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Immunopathogenic studies on Inflammation and bone destruction in Rheumatoid arthritis

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TO SHIRIDI SANSTHAN
ABSTRACT

Rheumatoid arthritis (RA) is a chronic systemic joint disease characterized by synovial inflammation, leading to destruction of cartilage and bone. Local recruitment of immune cells and defective apoptosis results in chronic inflammation with increased synovial citrullination and local hypoxia. Synovial inflammation by itself can promote destruction of bone by modulating the RANKL/RANK/OPG system. Recently it has been suggested that the two mechanisms are at least partly uncoupled.

The aim of this thesis was to better understand pathogenic mechanisms responsible for inflammation and bone destruction and to study their modulation by effective anti-rheumatic treatment.

We demonstrated that T cells-resistance to apoptosis and protein citrullination are important features of the rheumatoid joints, potentially contributing to perpetuation of local inflammation. Intra-articular glucocorticoids fail to modulate synovial apoptosis but successfully decreased synovial citrullination both in vivo and in vitro. Modulation of synovial citrullination appears to be a drug specific effect rather than a consequence of decreased inflammation as far as methotrexate showed no effect when tested both in vivo and in vitro.

We have identified synovial RANKL expression and hypoxia as two important factors mediating bone destruction independent of inflammation. First we showed that high levels of the RANKL/OPG ratio characterize early-untreated RA. Methotrexate treatment modulates synovial expression of RANKL and protects against future radiographic progression independent of the anti-inflammatory effect. The direct effect of methotrexate on bone metabolism was confirmed in vitro where dual mechanisms of action were identified consisting in both RANKL modulation and direct cellular effects on osteoclasts. Second we demonstrate that hypoxia modulates RANKL and OPG expression in osteoblasts through a HIF-2α mediated mechanism. Hypoxia promotes osteoclastogenesis and bone destruction acting both through up-regulation of the RANKL/OPG ratio and direct cellular effects on osteoclasts. The additive effect of hypoxia and inflammation observed in our in vitro osteoclastogenesis assays further support the idea that inflammation is not the only mechanism responsible for bone destruction in RA.

In conclusion we have identified T cell resistance to apoptosis, citrullination, synovial expression of RANKL and hypoxia as important denominators of chronic inflammation and bone destruction in RA. Specific targeting of these molecular pathways might provide further insight in the complex pathogenesis of the disease and lead in the future to new therapeutic concepts.
LIST OF PUBLICATIONS

I. Dimitrios Makrygiannakis, Shankar Revu, Pertra Neregård, Erik af Klint, Omri Snir, Cecilia Grundtman, and Anca Irinel Catrina. Monocytes are essential for inhibition of synovial T-cell glucocorticoid-mediated apoptosis in rheumatoid arthritis. 
*Arthritis Research & Therapy* 2008, 10:R147.

*Arthritis Research & Therapy* 2012, 14:R20

III. Shankar Revu, Petra Neregård, Erik af Klint, Marina Korotkova, Anca Irinel Catrina. Methotrexate inhibits RANKL and prevents osteoclastogenesis in early, untreated RA. 
Submitted manuscript

Submitted manuscript

* authors equally contributed
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ACPA</td>
<td>Anti citrullinated protein antibody</td>
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<tr>
<td>BCL</td>
<td>B cell lymphoma</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
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<tr>
<td>CD</td>
<td>Cluster differentiation</td>
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<tr>
<td>C-TAD</td>
<td>C-terminal activation domain</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DMARD</td>
<td>Disease modifying anti-rheumatic drug</td>
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<tr>
<td>DMOG</td>
<td>Dimethylxaloylglycine</td>
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<tr>
<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
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<td>FLS</td>
<td>Fibroblast-like synoviocytes</td>
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<td>GCR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HIFα</td>
<td>Hypoxia inducible factor alpha</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HRE</td>
<td>Hypoxia responsive element</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MTX</td>
<td>Methotrexate</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NSAID</td>
<td>Nonsteroid anti-inflammatory drug</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<tr>
<td>PAD</td>
<td>Peptidylarginine deiminase</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase</td>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
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<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa B</td>
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<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
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<tr>
<td>SE</td>
<td>Shared epitope</td>
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<td>SFMC</td>
<td>Synovial fluid mononuclear cells</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>TRAF</td>
<td>TNF-receptor associated factor</td>
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<td>TRAP</td>
<td>Tartrate resistant acid phosphatase</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl-mediated dUTP nick end labeling</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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1. History
The term Rheumatoid arthritis (RA) was first coined by British Rheumatologist Dr. Alfred Baring Garrod in 1859. The foremost clinical description of RA was described from the studies in female population at Salpetiere hospital, Paris [1].

