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Post-translational and therapy-induced modifications in rheumatoid arthritis

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The cover illustrates a personal simplified approach representative for the 4 papers of this thesis. Photos represent RANKL expression in the synovial tissue of an RA patient before and after and intraarticular knee injection of triamcinolone hexacetonide.

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To rheumatological patients

ABSTRACT
Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial proliferation and excessive mononuclear infiltration leading to destruction of bone and cartilage, with subsequent joint deformities and physical disability. Even though RA etiology is still unknown, autoantibody driven pathogenic mechanisms are considered to be important, with strong evidence for the pathogenic potentials of antibodies directed against citrullinated antigens. Several drugs are used for RA treatment such as non-steroidal anti-inflammatory drugs, glucocorticoids, disease modifying anti-rheumatic drugs and biological agents. Intra-articular glucocorticoids, potent anti-inflammatory compounds, are successfully used by clinicians as an adjuvant therapy for RA, but there is limited information regarding their molecular mechanisms of action. This thesis work investigates the relevance of the post-translational modification citrullination in RA pathogenesis and inflammation and the effect of intra-articular glucocorticoids in RA inflammation and associated bone destruction.

Approximately 60% of RA patients are characterized by presence of anti-citrullinated protein antibodies (ACPAs), associated with worse disease prognosis. ACPA development can be a result of a gene-environment interaction between HLA-shared epitope and smoking. We demonstrated that both citrullinated proteins and peptidylarginine deiminase 2 enzyme expression is increased in bronchoalveolar lavage cells of smokers in comparison to non-smokers, which could be an early step in the pathogenesis of ACPA positive RA. In RA inflamed synovial tissue citrullinated proteins - potential targets of ACPAs - are present. We demonstrated the presence of citrullinated proteins in a wide range of inflammatory tissues, which suggests that citrullination is not specific for synovial inflammation but more generally associated with inflammation. Our findings suggest that additional factors than presence of citrullinated proteins determine the association between presence of ACPAs and synovial inflammation.
Decreased apoptosis is linked to RA synovial inflammation. We demonstrated that intra-articular glucocorticoids modify RA synovial inflammation by decreasing T cell numbers and we investigated if this was linked to T cell apoptosis. We found RA synovial T cells resistant to glucocorticoid induced apoptosis both at the synovial tissue and fluid level, a specific feature for RA attributed to interactions with monocytes in the inflammatory synovial environment. Synovial inflammation in RA is linked to bone destruction, a dynamic process balancing bone matrix synthesis by osteoblasts and bone resorption by osteoclasts, mediated through the receptor activator of NFκB ligand (RANKL)-osteoprotegerin (OPG) system. We demonstrated that intra-articular glucocorticoid treatment decreased synovial RANKL expression without changes in OPG expression. The synovial RANKL/OPG ratio thus decreased following treatment, suggesting a bone protective effect of intra-articular glucocorticoids in the rheumatoid joint.

In conclusion, we have demonstrated that citrullination might be a relevant mechanism for RA pathogenesis in the context of gene-environment interactions, while it is not an exclusive characteristic of RA inflammation. RA synovial inflammation and associated bone destruction is modified by intra-articular glucocorticoids, while RA synovial monocytes render T cells glucocorticoid induced apoptosis resistant, suggesting that combination treatment with monocyte targeted therapies could be beneficial.

PUBLICATIONS

This thesis is based on the following papers, which will be referred in the text by their Roman numbers.

I. Dimitrios Makrygiannakis, Monika Hermansson, Ann-Kristin Ulfgren, Anthony P. Nicholas, Albert J.W. Zendman, Anders Eklund, Johan Grunewald, C. Magnus Skold, Lars Klareskog, Anca Irinel Catrina
Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells
Annals of the Rheumatic Diseases (2008) Apr 15; [E-pub ahead of print].

II. Dimitrios Makrygiannakis, Erik af Klint, Ingrid E Lundberg, Robert Löfberg, Ann-Kristin Ulfgren, Lars Klareskog, Anca Irinel Catrina
Citrullination is an inflammation-dependent process
Annals of the Rheumatic Diseases (2006) Sep;65(9):1219-22.

III. Dimitrios Makrygiannakis*, Erik af Klint*, Sergiu-Bogdan Catrina, Ileana Ruxandra Botusan, Elin Klareskog, Lars Klareskog, Ann-Kristin Ulfgren, Anca Irinel Catrina
Intraarticular Corticosteroids Decrease Synovial RANKL Expression in Inflammatory Arthritis
Arthritis and Rheumatism (2006) May;54(5):1463-72.

*These authors contributed equally

IV. Dimitrios Makrygiannakis, Petra Neregård, Erik af Klint, Omri Snir, Cecilia Grundtman, Anca Irinel Catrina
Interactions between monocytes and T cells are essential for inhibition of synovial T cell glucocorticoid mediated apoptosis in rheumatoid arthritis
Submitted manuscript.

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LIST OF ABBREVIATIONS

ACPAs: anti citrullinated protein antibodies
AMC: anti modified citrulline
Apaf: apoptotic protease activating factor
BAL: broncho alveolar lavage
Bcl: B cell lymphoma
Bid: BH3 interacting domain death agonist
CCP: cyclic citrullinated peptide
CD: cluster of differentiation
CTLA: cytotoxic T lymphocyte antigen
DIABLO: direct IAP binding protein with low pl
DMARDs: disease modifying anti-rheumatic drugs
DNA: deoxyribonucleic acid
FADD: Fas associated death domain
FLIP: FADD-like IL-1β-converting enzyme inhibitory protein
GCR: glucocorticoid receptor
HLA: human leukocyte antigen
IAP: inhibitor of apoptosis protein
ICAM: intercellular adhesion molecule
Ig: immunoglobulin
IL: interleukin
MCP: metacarpophalangeal
MHC: major histocompatibility complex
MTP: metatarsophalangeal
NFκB: nuclear factor-κB ligand
NSAIDs: non steroidal anti-inflammatory drugs
OPG: osteoprotegerin
p53: protein 53
PAD: peptidylarginine deiminase
PIP: proximal interphalangeal
RA: rheumatoid arthritis
RANK: receptor activator of nuclear factor-κB
RANKL: receptor activator of nuclear factor-κB ligand
RF: rheumatoid factor
SE: shared epitope
SF: synovial fluid
Smac: second mitochondria-derived activator of caspases
Th: T helper
TNF: tumor necrosis factor
TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VEGF: vascular endothelial growth factor

GENERAL INTRODUCTION

The aim of this thesis is to enlighten some aspects of the pathogenesis of rheumatoid arthritis (RA) relevant to the post-translational modification citrullination and to investigate the mechanism of action of intra-articular glucocorticoids in reducing RA synovial inflammation.

BACKGROUND

Rheumatoid arthritis, what is it?

RA is a chronic systemic inflammatory disease that predominantly manifests in the synovial membrane of the diarthrodial joints. The chronic inflammatory process induces changes in the cellular composition of the synovial membrane, resulting in its hyperplasia and consequent structural damage of cartilage and bone (Choy and Panayi, 2001), (McInnes and Schett, 2007). Extra-articular disease affecting a variety of organs is not uncommon in RA patients and is a significant factor in morbidity and mortality of people with RA (Turesson and Jacobsson, 2004). The severity of RA encompasses a wide spectrum (Ollier et al., 2001), causing varying degrees of joint destruction and extra-articular organ involvement. The term RA was first suggested in 1859 by Alfred Baring Garrod, who emphasized the differences from gout and rheumatic fever (Fraser, 1982). The currently most used definition of rheumatoid arthritis is provided from the 1987 American College of Rheumatology classification scheme (Arnett et al., 1988) (Table 1).

Epidemiology of RA

RA is the most common chronic inflammatory disease, which more frequently affects females (Silman, 1994). The disease exhibits an age peak onset around the sixth decade of life and shows an annual incidence rate of approximately 3-4/10000 for women and 1.5-2/10000 for men (Symmons et al., 1994), (Soderlin et al., 2002), (Uhlig et al., 1998). With few exceptions in some countries of the world, RA shows a prevalence varying between 0.5-1% of the total population (Symmons, 2002).

The normal and the inflamed RA synovium

Diarthrodial (or synovial) joints represent the most common type of articulations. A diarthrodial joint consists of adjoining bones cushioned by hyaline cartilage, being both nourished and lubricated by synovial tissue. The synovial tissue covers all intra-articular structures with the exception of intra-articular cartilage surfaces (Simkin, 2003). The synovial membrane consists of two layers: the lining (or intimal) and the sublining (or subintimal) layer. The lining layer consists of 2 cell levels consisting of 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 limited amounts of mononuclear cells. The synovial tissue and the cartilage are in contact with the synovial fluid (SF), a plasma transudate supplemented with saccharide-rich molecules produced by type B synoviocytes (Maini and Feldmann, 2004).

Table 1: The American College of Rheumatology classification criteria for RA (Arnett et al., 1988)

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously with soft tissue swelling or fluid (not bony overgrowth alone) observed by physician. The 14 possible areas are right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle and metatarsophalangeal (MTP) joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs or MTPs is accepted without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces or in juxtaarticular regions observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the results have been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)
For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite or probable rheumatoid arthritis is not to be made.	

During RA, profound changes happen in the synovial joint. The synovial tissue becomes hyperplastic and the SF may increase in volume and contains excessive amounts of inflammatory cells. Hyperplasia of the synovial tissue regards both the lining and the sublining layer. Lining hyperplasia and formation of an aggressive front at the cartilage-

bone junction is characterized with the term ‘pannus’ and leads to the characteristic bone erosions in RA. In the sublining layer during RA there is massive mononuclear infiltration and blood vessel formation (Maini and Feldmann, 2004). In Figure 1 the differences between the normal (A) and the RA (B) synovial tissue are shown.

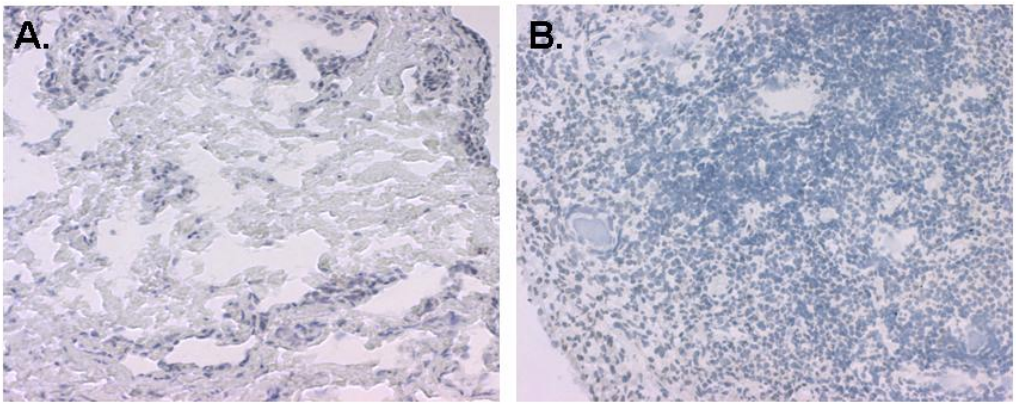

Figure 1. Histological features of normal (A) and RA (B) synovial tissue

Cell populations resident in the inflamed RA joint

The RA synovium is characterized by a variety of cell populations that consist both by migrated and resident cells.

