mechanism, a feature that is specific for RA-derived synovial T-cells. We propose that this might be overcome by the combination of locally administrated glucocorticoids with monocyte-targeted therapies rather than T-cell apoptosis-inducing therapies (Paper III)
- LL-37 is expressed in the inflamed rheumatoid synovial membrane and is modulated by distinct anti-rheumatic agents (Paper IV)

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Synovial arthroscopy is a useful tool in investigating the site of active inflammation in chronic joint diseases. We have contributed with the experience of our centre to the available data in the field. It is important to note that in this context there are few Rheumatology centres around Europe currently performing arthrosocopies and therefore thorough validation and standardization of different procedures is highly needed. We have in our first paper described in detail our experience in performing arthroscopies and consider this an important part of the current ongoing collaboration between centres to achieve consensus and to eventually develop common protocols. This is an important task for the future as the development of new anti rheumatic drugs as well as the need for identification of new therapeutic and diagnostic biomarkers urge access to large samples of biopsy material that should be collected in similar ways in order to allow a meaningful analysis. One problem with clinical invasive studies is usually the small number of patients possible to collect, as was the case also in our hands. Because of this we have chosen to make use of internal controls where patients constitute their own controls.

My thesis has also provided insight in how one should use arthroscopies and synovial biopsies in order to not only describe clinically the effect of various anti rheumatic drugs but also to map new and some time unexpected mechanisms of action of these drugs. The glucocorticoids failure to induce apoptosis was somewhat surprising as dexamethasone is used as a prototype for apoptosis induction in vitro in a large array of cellular systems. This added new knowledge to our current understanding on how lymphocytes gain important survival advantages just by interaction with other cellular components of the synovial inflamed joint. Also differences in results when different cell systems are used (biopsies versus mixed cell cultures versus single type cell cultures) further stress the importance of having access to systems as similar as possible with the in vivo situations. In this respect synovial biopsies offer one of the closest model to the in vivo situation. Further work is needed to map the factor(s) responsible for the apoptosis resistant phenotype of the synovial lymphocytes. If successful, such work would potentially allow identification of factors that might be new targets for therapeutic modulation.

Using same methodology as above we characterized in detail synovial expression of novel potentially inflammatory mediators, namely LT- α and LL-37. While some isolated reports on LT- α synovial distribution were published before, our study provides a more thorough investigation in a large number of biopsies. Despite some distinct modulation by etanercept, in accordance with what we were expected from in vitro experiments, we conclude that most probably LT- α is not a major denominator of the synovial inflammation in RA but merely a bystander molecule. Despite this being apparently a negative result it will still help us to better understand how drugs that have a certain cytokine inhibitory profile when studied in vitro can have a different clinical efficacy and safety profile in vivo. Analysing complex structures such as biopsies in parallel with more simplified cellular systems offers better possibilities in both designing new drugs and understanding the mechanisms of action of “classic” drugs.

Recent reports on the potential relevance of the anti microbial peptide LL-37 as a modulator of inflammation and adaptive immunity prompted us to investigate its role in arthritis. Despite synovial membrane being a privileged area in terms of susceptibility to infections, we did not find a high expression of LL-37 in healthy synovium. It might be so that AMPs others than LL-37 are expressed in the joints. However the clear up regulation of LL-37 in context of inflammation and its specific down regulation by some but not all anti rheumatic therapies points to a potential pathogenic role for this molecule in the context of synovial inflammation. Future research in this area is needed. Recently it has been shown that modification of LL-37 by the process of citrullination confers higher chemotactic activity against mononuclear leukocytes as compared to native LL-37, a process that might play a role in chronic obstructive pulmonary disorders. Preliminary data from our laboratory shows extensive expression of LL-37 in the lungs of patients with RA. If this is citrullinated or not, pro inflammatory or not, important for disease pathogenesis or not, remains to be demonstrated and will be a focus of future research.

The studies presented in my thesis have contributed to the knowledge on how synovial arthroscopy can be used to study mechanisms of action of anti rheumatic drugs and to identify new synovial biomarkers. However much more work is needed in the future in order to translate this knowledge in clinical practice and to use it for the benefit of the patients.

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