1.1 Epidemiology of RA
The prevalence of RA varies from 0.5-1% population worldwide [2], at earlier ages in Native Americans and later in European countries [3, 4]. In north European countries like Sweden the prevalence rate is reported as 0.5-0.9% [5]. RA is more prevalent in women than men and the disease incidence increases with age up to 70 years [6].

1.2 Etiology
Etiology of RA is not clearly known, but it is believed that RA results from the complex interaction between different risk factors including abnormal immune response, environmental factors, and genetic susceptibility. Genetic factors like HLA-DR and the shared epitope has been reported as the major risk factor of RA [7]. Some other non HLA genetic factors such as polymorphism in genes PTPN22 and CTLA4 have been recently reported as risk factors that might trigger the autoimmune disease [8]. Non genetic factors such as gender, smoking, socioeconomic status, infectious agents have long been studied as potential etiological factors of RA [5], with smoking being the most well established non-genetic risk factor.

1.3. Background and description
RA is a severe chronic autoimmune disease and is characterized by synovial joint inflammation leading to the destruction of articular cartilage and sub-chondral bone. Besides its main localization to the synovial joints, RA can also affect other tissues. Chronic persistent joint inflammation is due to both synovial accumulation and proliferation of inflammatory cells (macrophages and lymphocytes) and decreased apoptosis of resident cells in the synovium. This leads to excessive number of infiltrating inflammatory immune cells and production of pro-inflammatory cytokines that further amplify local inflammation and promote synovial hypertrophy with consecutive local hypoxia [9, 10].
2. Clinical manifestation
RA clinically affects joints with local inflammation leading to swollen and tender joints and sometimes involvement of other tissues such as skin, lungs, heart and kidneys.

2.1 Diagnosis and classification
The diagnosis of RA is still today a clinical diagnosis based on a core set of clinical signs and symptoms and a series of laboratory findings. In order to homogenize study populations in clinical studies several sets of criteria for classification of RA emerged. In 1987 American College of Rheumatology (ACR) proposed criteria for classification of RA (table 1) [11].

<table>
<thead>
<tr>
<th>Table.1. 1987 ACR criteria for rheumatoid arthritis</th>
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<tbody>
<tr>
<td>1 Morning stiffness of the joints, lasting for 1 hour before maximal improvement.</td>
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<tr>
<td>2 Involvement of arthritis atleast 3 joint areas soft tissue swelling or fluid retention. Possible areas are right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle and metatarsophalangeal (MTP) joints.</td>
</tr>
<tr>
<td>3 Arthritis of hand joints, atleast 1 area swollen as mentioned above (criteria.2).</td>
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<tr>
<td>4 Symmetric arthritis, simultaneous involvement of the same joint areas on both sides of the body.</td>
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<tr>
<td>5 Presence of rheumatoid nodules, subcutaneous nodules over bony prominences or extensor surfaces or in juxta-articular regions.</td>
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<tr>
<td>6 Presence of serum rheumatoid factor.</td>
</tr>
<tr>
<td>7 Radiographic changes in hand and wrist which include erosions or unequivocal bony decalcification.</td>
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The ACR guidelines included a classification criteria based on fully established manifestations of RA. However during last year's these criteria proved to have low sensitivity for the classification of RA in more early stages of the disease. Recently, an updated and improved ACR and European League Against Rheumatism (EULAR) criteria was proposed in 2010 [12, 13]. The ACR-EULAR criteria (Table 2), evaluation are mainly based on the scores from four different categories aimed for classification of
RA. In order to classify as RA, a score of at least 5 in the presence of at least one swollen joint is needed.