Monocytes/macrophages are abundant both in synovial tissue and SF. These cells express lineage markers such as CD14, CD68 and CD163 and appear to play a pivotal role in RA as they have an activated phenotype with overexpression of major histocompatibility complex class II molecules. Moreover synovial monocytes/macrophages are a principal source of cytokines, chemokines, chemoattractants and metalloproteinases, thus being important mediators of local inflammation and joint damage (Kinne et al., 2000).

T cells are present in SF but also in the synovial tissue, either as scattered cells in the inflamed sublining layer or in lymphoid aggregates. They express the lineage marker CD3 and belong either to the CD8 or CD4 subtype, with the majority being CD4 positive (Cope, 2002). CD4 T cells can be divided to Th1 cells that deal with the activation of

macrophages and Th2 cells that are responsible for B cell activation and antibody based immunity (Coffman, 2006). Recently more subsets of CD4 T cells have been described, Th17 cells having pro-inflammatory properties (Bettelli et al., 2007) and T regulatory cells that mediate immune homeostasis (Tang and Bluestone, 2008). RA synovial T cells exhibit an activated phenotype but are anergic (Cope, 2002).

Fibroblasts in the RA inflamed synovium can be found both in the lining and the sublining layer. Lining layer fibroblasts differ both from normal and sublining RA fibroblasts as they display numerous features of activation that result in an aggressive, invasive behaviour. They also have features similar with transformed cells, are able to secrete large amounts of cytokines, and have invasive properties, being responsible for the invasive character of the RA synovium (Pap et al., 2000).

Dendritic cells in RA synovium have been described as having a mature, differentiated phenotype, expressing high levels of HLA-DR and CD86 and have been observed to associate with T cells in lymphoid aggregate structures. Since dendritic cells are regarded as the professional antigen presenting cells of the immune system they are likely to play a significant role in breaking tolerance of self-reactive lymphocytes and supporting autoimmune responses (Cravens and Lipsky, 2002).

B cells represent a relatively small proportion of RA synovial cells but the capacity of plasma cells to secrete RA associated autoantibodies such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) make them very relevant for disease pathogenesis. Moreover rheumatoid synovitis recapitulates the pathways of lymph node formation and B cells play a key role in this process (Weyand et al., 2005).

Endothelial cells seem also to play an important role in RA as they are activated, express adhesion molecules and present chemokines, leading to leukocyte migration from the blood into the tissue. The endothelium expresses various cytokines, cytokine receptors and proteases that are involved in angiogenesis, proliferation and tissue degradation (Middleton et al., 2004).

Neutrophils are present predominantly in RA SF rather than synovial tissue. They may play an important role in RA by mediating cartilage destruction and generation of destructive oxygen radicals (Liu and Pope, 2004).

The cytokine network in RA

Cells can communicate and interact with each other through either direct cell to cell contact or through cytokines. Cytokines are local protein mediators involved in almost all important biological processes, including cell growth and activation, inflammation, immunity and differentiation. Thus it is not surprising that cytokines have a major role in RA. There is abundance of many cytokines in the rheumatoid synovium as it contains a spectrum of cells such as mainly monocytes/macrophages and fibroblasts but also T cells, plasma cells and endothelial cells all of which can produce cytokines. RA may be envisaged as a disease where there is imbalance between the production of pro-

inflammatory and anti-inflammatory cytokines (Andreakos et al., 2002), (McInnes and Schett, 2007).

FROM GENES AND ENVIRONMENT TO ANTI-CITRULLINE IMMUNITY IN RA

Etiology of RA

The etiology of RA is not known still, but genetic susceptibility and environmental factors are important factors for development of this complex disease. However we should not forget that RA is a heterogenic disease, with heterogeneity both regarding phenotypic expression of disease as well as in the risk factor make-up (Padyukov et al., 2004), (Klareskog et al., 2006), (van der Helm-van Mil et al., 2007a).

In the pathogenesis of RA genetic factors play an important role and account for approximately 60% of the variation in liability to disease (MacGregor et al., 2000). The most important genetic risk factor for RA is found within the HLA system. Research on the relationship between HLA and susceptibility to RA began in 1969, with the observation that lymphocytes from patients with RA were unreactive in mixed lymphocyte culture reactions against cells from other patients with RA (Astorga and Williams, 1969). In 1976 it was proposed that the low responsiveness in RA was based on the sharing of genes within the HLA region (Stastny, 1976) and 2 years later it was shown that HLA-DRw4 is more frequently present in rheumatoid factor positive patients than controls (Stastny, 1978). The presence of HLA-DR expressing antigen presenting cells in synovial tissue and their interactions with T cells was thus proposed as an important feature of RA pathogenesis (Klareskog et al., 1981). Later studies showed that several other HLA-DRB1 alleles were also associated with the disease. The products of these alleles appeared to share an amino acid sequence at position 70-74 in the third hypervariable region of the DRβ1 chain of the HLA-DRB1 molecule (QKRAA, QRRAA or RRRAA). These residues are part of the ridge of the peptide-binding site, therefore it has been postulated that the shared epitope (SE) motif itself is directly involved in the pathogenesis of RA by allowing the presentation of an arthritogenic peptide to T cells (Gregersen et al., 1987).

Besides genes, hormonal influences seem to play an important role in RA. More women than men suffer from RA with a ratio of 2-3 to 1 (Silman, 1994) and up to 75% experience a lower disease activity during pregnancy while a flare is common *post partum* indicating a role for sex hormones in disease (Olsen and Kovacs, 2002). Other disease-inducing triggers that have been suggested are obesity and blood transfusions (Symmons et al., 1997). Several studies have suggested microbial antigens to be triggers for RA development (Takahashi et al., 1998), (Origuchi et al., 1995) but this associations still remain to be clarified.

Environmental factors are also relevant for the development of RA. Occupational exposure to mineral oils is an example (Sverdrup et al., 2005). Moreover, it was already in the 50s evident that in coal workers with pneumoconiosis there is a close association with RA (Caplan, 1959). Later studies showed an association between silica exposure and

RA (Klockars et al., 1987), (Calvert et al., 2003), (Stolt et al., 2005). Asbestos exposure is another risk factor for RA (Olsson et al., 2004).

But the strongest environmental factor associated with RA is smoking (Vessey et al., 1987), (Silman et al., 1996), (Uhlig et al., 1999), (Criswell et al., 2002). Other studies have shown that the intensity and duration of smoking correlates with the risk of developing RA and that the increased risk remains even after some years from smoking cessation (Stolt et al., 2003), (Karlson et al., 1999). Several studies have shown that smoking is a risk factor for RA in rheumatoid factor positive patients (Heliovaara et al., 1993), (Uhlig et al., 1999), (Stolt et al., 2003), (Costenbader et al., 2006) but the most striking finding regarding this was the demonstation of a gene-environment interaction between smoking and the SE for rheumatoid factor positive but not negative RA (Padyukov et al., 2004). Smoking is also a risk factor for extra-articular manifestations of RA (Nyhall-Wahlin et al., 2006). How smoking might be related to pathogenesis of RA is a topic that will be discussed later during the introduction and the results/discussion part.

The post-translational modification citrullination

The time I started my PhD in 2004 the relevance of citrullination in RA was a field that was receiving increasingly more interest. I will talk here about what citrullination is, why it is an important modification in the context of RA and why it is relevant in the context of gene-environment interactions in pathogenesis of RA.

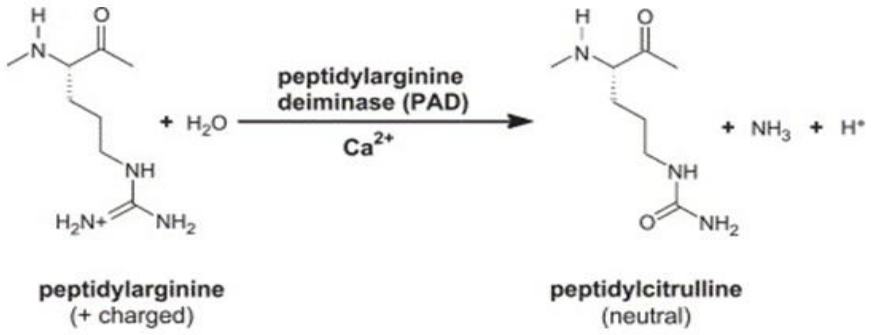

Figure 2: The citrullination modification

Citrullination is a post-translational modification during which the charged peptidylarginine is deiminated to the neutral peptidylcitrulline in an enzymatic process mediated by a series of enzymes known as peptidylarginine deiminases (PADs) (Figure 2) (Nijenhuis et al., 2004). The activity of PAD enzymes is dependent on high

intracellular levels of calcium, higher than the levels existing in living cells. In this context it has beeen demonstrated that certain apoptotic events have to happen in order that intra-cellular proteins get citrullinated (Asaga et al., 1998). Similarly PAD enzymes during cell apoptosis could leak to the extra-cellular space where the demanded calcium levels exist and result in the citrullination of extra-cellular proteins (Nijenhuis et al., 2004).

The precise physiological function of citrullination is not fully understood yet. We know that deimination changes the charge of critical residues of the protein and this leads to changes in intra- and inter-molecular interactions, which could lead to altered protein folding, enhanced degradation by proteases and exposure of cryptic epitopes (Klareskog et al., 2008). Citrullination is a modification occurring in a number of normal and pathological conditions, about which I will talk in later parts of the introduction. Right now I will focus on the relevance of antibodies against citrullinated peptides in the pathogenesis of RA.

Autoantibodies in RA - Relevance to citrullination

The autoimmune nature of RA is reflected by the presence of autoantibodies, among other factors. The classic antibody associated with RA is rheumatoid factor (RF), an antibody that is directed to the Fc portion of IgG (Dorner et al., 2004). Waaler (Waaler, 2007) and Rose (Rose et al., 1948) first described this autoimmune phenomenon in detail as a clue to self-reactivity in patients with RA, while Kunkel first described its antibody nature (Kunkel et al., 1959). This antibody has the disadvantage in being used in diagnosis of RA that it is not uniquely present in RA patients but can also be found in patients with other autoimmune and infectious diseases as well as in healthy (usually elderly) persons (Shmerling and Delbanco, 1991).

On the other hand, anti citrullinated protein antibodies (ACPAs) have received increasing attention lately. ACPAs can be found in approximately 60% of RA patients (Schellekens et al., 2000) and have the obvious advantage in comparison with RF that they are highly specific for RA (rare in other inflammatory conditions and the normal population) (Schellekens et al., 1998), (Schellekens et al., 2000).

Besides their diagnostic applications, clinical and experimental observations provide evidence that ACPAs may be related to important pathophysiologic processes in RA:

1. ACPAs can be found in blood samples of individuals who will develop RA years before onset of disease and increased frequencies as well as higher concentrations of antibodies were observed as the individuals approached onset of RA (Nielen et al., 2004), (Rantapaa-Dahlqvist et al., 2003). Most individuals who will develop ACPA positive RA have already developed their autoantibody status before disease onset (Nielen et al., 2004), (Rantapaa-Dahlqvist et al., 2003).
2. The presence of ACPAs in RA is associated with greater disease activity (Kastbom et al., 2004), (Vallbracht et al., 2004) and with more severe joint destruction (Vallbracht et al., 2004), (Ronnelid et al., 2005) with ACPA positivity at the time of diagnosis being a predictor of more aggressive disease course (Kastbom et al., 2004). Moreover ACPAs are associated with extra-articular manifestations of RA (Turesson et al., 2007).

3. A sustained presence of IgM ACPAs in RA patients can be interpreted as a continuous activation of ACPA reactive B cells within the course of disease (Verpoort et al., 2006). Moreover ACPAs seem to be produced topically in the inflamed joint compartment, the target organ of RA (Vossenaar et al., 2004c).
4. Another line of evidence for the pathogenic role of ACPAs in RA comes from experiments with animal models of RA. Monoclonal antibodies to citrullinated fibrinogen are not able to cause arthritis in naïve animals with no joint lesions, but are able to enhance collagen induced arthritis (Kuhn et al., 2006).

The term ACPAs includes a variety of different antibodies directed against different citrullinated peptides. In common clinical practice measurement for ACPAs is performed with the help of the anti-cyclic citrullinated peptide (anti-CCP) assay which displays a high specificity for RA (Schellekens et al., 2000) but is detecting antibodies against an artificial citrullinated peptide. Several reports have described autoantibodies against natural citrullinated proteins in RA sera (Burkhardt et al., 2005), (Masson-Bessiere et al., 2001), (Sebbag et al., 2006), (Kinloch et al., 2005), (Vossenaar et al., 2004a), (Bang et al., 2007). In following parts of the thesis I will use the term ACPAs except if I need to be more specific.

Genes and ACPAs
The relationship between the strongest genetic determinant of RA, SE, and the most reliable serologic marker for the disease, ACPAs, has been a field of investigations during the last years. SE alleles are associated with ACPA positive but not ACPA negative RA (Huizinga et al., 2005), (Klareskog et al., 2006), in other words these studies refined the role of SE as a risk factor for RA showing that SE confers to a particular phenotype of RA. Another study refined the role of SE even more by showing that SE alleles do not confer risk for RA directly, but contribute to the development of ACPAs (van der Helm-van Mil et al., 2006). So it is ACPAs that explain the association between the SE alleles and RA. Strikingly, in the same study, ACPA(+)-SE(+) patients had higher levels of ACPAs than ACPA(+)-SE(-) patients.

The observation that SE alleles confer a risk only for ACPA positive RA suggests that the etiology of ACPA positive is different than the one of ACPA negative RA. Thus - and with all the differences regarding disease activity and bone erosions - they should be considered as different subsets of the disease. Interestingly, during recent years it has been shown for other genes or gene-gene interactions also that they confer either to only ACPA positive (Kokkonen et al., 2007), (Plenge et al., 2007), (Kallberg et al., 2007b) or to only ACPA negative RA (Verpoort et al., 2005), (Sigurdsson et al., 2007), (Lorentzen et al., 2007). In the same line, environmental risk factors have been found to differ between ACPA positive and negative RA (Klareskog et al., 2006), (Pedersen et al., 2006), (Pedersen et al., 2007).

A gene-environment interaction and ACPAs
As I mentioned before, smoking is the strongest environmental factor linked to RA while HLA-DRB1 SE alleles are a strong genetic predisposing factor for RA. Our group identified a striking gene-environment interaction between smoking and presence of the

HLA-DRB1 SE alleles as a risk factor for only ACPA positive RA (Klareskog et al., 2006). The results from our study where confirmed by two more studies from the Netherlands (van der Helm-van Mil et al., 2007b) and Denmark (Pedersen et al., 2007) while a third study from North America (Lee et al., 2007) obtained less clear results regarding the SE-smoking interaction, thus suggesting that there could be other confounding factors obscuring the associations observed in our study. The relative risk of developing ACPA positive RA was much higher for smokers carrying two copies of the HLA-DRB1 SE alleles than for non-smokers with no SE alleles, from a range of 20 (Klareskog et al., 2006) to 50 (Pedersen et al., 2007) times higher. On the other hand, no increased risk was observed in developing ACPA negative RA. In a more recent study, dose-dependent correlations in this gene-environment interaction were found, showing that heavy smokers carrying 2 SE alleles have 50 times higher relative risk for developing ACPA positive RA than non-smokers SE negative (Kallberg et al., 2007a).

The above findings describe a striking gene-environment interaction associated with an immunologically defined subgroup of RA. These findings are the basis from which molecular and immunological events should be studied in order to elucidate this interaction. In this context, in our study (Klareskog et al., 2006) we showed that smoking is associated with increased presence of citrullinated proteins in bronchoalveolar lavage (BAL) cells of smokers in comparison to non-smokers, but in a very limited number of individuals.

Exactly here comes the need for performing our first study (Paper I). We considered essential to extend our previous limited findings in a new prospective study including larger numbers of individuals but also to introduce a second lung compartment – bronchial mucosa – than just the alveolar compartment (BAL cells) in our study. We wanted also to study more events regarding citrullination, such as expression of the PAD2 and PAD4 enzymes which are the main PAD enzymes associated with RA (Foulquier et al., 2007) but also to study expression of citrullinated proteins with an additional antibody of particular interest in RA(Nicholas et al., 2003), (De Rycke et al., 2005).

THE POTENTIAL TARGETS OF ACPAs: CITRULLINATED PROTEINS

I discussed above the strong pathogenic potentials of ACPAs in RA. But we know as well that citrullinated proteins, which are the candidate targets for those antibodies, are not targeted by ACPAs in every instance in RA patients. A striking example is the presence of citrullinated proteins in the skin (Tsuji et al., 2003) but skin is not a target organ in RA. On the other hand ACPAs are locally produced in the inflamed joint compartment in RA (Vossenaar et al., 2004c) - the target organ of RA - where citrullinated proteins also exist (Baeten et al., 2001).
So let’s take a closer look in the presence of citrullinated proteins in health and disease.

Citrullinated proteins in health

Citrullination is catalyzed by the PAD enzymes of which five different isotypes exist with a wide expression in the human body (Nijenhuis et al., 2004). Citrullination occurs under normal conditions, for example in the epidermis citrullination is of importance for structure, as citrullinated proteins bind to each other forming dense macrofibrils (Senshu et al., 1999), (Mack et al., 1993) but get also cross-linked during the process of reorganization of cornified filaments (Ishida-Yamamoto et al., 2002). Another site of citrullination in the epidermis is the hair follicle, where citrullination results in the formation of rigid structures that facilitate growth of the hair fiber (Steinert et al., 2003). Citrullinated proteins can be also found in the central nervous system of humans, with large expression in infants while their presence in adult brains is decreased and possibly associated with the formation of the myelin sheath (Musse et al., 2006). Citrullination in oocytes has been associated with fertility (Esposito et al., 2007). Citrullination might also play a role in gene regulation as PAD4 catalyzes citrullination of histones (Arita et al., 2006).

Citrullinated proteins in disease - focus on the inflamed synovium

Besides RA, the other disease where the role of citrullination was quite extensively studied is multiple sclerosis. In brains of multiple sclerosis patients there is more expression of citrullinated proteins than in healthy individuals (Wood et al., 1996) which could result in disease pathogenesis through increased susceptibility of the myelin sheath to cleavage and consequent exposure of an immunodominant epitope (D'Souza and Moscarello, 2006). However multiple sclerosis is not associated with the appearance of ACPAs (de Seze et al., 2001). Knocking out PAD2 in animal models of multiple sclerosis resulted in a dramatic reduction in central nervous system citrullination but could not impair development of disease (Raijmakers et al., 2006).

Could the exclusive presence of ACPAs in RA be associated with the existence of citrullinated proteins in RA synovium? The first study that investigated this issue comparing synovial tissue from RA and non-RA arthritides showed the exclusive presence of citrullinated proteins in RA synovial tissue and the co-localization of anti-citrulline and ACPA reactivity in RA synovial tissue (Baeten et al., 2001). This study seemed to explain why ACPAs exist only in RA. The development of an antibody that facilitated the detection of all citrullinated proteins in a tissue (Senshu et al., 1992) set the fore-mentioned explanation in doubt as two later studies showed that the presence of citrullinated proteins is a general characteristic of the inflamed synovium and not an exclusive characteristic of the RA synovium (Vossenaar et al., 2004c), (Chapuy-Regaud et al., 2005).

In the light of these observations we decided to conduct our second study (Paper II). The presence of citrullinated proteins in the inflamed synovium of both RA and non-RA arthritides as well as in inflammation in the central nervous system led as to the assumption that citrullination is a more general phenomenon occurring in the context of inflammation. Thus we decided to study the presence of citrullinated proteins in inflamed muscle, intestinal and tonsil tissue. Moreover we considered essential a comparison of inflamed tissues with non-inflamed controls, as the existence of citrullinated proteins in

many healthy situations as well as the existence of citrullinated proteins both in healthy and inflamed brain points out to the necessity of this comparison. In this context we compared RA with healthy synovial tissue, inflamed (polymyositis) with non-inflamed muscle tissue and intestinal inflammatory bowel disease tissue (both Crohn's and Ulcerative Colitis) from macroscopically inflamed and non-inflamed areas.