Table 2. 2010 ACR-EULAR classification criteria for rheumatoid arthritis

<table>
<thead>
<tr>
<th>Category 1 (Joint involvement)</th>
<th>Score</th>
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<tr>
<td>a. 1 large joint (shoulders, elbow, hip, knee and ankle)</td>
<td>0</td>
</tr>
<tr>
<td>b. 2-10 large joints</td>
<td>1</td>
</tr>
<tr>
<td>c. 1-3 small joints (with or without involvement of large joints including MTP, PIP and wrists)</td>
<td>2</td>
</tr>
<tr>
<td>d. 4-10 small joints (with or without involvement of large joints)</td>
<td>3</td>
</tr>
<tr>
<td>e. &gt;10 joints (at least one small joint)</td>
<td>5</td>
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| Category 2 (Serology, at least 1 test result is needed for classification)                     |       |
| a. Negative RF and negative ACPA                                                              | 0     |
| b. Low-positive RF or low-positive ACPA                                                        | 2     |
| c. High-positive RF or high-positive ACPA                                                      | 3     |

| Category 3 (Acute-phase reactants, at least 1 test result is needed for score classification)  |       |
| a. Normal CRP (C-reactive protein) and normal ESR (erythrocyte sedimentation rate-0)         | 0     |
| b. Abnormal CRP or normal ESR 1                                                               | 1     |

| Category 4 (Duration of symptoms, self reported)                                               |       |
| a. <6 weeks                                                                                   | 0     |
| b. ≥6 weeks                                                                                   | 1     |

2.2 RA evaluation and monitoring

The assessment of a patient with RA is done by physical evaluation of tender and swollen joints, estimation of systemic inflammation by measurements of acute phase reactants like erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), serum detection of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [14]. Global assessment of disease activity can be done by clinical composite scores such as the disease activity score 28 (DAS28). DAS score is commonly used to monitor the activity of disease and response to treatment in the clinical trials to define responders, moderate responders and non responders [15]. Monitoring of involved joints by X-rays, ultrasound and MRI are additional useful tools and may reveal the progression of disease at a more intimal level than clinical evaluation [16].
2.3 Treatment
Several treatment options are available in the management and control of RA. The aim of the treatment is to attain clinical remission or low disease activity as early as possible [17, 18]. In the absence of early and aggressive treatment, RA is chronic and severe in some cases. When left untreated the disease tend to develop to cause considerable morbidity with consecutive increased disability and mortality as compared to general population [19]. Currently, there are different classes of anti-rheumatic drugs available for RA treatment. In 2008 ACR provided a set of recommendations for the use of different anti-rheumatic treatment options for RA and management of the disease [20] that to great extent are still valid today. Anti-rheumatic drugs include: non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), glucocorticoids (GCs) and biologicals. The clinical efficacy and mode of action of these drugs are addressed in detail in the section of therapeutic interventions.

3. Current etiopathogenic hypothesis
Complex immune-mediated inflammatory mechanisms are involved in the pathogenesis of RA. The initiation of disease is thought to be due to the interplay between genetic susceptibility and environmental factors leading to specific immune reactions. Evidences from twin studies show that genetic factors contribute approximately 60% for the disease pathogenesis [21]. In a twin gene study it is reported that the concordance rate of disease susceptibility is higher in monozygotic twins (12-15%) than dizygotic twins (3-4%) [22]. It is well known that class II major histocompatibility complex (MHC) or Human Leukocyte Antigen (HLA) and shared epitope (SE) are major risk factor for RA [23]. Some other non HLA-genes have also been identified in the pathogenesis of RA but have a relatively lower risk susceptibility compared to individuals that carry the HLA-DR haplotype. These include a functional variant of intracellular protein PTPN22, transcriptional factor STAT4, and co-stimulatory receptor CTLA-4 that may be involved in T and B cell activation [24, 25].

It is believed that RA can initiate when specific interactions occur between these genes and environmental factors such as interaction between smoking and SE particularly in seropositive patients [26]. According to this hypothesis smoking is able to induce postranslational modification such as citrullination in the lungs of healthy smokers.
the presence of right SE allele citrullinated proteins are more firmly and better presented as autoantigens on the surface of antigen-presenting cells. This further leads to immune activation of T cells and eventually B cells with production of specific antibodies (ACPA) in the serum of individuals that are yet not having any joint complaints and are still healthy. In this model a second hit leading to increased synovial citrullination over a certain threshold is needed, allowing circulating ACPA to localize to the joint and initiate local immune reactions. This in turn results in chronic inflammation due to increased cell recruitment and decreased cell clearance (defective apoptosis) leading to synovial hypertrophy with consecutive cartilage and bone destruction.