TRYING TO SUPPRESS RA INFLAMMATION: RA TREATMENT

Pathogenesis and treatment in RA are two terms closely related to each other. On one hand exploring pathogenic mechanisms of the disease is the starting point from where development of new effective drugs can be achieved. On the other hand exploring the molecular mechanisms of action of a drug in a disease can bring new knowledge regarding pathogenic mechanisms and consequently result in development of new even more effective drugs. Moreover knowledge of the molecular mechanisms of action of drugs can result in the establishment of effective combination therapies in order that more than one important pathogenic mechanisms of the disease are modified. What is nowadays generally accepted is the importance of an aggressive treatment early during disease course in order to prevent joint damage and control disease activity (Breedveld and Kalden, 2004).

Pharmacological treatment of RA can be divided to the following categories:

NSAIDs

Non steroidal anti-inflammatory drugs (NSAIDs) have been used extensively for the treatment of RA owing to their quick onset of analgesic effects and mild anti-inflammatory properties. Despite the potential association with various side effects, they still remain as one of the most widely prescribed classes of medication worldwide. Their prompt clinical response lasts for only as long as the drug is taken. They do not alter the disease course or prevent joint destruction. Their action is based on inhibition of the cyclooxygenase enzyme thus inducing a decrease in prostaglandin synthesis (Lee and Kavanaugh, 2003).

DMARDs

Disease modifying anti-rheumatic drugs (DMARDs) are defined as medications that retard or halt the progression of disease (O'Dell, 2004). DMARDs have the potential to reduce or prevent joint damage and preserve joint integrity and function. The DMARDs commonly used in RA include methotrexate, sulfasalazine, leflunomide and hydroxychloroquine (2002). Methotrexate is the most frequently used DMARD. Its precise mechanism of action in RA is unclear, but it seems that it prevents purine and pyrimidine synthesis and inhibits cellular proliferation of lymphocytes. Methotrexate is able to increase apoptosis, modify cytokine levels, cellular adhesion and to indirectly inhibit osteoclast formation (Wessels et al., 2008). Sulfasalazine is an alternative option (Svartz, 1948), (McConkey et al., 1980) able to modulate immune responses and angiogenesis (Lee and Kavanaugh, 2003), (Trollmo et al., 2007). Hydroxychloroquine is

an antimalarial drug and is used mainly for mild RA, able to modulate macrophage function and cytokine secretion (Lee and Kavanaugh, 2003). Leflunomide is able to inhibit synthesis of pyrimidine nucleotides in immune cells and to reduce TNF and IL-1 (Alldred and Emery, 2001).

Biologicals

The term biologicals regards pharmacological agents that are similar to endogenous molecules that occur naturally in the body, such as antibodies and soluble receptors (Andreakos et al., 2002). Anti-TNF treatment is used in RA with high efficacy. Three TNF blocking agents are available in clinical practice: infliximab (a chimeric monoclonal antibody) (Elliott et al., 1994), (Lipsky et al., 2000), etanercept (a recombinant TNF receptor - Fc fusion protein) (Weinblatt et al., 1999), (Klareskog et al., 2004) and adalimumab (a fully human anti-TNF antibody) (den Broeder et al., 2002), (van de Putte et al., 2003). Modes of action for anti-TNF treatment include inhibition of the inflammatory 'cytokine cascade' mediated by TNF, complement mediated lysis of TNF expressing cells and effects in neovascularization, endothelial activation and leukocyte recruitment (Maini and Feldmann, 2002), (Choo-Kang et al., 2005). A recombinant IL-1 receptor antagonist (Bresnihan et al., 1998) and tocilizumab, an anti-IL6 receptor antibody (Smolen et al., 2008) have also shown efficacy in RA treatment. For refractory RA cases non-responsive to anti-TNF treatment, Rituximab, a monoclonal anti-CD20 antibody able to deplete B cells by antibody dependent cell mediated cytotoxicity (Edwards et al., 2004), (Emery et al., 2006) and Abatacept, a CTLA4-Ig fusion protein able to block T cell activation (Kremer et al., 2003), (Genovese et al., 2005) are used with good results.

Glucocorticoids

Glucocorticoids are a highly effective treatment for RA (Hench et al., 1949), a treatment that in 1950 was awarded with the Nobel Prize, the only rheumatology associated treatment ever to achieve that. Glucocorticoids are a very popular anti-rheumatic therapy, around 60% of patients with RA are treated more or less continuously with glucocorticoids (Buttgereit et al., 2004). Glucocorticoids are effective in reducing disease activity parameters already in low doses (less or equal to 7.5 mg oral prednisolone) and associated with few side effects in these low doses (Kirwan, 1995), (Svensson et al., 2005). Combination therapy with high-dose (60 mg oral prednisolone) step-down glucocorticoids, sulfasalazine and methotrexate was superior to sulfasalazine monotherapy, but this effect was lost on withdrawal of prednisolone, suggesting the importance of glucocorticoids in response (Boers et al., 1997).

The effects of glucocorticoids are considered to be mediated by two different molecular mechanisms of action: 1. the classical genomic mechanism of action caused by the cytosolic glucocorticoid receptor (cGCR) and 2. non-genomic effects that can be divided to: a. non-genomic effects initiated by the cGCR, b. membrane-bound glucocorticoid receptor (mGCR) mediated non-genomic effects and c. non-specific, non-genomic effects caused by interactions with cellular membranes. The classical genomic mechanisms can be divided into two processes: a. Transrepression, which is responsible for a large number of desirable anti-inflammatory and immunomodulating effects and b.

Transactivation, which is associated with frequently occurring side effects as well as with immunosuppressive activities (Stahn et al., 2007). Non-genomic actions are rapid and are seen usually in high doses of glucocorticoids while genomic ones are quite slower and are evident in low doses as well (Buttgereit et al., 2004). The effectiveness of glucocorticoids in rheumatoid arthritis is based on a variety of effects on immune cells (Buttgereit et al., 2005), the most important of them are shown in Table 3.

Table 3: Effects of glucocorticoids on immune cells (Buttgereit et al., 2005)	
Monocytes/macrophages	- ↓ number of circulating cells (↓ myelopoiesis, ↓ release) - ↓ expression of MHC class II molecules and Fc receptors - ↓ synthesis of pro-inflammatory cytokines (IL-2, IL-6, TNF and others) and prostaglandins
Fibroblasts	- ↓ proliferation - ↓ production of IL-1 and prostaglandins
T cells	- ↓ number of circulating cells - ↓ production and action of IL-2
Granulocytes	- ↓ number of eosinophile and basophile granulocytes - ↑ number of circulating neutrophils
Endothelial cells	- ↓ vessel permeability - ↓ expression of adhesion molecules - ↓ production of IL-1 and prostaglandins

The first randomized controlled trials for glucocorticoid use in RA not only regarding clinical symptoms , but also regarding joint erosion, have been contradictory, with some studies showing a superior effect of cortisone than aspirin (1959), (1960), while other studies did not show any difference (1957), thus opening a still valid debate regarding the efficacy of glucocorticoids on joint destruction. More recent data suggests again the protective effect of glucocorticoids on bone erosions in RA (Kirwan, 1995), (Svensson et al., 2005).

Potential side effects of glucocorticoids have raised concerns regarding their clinical use. Glucococorticoids may have a variety of side effects from different organs, less or more frequent, serious or not, reversible or not, whose occurrence also depends on the dose of glucocorticoids used (Boers, 2004). But from all side effects attributed to glucocorticoids, the one that causes most concerns is osteoporosis (Reid, 1997).

Intra-articular glucocorticoids

Even from the first reports on the effects of glucocorticoids in patients with RA it was obvious that side effects were going to be a limiting factor in their use. In order to avoid the side effects of systemic glucocorticoids, intra-articular glucocorticoids started getting tested in RA patients, with a prompt alleviating effect in a RA patient being reported

already in 1951 (Hollander et al., 1951). Today intra-articular glucocorticoids represent a successful adjuvant therapy for RA (Rozental and Sculco, 2000).

It is generally accepted that the controversy regarding use of glucocorticoids in RA is a result of our lack of the necessary studies (Boers, 2004). The lack of appropriate studies regarding intra-articular glucocorticoids is even bigger, with most of the few studies investigating molecular mechanisms of action of intra-articular glucocorticoids in RA being performed during the time of this PhD (Wittkowski et al., 2007), (Weitoft et al., 2005), (Korotkova et al., 2005), (Magnusson et al., 2007), (Eklow et al., 2008), (af Klint et al., 2005).

This scarcity of characterization of molecular mechanisms of action of intra-articular glucocorticoids was the basis for Paper III and Paper IV.

INTRA-ARTICULAR GLUCOCORTICOIDS AND THE LINK BETWEEN REDUCTION OF INFLAMMATION AND EFFECTS ON BONE METABOLISM: ROLE OF THE RANKL/OPG SYSTEM

The skeletal complications of RA include focal bone erosions and juxta-articular osteopenia at sites of active inflammation as well as systemic osteopenia, resulting in significant joint deformities and disability in addition to an increased risk for bone fractures (Gough et al., 1994), (Goldring and Gravallese, 2000). Osteoclasts, multinucleated cells of the monocyte/macrophage lineage are the key mediators of bone loss in RA (Hofbauer and Heufelder, 2001). The link between synovial inflammation and bone absorption in RA (Conaghan et al., 2003) is the RANKL/OPG system (Gravallese et al., 2000), (Haynes et al., 2001), (Schett, 2007).

This system consists of: 1. a cytokine, receptor activator of nuclear factor κB ligand (RANKL) (also called osteoprotegerin ligand – OPGL, osteoclast differentiation factor – ODF or tumor necrosis factor related activation induced cytokine – TRANCE), which is a member of the TNF ligand superfamily and which exists in a membrane-bound and a soluble form, 2. the transmembrane receptor RANK and 3. the decoy receptor osteoprotegerin (OPG) (Hofbauer and Heufelder, 2001).

RANKL is an important mediator in both bone metabolism and immunological processes in the synovium/bone area. RANKL is produced by osteoblastic lineage cells (Nakamichi et al., 2007), activated T cells (Kong et al., 1999a) and fibroblasts (Kotake et al., 2001) and interacts with RANK receptor which can be found in osteoclast precursors in order to promote osteoclastogenesis and osteoclast survival (Kotake et al., 2001), (Fuller et al., 1998), in monocytes in order to promote their effector function (Seshasayee et al., 2004) and their chemotaxis (Mosheimer et al., 2004) and in dendritic cells in order to promote their adjuvant properties during antigen presentation but also survival while they enhance T cell activation (Josien et al., 2000), (Anderson et al., 1997). Proinflammatory cytokines such as TNF and IL-1 are able to upregulate RANKL expression (Hofbauer et al.,

1999b). OPG is secreted by endothelial cells (Collin-Osdoby et al., 2001), B cells (Li et al., 2007), dendritic cells (Schoppet et al., 2007) and osteoblasts (Hofbauer et al., 1999a).

Lessons on the importance of the RANKL/OPG system in bone metabolism but also immunological processes come from the field of knock-out animals as well. RANKL knock-out mice exhibit on one hand osteopetrosis and on the other hand lymph node agenesia, thymus hypoplasia and defects in the development of B and T cells (Kong et al., 1999b). OPG knock-out mice develop early onset osteoporosis (Bucay et al., 1998) and defects in B cell development (Yun et al., 2001).

An increase in the RANKL/OPG ratio has been associated with increased bone resorption both in *in vitro* (Hofbauer et al., 1999a) and clinical studies (Geusens et al., 2006). Immunohistochemical studies at the synovial tissue level have identified the expression pattern for both RANKL and OPG in RA. RANKL is present mainly in lymphoid aggregate areas and is associated with active synovitis (Crotti et al., 2002) while OPG is mainly expressed by endothelial cells and is associated with inactive synovitis (Haynes et al., 2003), findings that could also suggest that active synovitis is associated with a high RANKL/OPG ratio.

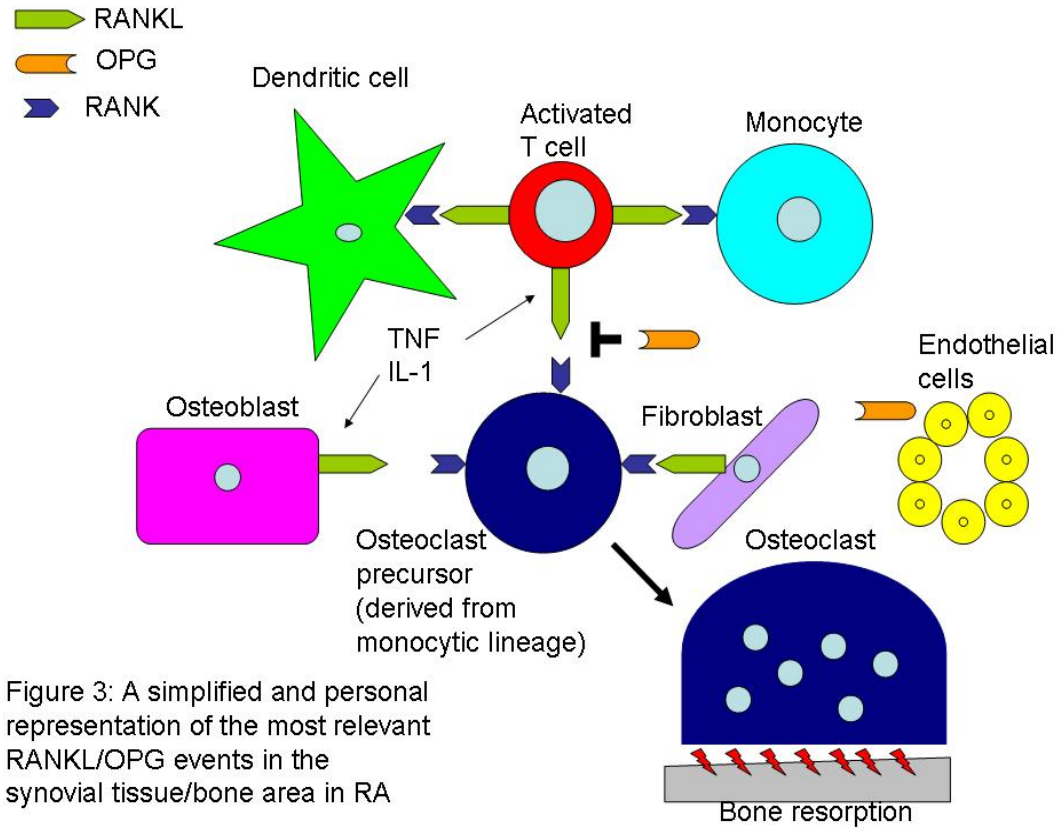

Figure 3: A simplified and personal representation of the most relevant RANKL/OPG events in the synovial tissue/bone area in RA

Alterations of the RANKL/OPG system are associated both with the bone protective effect of anti-rheumatic treatment and with anti-osteoporotic treatment. Anti-TNF treatment is retarding radiographic progression in RA (Lipsky et al., 2000), which can be explained by an increase in the synovial expression of OPG (Catrina et al., 2006). RANKL inhibition with the monoclonal antibody denosumab is a new effective therapy in the treatment of osteoporosis (McClung et al., 2006).

A schematic representation of the most relevant interactions regarding the RANKL/OPG system in the RA synovial tissue and pannus/bone area are shown in Figure 3.

We decided to perform our third study (paper III) as very limited data on the effects of intra-articular glucocorticoids on joint erosions existed. Thus the effects of intra-articular glucocorticoids on the RANKL/OPG system at the synovial tissue level were studied.

SYNOVIAL INFLAMMATION AND APOPTOSIS – FOCUS ON T CELLS

What is apoptosis?

Apoptosis, a word derived from the Greek language, is composed of two words, apo- (from) and –ptosis (falling), which could be translated in English as 'falling from somewhere'. Apoptosis is a type of programmed cell death that does not induce bystander cell death, inflammation or tissue scaring. In mammalian cells two major apoptotic pathways exist, which have as a common link the activation of caspases. The death-receptor apoptotic pathway is triggered by members of the death receptor superfamily, such as CD95 (Fas) and TNF receptor I. Binding of the ligand to the death receptor results in caspase-8 activation with the help of the intracellular adaptor molecule FADD, while this activation can be blocked by FLIP. The mitochondrial apoptotic pathway is used extensively in response to extracellular cues and internal insults such as DNA damage. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of the mitochondira where they compete for release of cytochrome c. If the pro-apoptotic members win, cytochrome c is released and associates with Apaf-1 and then procaspase-9 to form the apoptosome. Minimal cross-talk between the death receptor and the mitochondrial pathway takes place through the pro-apoptotic Bcl-2 family member Bid. The death receptor pathway through caspase-8 and the mitochondrial pathway through the apoptosome converge at the level of caspase-3 activation. Caspase-3 activation is antagonized by the IAP proteins, which are antagonized by the mitochondrial protein Smac/DIABLO. Caspase-3 activation leads to apoptotic cell death (Hengartner, 2000). An apoptotic cell has certain characteristics such as cell shrinkage, membrane blebbing, DNA fragmentation, nuclear condensation and formation of apoptotic bodies (Elmore, 2007).
A schematic representation of the apoptotic pathways is shown in figure 4 (adapted from (Hengartner, 2000)).

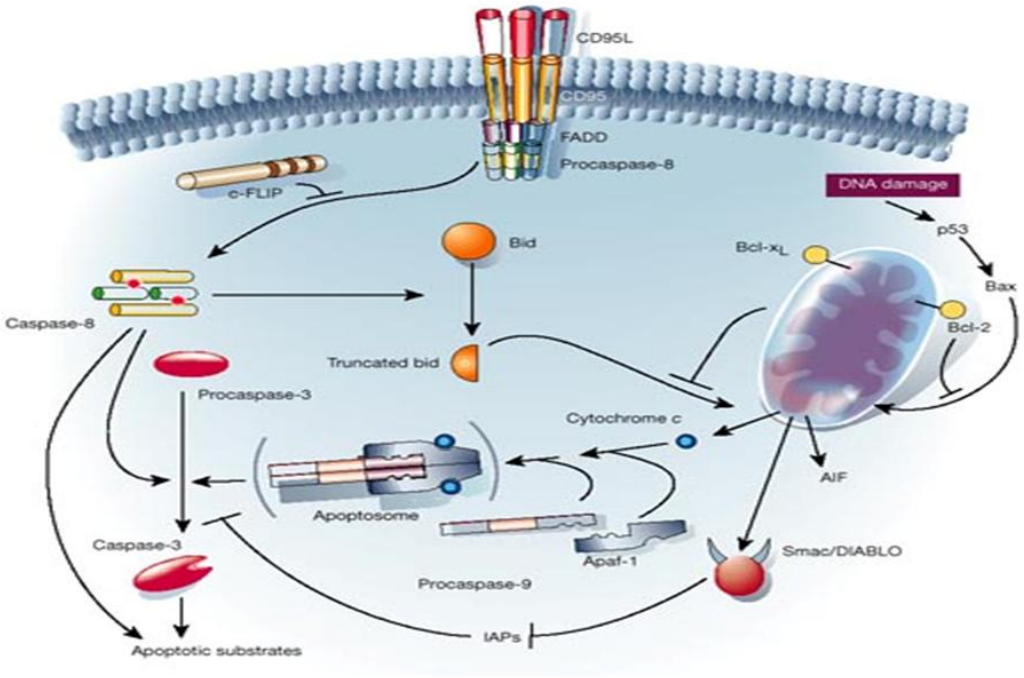

Figure 4: The two major apoptotic pathways [adapted from (Hengartner, 2000)]

Apoptosis in RA synovium

The excessive synovial infiltration and proliferation of mononuclear cells causing hyperplasia which characterizes RA is partly due to a defective apoptotic process (Liu and Pope, 2003). Studies using the TUNEL technique, which labels DNA fragments (Ceponis et al., 1999) and electron microscopy which identifies the apoptotic morphology (Matsumoto et al., 1996) showed low apoptotic levels in RA synovial tissue, between 1-3% of cells. Resistance to apoptosis at the synovial level in RA could be a result of different parameters.

Numerous investigations have focused on the death receptor pathway in RA. The intriguing fact is that in the RA synovium there is both expression of Fas and Fas-ligand (Asahara et al., 1996), so the molecules active in death receptor pathway mediated apoptosis are present in RA. One explanation for the reduced apoptosis could be the existence of the anti-apoptotic forms soluble Fas and soluble Fas-ligand in the rheumatoid joints (Hasunuma et al., 1997), (Hashimoto et al., 1998). High expression of FLIP could be one more reason for deficiencies in the death receptor pathway in RA (Perlman et al., 2001), as evidenced by the association of high FLIP expression with low levels of apoptosis in the rheumatoid synovium (Catrina et al., 2002). NF-κB is a transcription factor blocking Fas mediated apoptosis (Yamasaki et al., 2001) and it is found expressed in high levels in RA synovium (Handel et al., 1995). Another molecule that has been shown to inhibit Fas mediated apoptosis is sentrin, which is also up-regulated in RA synovium (Franz et al., 2000).

There is also evidence for a role of the mitochondrial pathway in apoptosis resistance in RA. For example, enhanced expression of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL but not pro-apoptotic members has been shown in the rheumatoid synovium (Sugiyama et al., 1996), (Busteed et al., 2006).