Production of autoantibodies and in particular ACPA is a central event in the pathogenesis of RA. ACPA are highly specific for RA [27] and could be detected in the blood long before the onset of the disease. ACPA detection is currently used as a useful tool in the diagnosis and prognostic of RA. Their role in disease pathogenesis have recently been highlighted by their possibility to induce activation of monocytes through a Fcγ dependent mechanism [28] but also more interestingly through the potential direct interaction between these antibodies and citrullinated targets on cell surfaces [29, 30]. Beside ACPA, rheumatoid factors (RF) are autoantibodies directed against Fc portion of IgG and are commonly found in RA patients with somewhat lower specificity as compared to ACPA [27].

4. Pathophysiology of synovium
Joint synovial membrane is a soft tissue found between articular cavity and the capsule of the diarthrodial joints and synovium is the primary site of inflammation in RA.

4.1 Healthy and RA synovium
Normal synovium consists of two compartments the lining and sublining. The lining compartment is restricted to two to three cellular layers of macrophage-like or type A synoviocytes and fibroblast-like or type B synoviocytes in a loose extracellular matrix. The later is responsible for synthesis of collagen, fibronectin and hyaluronic acid that facilitate lubrication and maintenance of cartilage surface. The sublining layer contains vascular, fatty and connective tissue with sparsely dispersed mononuclear cells. In
inflammatory conditions such as RA, synovial lining layer becomes hyperplastic and thickened with more layers containing both cell types (A and B synoviocytes) leading to pannus formation that progresses to the adjacent joint bone [31]. In the sublining layer there is imported cell infiltration with activated macrophages, dendritic cells (DCs), natural killer (NK) cells, and T and B cells.

Macrophages are the dominant cell type in RA synovitis and considered master regulators of the local inflammation. In addition differentiated CD4+ T cells of Th1 cell phenotype and CD8+ T cells might also be present diffuse or more grouped in aggregates in the sublining layer [32]. Accumulation of activated cells leads to secretion of proinflammatory cytokines and chemokines that further amplify local inflammation and promote neovascularization and synovial hypertrophy with consecutive local hypoxia [10, 33]. Synovial hyperplasia results in increased distance between blood vessels and synoviocytes, further increasing the demand for oxygen consumption from dysregulated blood vessels. Figure 1 represents the difference between normal (A) and inflamed RA (B) synovium.

![Figure 1](image_url)

**Figure 1.** Microscopic image of haematoxylin (HTX) stained histology from normal (A) and RA (B) synovial tissue. Arrow indicates infiltration of inflammatory cells.

### 4.2 Synovial fluid (SF)

The normal joint contains very low levels of SF needed for lubrication and function of the periarticular cartilage around the joint. In active RA, the volume of SF increases and is accompanied by an increase in inflammatory cells, cytokines and filtrates of plasma derived proteins within the joint space. Neutrophils are the dominant cell type in inflammatory SF. Excessive accumulation of immune cells in SF occur due to increased recruitment and resistance to apoptosis [9, 34] with high levels of chemotactic factors such as chemokines, leukotriene B4 and activated C5a complement...
factors [35]. The lymphocyte population within the SF is mainly composed of CD8+ cells. Inflammatory SF contains activated cells, various inflammatory mediators and degradative enzymes that are responsible to damage cartilage and bone by chondrocytes and osteoclasts [36, 37]. Figure 2 illustrate the difference between normal synovial joint (A) and inflamed RA synovial joint (B).

![Figure 2](image)

**Figure 2.** Schematic representation of synovial joint (A) normal synovium and (B) inflamed RA synovium (Adapted from Vibeke Strand et al. *Nature Reviews* 2007).

### 4.3 Cellular and cytokine network in RA

At the synovial level a complex network of sequential activated immune cells and pro-inflammatory cytokines play an important role in initiation and propagation of chronic inflammation eventually leading to cartilage and bone destruction.