The importance of apoptosis in the pathogenesis of RA is underlined by the fact that effective therapeutic agents have been found to work partly through modifying cell apoptosis (Catrina et al., 2005), (Pope, 2002).

T cells in the rheumatoid synovium: status and apoptosis

T cells in the chronically inflamed synovial environment of RA have certain characteristics. They express a cell-surface phenotype that is suggestive of chronic immune activation, as CD45RO and CD69 (Thomas et al., 1992) but on the other hand they show signs of anergy as they are hyporesponsive to TCR ligation and their cytokine production is suppressed, due to the chronic effects of TNF in the rheumatoid joint environment (Cope, 2002). Another main characteristic of synovial RA T cells is that they are apoptosis resistant (Firestein et al., 1995), (Salmon et al., 1997). It has been suggested that factors such as chronic exposure to TNF (Cope, 2002), exposure to IL2 receptor γ chain signaling cytokines as well as inhibitory signals received through interaction with stromal cells (Salmon et al., 1997) might result in the apoptosis resistant phenotype of T cell in the rheumatoid synovium. This phenotype has been associated with overexpression of two pro-apoptotic molecules of the mitochondrial pathway, Bcl-2 and Bcl-xL (Salmon et al., 1997), (Firestein et al., 1995).

The idea for performing our fourth study (Paper IV) came when in a previous work of our group (af Klint et al., 2005) we noticed that intra-articular glucocorticoids are down-regulating T cell numbers in inflammatory arthritis. Although this study suggested that this down-regulation might be the consequence of reduced inflammatory cell trafficking to the joint, we hypothesized that apoptosis induction could be an additional mechanism. Glucocorticoids induce apoptosis of human thymocytes and activated human peripheral blood T cells *in vitro* (Kirsch et al., 1999), (Lanza et al., 1996).

AIMS

General aim

The general aim of the present thesis was to study the relevance of the post-translational modification citrullination in RA pathogenesis and inflammation and the molecular mechanisms of action of intra-articular glucocorticoids in reducing RA inflammation.

Specific aims

- To describe the presence of citrullinated proteins and PAD enzymes in lungs of smokers and non-smokers

- To describe the presence of citrullinated proteins in inflamed tissues from various diseases and non-inflamed tissues

- To study the effect of intra-articular glucocorticoids on the RANKL/OPG system in inflammatory arthritis

- To study the effect of intra-articular glucocorticoids on RA synovial T cell apoptosis

RESULTS AND DISCUSSION

PAPER I – The influence of smoking on citrullination in human lungs

As mentioned in the introduction, the aim for Paper I was to extend our previous limited findings regarding the influence of smoking in protein citrullination in human lungs (Klareskog et al., 2006) in a new prospective study including larger numbers of individuals but also to introduce a second lung compartment – bronchial mucosa – than just the alveolar compartment (BAL cells). We wanted also to study more events regarding citrullination, such as expression of the PAD2 and PAD4 enzymes which are the main PAD enzymes associated with RA (Foulquier et al., 2007) but also to study expression of citrullinated proteins with an additional antibody of particular interest for RA (Nicholas et al., 2003), (De Rycke et al., 2005). We used a cohort of healthy smokers and a cohort of healthy non-smokers where we obtained BAL cells and bronchial mucosal biopsies through bronchoscopy.

Regarding BAL cells, using the same antibody (anti modified citrulline - AMC) (Senshu et al., 1992) as in our previous study (Klareskog et al., 2006) we found a significant difference in the expression of citrullinated proteins: 56% of smokers vs 7% of non-smokers showed expression of citrullinated proteins in BAL cells while with the other antibody (F95) (De Rycke et al., 2005) there was again higher expression in smokers but it did not reach statistical significance. The higher expression of citrullinated proteins in smokers was paralleled by higher expression of PAD2 while no difference regarding expression of PAD4 was observed. Around 90% of BAL cells were macrophages.

Regarding bronchial mucosal biopsies, we could unfortunately not use the AMC antibody while with the help of the F95 antibody we found that 100% of smokers but also 85% of non-smokers were positive for citrullinated proteins, with similar amounts and distribution. On the other hand, the observed higher expression of PAD2 in BAL cells was observed in the biopsies as well. Similarly with BAL cells, no difference regarding expression of PAD4 was observed.

This study confirms the results of our previous study regarding expression of citrullinated proteins in the alveolar compartment (BAL cells) of smokers vs non-smokers. Moreover we demonstrated for the first time that smoking is associated with higher expression of the PAD2 enzyme in two different lung compartments, alveolar (distal airways) and bronchial mucosal (proximal airways).

In the alveolar compartment, higher expression of the PAD2 itself seems not to be enough as the citrullination reaction requires also intracellular calcium levels that can only exist in apoptotic cells in order that PAD enzymes get activated (Asaga et al., 1998). Smoking induces alveolar macrophage apoptosis (Aoshiba et al., 2001), a finding that provides a link between smoking, higher PAD2 expression and induction of citrullination. An alternative/complementary explanation might be that smoking induces sub-clinical inflammation in healthy smokers (Linden et al., 1993), that might lead to increased citrullination, as I will talk about in Paper II.

In bronchial mucosal biopsies, we observed similarly to the alveolar compartment higher expression of PAD2. The higher expression of PAD2 but not PAD4 in both lung compartments could be related to the different maturation phase of alveolar and mucosal macrophages induced by smoking (Skold et al., 1996), (Vossenaar et al., 2004b). However, we detected citrullinated proteins in biopsies of both smokers and non-smokers, which could be explained either from the presence of citrullinated proteins in epithelial structures (Tsuji et al., 2003), (Brouwer et al., 2006) or from the fact that lung mucosa represents a natural barrier of the human body and thus a constant ongoing degree of sub-clinical inflammation which could lead to citrullination (Paper II) might be present. This basal constitutional level of citrullination in both healthy smokers and non-smokers might preclude identification of an additive effect of smoking through higher expression of PAD2 enzyme. A recent study (Bongartz et al., 2007) investigated with the help of the AMC antibody the presence of citrullinated proteins in lung tissues from RA-associated lung disease, idiopathic interstitial lung disease and control lung tissue from patients with lung cancer. They did not find any association for smoking and presence of citrullinated proteins, a finding which agrees with our finding in lung tissue biopsies.

The alveolar (BAL cells) and the bronchial mucosal compartment represent two different compartments of human lungs, distal and proximal airways. Smoking induced citrullination in BAL cells takes place in an immunologically relevant compartment (Reynolds, 2000). In a genetically susceptible smoker citrullination of proteins in alveolar macrophages could result in enhanced presentation of the citrullinated proteins to T cells, as a result of the higher affinity of citrullinated proteins with the SE (Hill et al., 2003) but also of the effect of smoking in the antigen presentation capacities of BAL cells (Bratke et al., 2008). Activated T cells could migrate from the bronchoalveolar space to regional lymph nodes (Pabst and Binns, 1995) and there interact with B cells for the production of ACPAs. The fact that IgA ACPA immunity is seen early during the development of the anticitrulline immune response (Verpoort et al., 2006) indicates that immunity triggered from mucosal surfaces such as the lung maybe be of great relevance for RA ACPA immunity. A finding that is even more supported by the presence of ACPAs in patients with interstitial lung disease and no clinical evidence of connective tissue disease (Gizinski et al., 2006), while most of these individuals were smokers.

In conclusion, we identified a specific effect of smoking regarding expression of citrullinated proteins in the alveolar compartment which supports our previously proposed hypothesis for ACPA positive RA pathogenesis that involves genes, environment and immunity to post-translationally modified molecules (Klareskog et al., 2006). I will provide a more analytical hypothesis for the pathogenesis of ACPA positive RA after discussing Paper II.

Technical comments for Paper I

Currently in the field of citrullination there is some confusion regarding which antibodies are the best to use for detection of citrullinated proteins in tissues. The AMC antibody is an antibody recognizing citrullinated proteins that have been previously chemically modified (Senshu et al., 1992). It is an antibody that theoretically recognizes every

citrullinated protein in a tissue independently of the back-bone protein. On the other side there are antibodies proposed to show specificity for detection of citrullinated proteins in the RA synovium, such as the anti-L citrulline antibody (Baeten et al., 2001), although this is a point of debate (Smeets et al., 2002), and the F95 antibody (De Rycke et al., 2005). Moreover there are reports suggesting that the AMC has a more restricted recognition pattern. For example, in normal adult rat brains deiminated myelin basic protein and glial fibrillary acidic protein is recognized by the F95 (Nicholas et al., 2003) but not by the AMC (Asaga and Ishigami, 2001). Some other antibodies have been used for detection of citrullinated proteins (Vossenaar et al., 2004c) but we do not have information regarding their citrullinated target epitopes. In this context, we used both the AMC (broad spectrum antibody) and the F95 antibody (specific antibody) for detection of citrullinated proteins in BAL cells. Both antibodies demonstrated almost exclusive expression of citrullinated proteins in smokers as compared to non-smokers. However no perfect match was observed between the two stainings regarding sample identity, suggesting that the two antibodies recognize different citrullinated epitopes. Additional studies that will identify the exact epitopes that each antibody is recognizing should be performed, and production of antibodies against specific citrullinated epitopes would add a lot in the field of immunohistochemical detection of citrullinated epitopes in tissues and in a better understanding of the significance of those epitopes in disease pathogenesis.

Despite our success in using the AMC antibody in a variety of tissues (synovium, muscle, intestine, tonsil) in Paper II and in using it for BAL cells in this paper, when using it for lung biopsies unspecific staining in the control made evaluation of results impossible despite our efforts. The issue of non-specific staining in certain types of tissues with certain types of antibodies is a known and accepted milestone in immunohistochemistry. The problem might be related either to the stained tissue or the antibody used.

PAPER II – The association of citrullination with inflammation

The aim of Paper II was to investigate the presence of citrullinated proteins in a variety of inflammatory tissues while comparing with their presence in non-inflamed control tissues. We investigated the presence of citrullinated proteins with the AMC antibody in synovial tissue from RA in comparison to healthy synovial tissue, in muscle tissue from polymyositis in comparison to muscle tissue from individuals with normal muscle histopathology and in intestinal tissue from inflammatory bowel disease (both ulcerative colitis and Crohn's disease) where we compared the presence of citrullinated proteins in macroscopically inflamed and non-inflamed areas. We also stained for citrullinated proteins in chronically inflamed tonsil tissues.

We found presence of citrullinated proteins in all RA synovial tissues while in only 30% of healthy synovial tissues and in significantly lower amounts than in RA. The difference in presence of citrullinated proteins was even more pronounced in muscle tissue, with presence in all polymyositis tissues but in none of the control tissues. For inflammatory bowel disease, although citrullinated proteins were present in 70% of macroscopically inflamed samples vs 40% of non-inflamed samples, this difference was not statistically significant, while there was also no significance regarding amounts of expression of citrullinated proteins. Finally all inflamed tonsil tissues were positive for the presence of

citrullinated proteins. We studied as well presence of the PAD2 enzyme in intestinal and tonsil biopsies and we found that its presence followed the same distribution pattern as that of citrullinated proteins (Figure 5).

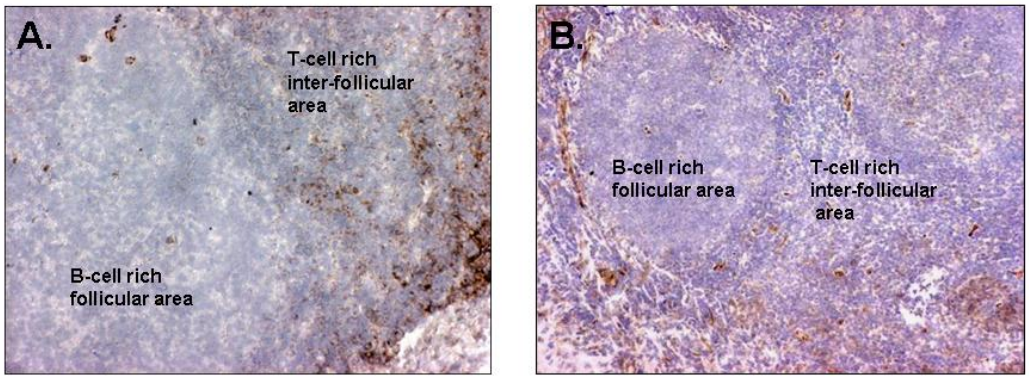

Figure 5. Presence of citrullinated proteins (A) and PAD2 enzyme (B) in inflamed tonsil

We demonstrate that citrullination is an inflammation associated phenomenon, by demonstrating the presence of citrullination in several inflammatory tissues, with little to no expression in control non-inflammatory tissues. The difference in presence of citrullinated proteins was obvious for synovial and muscle tissue, while the results for intestinal tissue are explained by the presence of microscopical inflammation we found in macroscopically non-inflamed, in accordance with previous observations (Floren et al., 1987), or by a constant sub-clinical inflammatory process in intestinal tissue, being a natural barrier of the human body. A recently published study (Neeli et al., 2008) provides further confirmation for our study, showing that inflammatory stimuli are able to induce citrullination of proteins.

An interesting finding of our study comes from the comparison of RA with healthy synovial tissue. Even if in much lower amounts, we identified presence of citrullinated proteins in 30% of healthy synovial tissues. This could be related to the sub-clinical inflammation in bearing joints (all healthy synovial biopsies were obtained from the knee joint) of healthy individuals, as a result of events such as minor injury and physical exercise. This finding is helpful in constructing a hypothesis regarding pathogenesis of ACPA positive RA, about which I will talk in the next part of the discussion.

The obvious question in the context of this and other publications that support the notion that citrullination is an inflammation associated phenomenon, is what is the importance of the presence of citrullinated proteins in the context of the development of ACPA positive RA. Citrullination of proteins in general is not enough for development of ACPA and consequently RA. So other factors seem to be of importance:

1. The presence of RA specific citrullinated epitopes, as has been suggested in two studies (Baeten et al., 2001), (De Rycke et al., 2005). Especially in the second study (De Rycke et al., 2005), presence of specific intracellular citrullinated proteins correlates with serum ACPA levels and determines the production of ACPAs in a SE restricted manner. Identification of the nature of those specific cittrullinated proteins seems essential.
2. The presence of ACPA antibodies against natural citrullinated proteins which are highly specific for RA such as antibodies to citrullinated Epstein-Barr virus nuclear antigen 1 (Pratesi et al., 2006), citrullinated a-enolase (Kinloch et al., 2005), citrullinated eukaryotic translation initiation factor 4G1 (Okazaki et al., 2006), citrullinated collagen type II (Burkhardt et al., 2005) might lead to the hypothesis that their citrullinated targets are also exclusive for the rheumatoid synovium. However, the examples of citrullinated fibrinogen and citrullinated vimentin are discouraging, as anti-citrullinated fibrinogen and anti-citrullinated vimentin antibodies are also highly specific for RA (Vander Cruyssen et al., 2006), (Vossenaar et al., 2004a), but citrullinated fibrin and citrullinated vimentin exist in non-RA synovial tissue as well (Chapuy-Regaud et al., 2005), (Tilleman et al., 2008). Citrullinated collagen cartilage expression seems also not to be RA specific (Omri Snir, personal communication).
3. B cell tolerance in patients with rheumatoid arthritis has been found to be impaired, which favors development of antibodies reactive to cyclic citrullinated peptide (Samuels et al., 2005).
4. Genetic predisposition for RA in terms of SE carriage with/without environmental factors such as smoking is the major determinant of the production of ACPA in RA, as already mentioned in the introduction part of this thesis. In this context, conversion of arginine to citrulline at the peptide side-chain position of vimentin interacting with the SE increased the peptide-MHC affinity (Hill et al., 2003) while citrullinated fibrinogen induced arthritis in DR4-IE transgenic mice and antibody reactivity against citrullinated fibrinogen but also other citrullinated proteins (Hill et al., 2008), findings that could provide the link between genes, citrullination, ACPA production and arthritis. This might also explain why in RA antibodies against natural citrullinated proteins such as citrullinated vimentin and fibrinogen exist, while the presence of those citrullinated proteins is not RA specific.

Hypothesis based on findings from paper I and II

Based on the data from papers I and II as well as on existing knowledge regarding the relevance of citrullination in RA, I would like to propose the following hypothetical model regarding pathogenesis of ACPA positive RA, which of course is not able to explain the pathogenesis of every case of ACPA positive RA:

Stage 1: Smoking results in higher expression of the PAD2 enzyme in the alveolar lung compartment and in higher apoptosis of BAL cells, possibly resulting in higher calcium influx and activation of PAD2, with subsequent citrullination of proteins in BAL cells. In

a genetically predisposed individual this will result in enhanced antigen presentation and activation of autoreactive T cells which could migrate to regional lymph nodes and stimulate B cells for the production of ACPAs. Thus in this hypothetic model the production of ACPAs is initiated outside the synovial compartment.
Stage 2: A second joint specific event, such as infection, trauma or excessive stress could lead to clinical or sub-clinical inflammation in the joint, influx of inflammatory cells and activation of PAD enzymes which results in citrullination of proteins. Citrullination of RA-specific autoantigens attracts ACPAs from the circulation, so they enter into the joint, bind to the specific citrullinated proteins and form immune complexes. On the other side, non-specific citrullination would result in the resolution of the synovial clinical or sub-clinical inflammation without further development to RA.
Stage 3: ACPA-citrullinated proteins immune complexes through binding to Fc receptors or the complement could result in the production of pro-inflammatory cytokines from inflammatory cells (Clavel et al., 2008) or further stimulation of antigen presenting cells, which in turn could result in further stimulation of T and B cells and shift the main region of ACPA production from the periphery to the synovial compartment. A vicious cycle with continuous production of ACPAs, pro-inflammatory cytokines, inflammatory cell recruitment into the joint, further PAD activation and further citrullination could result in the establishment and perpetuation of RA. In that context, the presence of various antibodies against citrullinated proteins exhibiting minimal cross-reactivity (Snir et al., 2007) in ACPA positive RA can be the result of an epitope spreading procedure (Vanderlugt and Miller, 2002).

PAPER III – The effect of intra-articular glucocorticoids on synovial inflammation and bone metabolism

The aim of Paper III was, in the context of the debate regarding bone metabolism effects of glucocorticoids, to investigate if intra-articular glucocorticoids could modulate synovial expression of the RANKL/OPG system in parallel with their anti-inflammatory effects at the synovial level.

Intra-articular glucocorticoids suppressed synovial inflammation, as macroscopically evaluated both by physician's assessment and imaging of the joint during arthroscopy. At the synovial tissue level, intra-articular glucocorticoids decreased T cells but not macrophages, while decreasing RANKL but not OPG levels, thus resulting in a significant down-regulation of the RANKL/OPG ratio after therapy. *In vitro*, glucocorticoids decreased surface expression of RA synovial fluid lymphocytes. In osteoblast-like cells, glucocorticoids increased RANKL and decreased OPG expression in non-TNF primed cells while they decreased RANKL (and did not affect OPG) expression in TNF primed cells.

Our cohort consisted of 13 patients with different arthritides (6 RA, 2 spondylarthropathies and 5 patients with unclassified oligoarthritis). The mixed arthritis cohort is to my personal opinion not a problem in studying the effects of intra-articular glucocorticoids on bone metabolism, as the concern for possible bone-destructive effects of this therapy is valid in every treated arthritic joint and thus a modification of the

RANKL/OPG system could be relevant for any type of arthritis. Besides, similar trends were seen in all of the 3 sub-cohorts as in the whole cohort regarding the ability of intra-articular glucocorticoids to down-regulate the synovial RANKL/OPG ratio.

The decrease in synovial RANKL expression after intra-articular glucocorticoid treatment could be a direct effect of the treatment or an indirect effect because of the decrease in synovial T cell numbers or because of the suppression of synovial inflammation. To exclude the possibility of being a secondary effect due to decreased T cell numbers, we calculated RANKL positive and CD3 positive cells in serial sections and the results were expressed as the percentage of CD3 positive cells that were RANKL positive. We found that the percentage of RANKL positive T cells also decreased following therapy. Thus RANKL synovial decrease is a direct effect of intra-articular glucocorticoids. On the other side, anti-TNF treatment directly increases synovial OPG (Catrina et al., 2006), thus a combined therapy consisting of both anti-TNF agents and intra-articular glucocorticoids could offer even more protection against bone destruction in RA.

Altough there is a long-standing debate regarding the effect of systemic glucocorticoids on bone metabolism there is evidence from several clinical studies that oral glucocorticoids are able to decrease joint erosions (Kirwan, 1995), (Hickling et al., 1998), (Wassenberg et al., 2005), (Svensson et al., 2005), (van Everdingen et al., 2002), (Jacobs et al., 2006) and juxta-articular osteoporosis in RA (Haugeberg et al., 2005). Regarding intra-articular glucocorticoids only limited data is available but this data provides proof for a direct protective effect for bone erosions in RA (Conaghan et al., 2003). Our study offers a molecular explanation of those findings.

An interesting point of our study is the *in vitro* effect of glucocorticoids on osteoblast-like cells. In non-TNF primed osteoblast-like cells glucocorticoids modulated the RANKL/OPG ratio towards bone destruction while in TNF primed cells, as an approach to mimic the pro-inflammatory environment of the RA joint, the RANKL/OPG ratio was modulated towards bone protection. These findings could offer a rationale for the effects of glucocorticoids in RA, depending on the presence or absence of pro-inflammatory mediators. Thus glucocorticoids could be bone-protective regarding bone erosions and juxta-articular osteoporosis which are in the vicinity of the pro-inflammatory RA synovial compartment, while the same therapy could be bone destructive in areas such as the lumbar spine which are usually not affected in RA (Grassi et al., 1998) and thereby should not be surrounded by the excess of pro-inflammatory mediators of the rheumatoid joint. This finding underlines the need for treatment strategies that would deliver glucocorticoids specifically in the inflamed tissue (Koning et al., 2006) in order to circumvent their side effects. Moreover, in the light of our *in vitro* results it was proposed that targeting local, tissue-specific glucocorticoid metabolism could be an approach for treatment of inflammatory diseases such as RA (Wilckens and Volkmann, 2007).

Paper IV – Glucocorticoid induced apoptosis of T cells in the inflamed RA synovial compartment

The aim of Paper IV was to assess if the decrease in synovial tissue T cells (as evidenced both in Paper III and (af Klint et al., 2005)) was mediated through apoptosis induction by intra-articular glucocorticoids. As mentioned in the introduction, glucocorticoids are known inducers of T cell apoptosis, mainly through the mitochondrial pathway (Herold et al., 2006).

Our first approach to this issue was to study if intra-articular glucocorticoids are able to modify apoptosis levels at the RA synovial tissue level. Our observation during the evaluation of those stainings was that lymphoid aggregates, synovial tissue formations rich in T cells (Cope, 2002), were virtually free of expression of apoptosis, both before and after treatment. The next step was to assess T cell apoptosis with dual immunofluorescence and we found that a minimal amount of T cells, both in lymphoid aggregates and scattered in the sublining layer, where apoptotic both before and after therapy. *In vitro*, we assessed apoptosis of non-isolated RA T cells in synovial fluid containing monocytes and lymphocytes but not fibroblasts. In contradiction with non-isolated synovial fluid T cells from spondylarthropathies, RA T cells were resistant to glucocorticoid induced apoptosis. Isolation of RA SF T cells rendered them prone to glucocorticoid induced apoptosis, underlining the importance of their interaction with monocytes.

Interaction of RA synovial T cells with fibroblasts and monocytes as well as the action of interleukine 2 receptor γ chain cytokines such as IL-15 has an anti-apoptotic effect (Salmon et al., 1997), (Wagner et al., 2004). We show in Paper IV that also RA synovial T cell glucocorticoid apoptosis resistance is a result of the interaction of T cells with monocytes, which could either demand cell-cell contact (Wagner et al., 2004) or could be mediated by cytokines excreted from monocytes such as IL-15 through up-regulation of anti-apoptotic proteins of the mitochondrial pathway (Salmon et al., 1997), (Miranda-Carus et al., 2006), (Bulfone-Paus et al., 1997). If we consider that intra-articular glucocorticoids do not succeed to decrease synovial tissue monocytes/macrophages, it seems necessary to combine intra-articular glucocorticoids with monocyte targeted therapies (Catrina et al., 2005).

Anergic RA synovial T cells have certain characteristics such as low levels of transcription of calmodulin (Ali et al., 2001) and insufficient intracellular calcium signaling (Carruthers et al., 2000), that are associated with resistance to glucocorticoid induced apoptosis (Dowd et al., 1991), (McConkey et al., 1989). Monocytes both in the synovial tissue and synovial fluid through production of TNF are possibly responsible for this aspect of RA synovial T cell resistance to glucocorticoid induced apoptosis as well (Ali et al., 2001), (Isomaki et al., 2001).

Thus if RA synovial T cells are resistant to glucocorticoid induced apoptosis, their decrease following intra-articular glucocorticoid treatment is most possibly because of a lower influx of T cells into the rheumatoid joint. Glucocorticoids have been found to reduce pro-angiogenic molecules such as VEGF in the rheumatoid synovium (af Klint et

al., 2005). They also down-regulate E-selectin and ICAM-1 (af Klint et al., 2005), (Youssef et al., 1996) which have an active role in respectively the initial and late phase of leukocyte emigration in the synovium. Moreover, glucocorticoids decrease T cell adhesion to synovial cells (Eguchi et al., 1992).

Value of combination therapy as indicated by Papers III and IV

Data from both Paper III and IV shows the possible value of combined therapies, as already mentioned before: combining intra-articular glucocorticoids with monocyte targeted therapies, such as anti-TNF treatment, could help in:

1. Sensitizing synovial RA T cells to glucocorticoid induced apoptosis (see discussion for Paper IV).
2. Suppressing bone absorption more effectively by regulating both RANKL and OPG expression (see discussion for Paper III).
3. Controlling RA synovial inflammation more effectively by down-regulating both T cells (Paper IV) and monocytes/macrophages (Catrina et al., 2005).

Thoughts on the thesis - Preliminary results

I hope that through this thesis we have enlightened some pathogenic and therapeutic aspects in RA. I think that Paper I provides convincing evidence that smoking induces citrullination in the alveolar lung compartment which in the context of genetical susceptibility could result in the production of ACPAs. Paper II shows that citrullination is an inflammation dependent phenomenon and helps in realizing that other factors that just the mere presence of citrullinated proteins could be responsible for the development of ACPA immunity in RA. Papers I and II together help in building a hypothesis regarding development of ACPA positive RA, which starts from citrullination in the lungs and tries to explain the pathogenic mechanisms in the inflamed RA synovium, with the hope that future research will contribute even more in a better understanding of those pathogenic procedures. This thesis went on with studying how intra-articular glucocorticoids can modulate synovial inflammation, bone metabolism, in the context of the RANKL/OPG system, and synovial T cell apoptosis. I think that paper III provides an important message to the clinician: treatment of inflammatory arthritis with intra-articular glucocorticoids can be performed without being too concerned of bone-destructive effects of this treatment, but rather with the potentials of bone-protective effects. Paper IV shows that monocyte-T cell interactions are crucial in the resistance of RA synovial T cells to glucocorticoid induced apoptosis. The conclusion provides another important clinical message: if we want to further suppress synovial inflammation by inducing T cell apoptosis we should combine intra-articular glucocorticoids with monocyte targeted therapies. Studying some interesting points raised in Papers III and IV would further help in better understanding of the pathogenesis and more efficient treatment of RA. The clarification of the paradoxical effects of glucocorticids in bone metabolism depending

on the presence or absence of an inflammatory environment and the clarification of the manner that RA synovial monocytes rescue T cells from glucocorticoid induced apoptosis (cell contact vs soluble factors) are such points.

In the context of this thesis, and having in mind the lessons regarding pathogenetic mechanisms of RA that we can learn from studying the molecular mechanisms of action of pharmaceutical treatment, we decided to study the effect of methotrexate, intra-articular glucocorticoid, infliximab and etanercept treatment on citrullinated proteins found to be specific for the RA synovium in comparison with the synovium of non-RA arthritides (F95) (De Rycke et al., 2005) and the main PAD enzymes at the RA synovial tissue level, PAD2 and PAD4 (Foulquier et al., 2007). We tried also to correlate citrullinated protein and PAD enzyme expression with the synovial inflammation score before and after therapy as well as to compare expression of citrullinated proteins and PAD enzymes in RA tissue before and after therapy with their expression in healthy synovial tissue.

F95 expression was almost exclusive in RA compared to healthy synovial tissue. PAD2 was increased in RA before and after treatment, while PAD4 only before treatment compared to healthy synovium. Methotrexate and TNF-antagonists did not alter PAD and F95 expression, with the exception of PAD4 decrease with infliximab. Intra-articular glucocorticoids decreased total and intracellular F95 and PAD4 expression. F95, PAD2 and PAD4 expression correlated with synovial inflammation before while only PAD4 expression correlated with synovial inflammation after treatment.

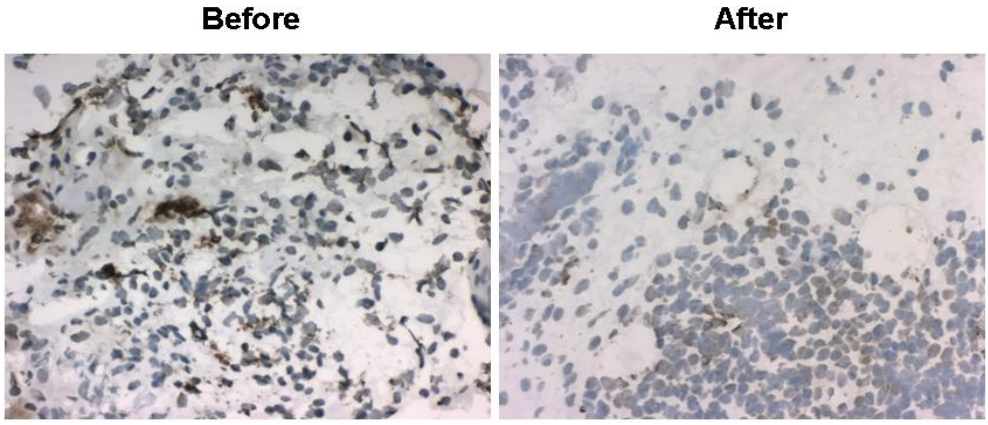

Figure 6 – The effect of intra-articular glucocorticoids on intra-cellular RA synovial citrullinated proteins detected with the F95 antibody

Thus it seems that the expression of F95 and PAD2 is relevant for RA pathogenesis with both markers being only partly associated with synovial inflammation. PAD4 expression seems to be more associated with synovial inflammation. In general, it seems that despite some modulation in citrullination parameters with current anti-rheumatic treatment, it is impossible to totally reverse the disease pathogenic mechanism associated with the presence of specific citrullinated proteins in the rheumatoid synovium. However, intra-articular glucocorticoids seem the most potent therapy in this aspect and this is a finding encouraging their use in clinical practice.

THESIS CONCLUSION

Citrullination is a post-translational modification relevant in the pathogenesis of RA in the context of gene-environment interactions while it is not an exclusive characteristic of the RA inflamed synovium. Treatment of synovial inflammation with intra-articular glucocorticoids provides a bone protective effect in the inflammatory joint compartment while combination treatment with monocyte targeted therapies could help in overcoming RA synovial T cell resistance to glucocorticoid induced apoptosis.

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