#### 4.3.1 Monocyte/Macrophages

Monocytes are recruited from peripheral blood and migrate to tissues as macrophages. They are abundant in both synovial tissue and SF leading to synovial hyperplasia. Monocytes and macrophages are phagocytic cells upon activation by inflammatory stimuli through Toll like receptors (TLRs) and serve as link for both innate and adaptive immune functions [38]. These cells express different lineage markers like CD14, CD68, and CD163 and activation phenotypic marker like MHC class II surface molecule. The monocyte-macrophage system is the major source of pro-inflammatory cytokine production [39]. Some of the important secreted cytokines include TNFα, IL-
1, IL-6, IL-12, IL-15, IL-23, GM-CSF and chemokines with both autocrine and paracrine actions [40]. Anti-inflammatory cytokines such as IL-10, TGF-β and IL-1Ra, are also present in the rheumatoid joint but not able to suppress inflammation presumably overwhelmed by the amount of pro-inflammatory local events. Monocytes can also act as osteoclast precursor cells, which upon stimulation with macrophage colony stimulating factor (M-CSF) express RANK receptor and induce osteoclastogenesis in the presence of osteoclast differentiation factor RANKL [41, 42]. Pro-inflammatory cytokines like IL-1 and TNFα have been reported to induce osteoclastogenesis from monocytes precursors with consecutive bone resorption in the presence of permissive levels of RANKL [43, 44].

4.3.2 Fibroblasts

Fibroblasts-like synoviocytes (FLS) are found in the lining and in the sublining layer of the synovitis. FLS from RA patients display a unique phenotype [45]. FLS are non-phagocytic, non immune cells, able to secrete a variety of cytokines and exhibiting some features similar to transformed cell features [45]. FLS are an important source of cytokines and chemokines such as IL-6, IL-15, IL-8, CCL5 and CXCL1 that actively contribute to recruitment of immune cells (neutrophils, T and B cells) to the inflamed synovium [46]. In RA, FLS are the major source of proteolytic enzymes like cathepsins, matrix metalloproteinases (MMPs) and acid metabolites that directly invade tissue matrix and destroy the cartilage [47]. Upon activation FLS can express RANKL that might contribute to the local induction of osteoclast and bone destruction [48].

4.3.3 Lymphocytes

Lymphocytes include T-cells, B-cells and Natural killer cells (NK) and are present mainly in the sublining layer rather than lining layer of the synovial tissue. Synovial T cells of RA express a phenotype suggesting chronic immune activation, but they are anergic and resistant to apoptosis [49]. They are present either as aggregates sometimes resembling germinal centers or diffusely widespread in the synovium. They express CD3 and can be further subdivided in CD4+ T-helper (Th), CD4+T-regulatory cells (T-reg), or CD8+ cytotoxic T cell (Tc-cell). CD4+ Th cells are the dominant subtype of T cells in the inflamed synovium. Depending on the cytokines present in a specific environment Th cells further differentiate into Th1, Th2 and Th17 phenotypes [49]. CD8+ Tc cells are cytotoxic or killer cells that can bind to MHC class I molecule. They
are present in the inflamed joint but thought to have a secondary role in disease pathogenesis.

B-cells and plasma cells are mainly seen in the sublining layer of the synovium and reside together with T cell population. B-cells are essential components of humoral immunity, and are characterized by B-cell receptor (BCR) expression and are able to bind directly to antigen to activate immune response. Their contribution in RA pathogenesis is by production of autoantibodies like RF and ACPA [50-52]. Synovial B cells secrete cytokines like TNFα, IL-2, IL-4, IL-6 and IL-12 contributing to local inflammation by activating dendritic cells and T cells [53, 54].

NK cells are CD3 negative, CD16 or CD 56+ and can be identified in the SF of established RA. Their activity and cytotoxicity is enhanced by IL-2 and IL-15 cytokines [55, 56]. Besides cytotoxicity NK cells produce cytokines like IFN-γ, TNFα, IL-4, IL-13, and IL-10 [57]. Recent studies reported that NK cells in the SF could trigger the differentiation of osteoclast and bone destruction in arthritis when co-cultured with monocytes through a process dependent on RANKL and M-CSF induction [58].

4.3.4 Neutrophils

Neutrophils are the most predominant cell type and constitute about 70% of the cell population in the inflamed SF and to a lesser extent in the synovial tissues of RA. [35]. Upon migration to specific sites of inflammation they release proteases and oxygen free radicals leading to the destruction of cartilage and other tissues [35, 59]. Neutrophils regulate both the innate and adaptive immunity and their function is mediated through TLR activation [60, 61] and secretion of cytokines like IL-1α and IL-1β in RA [62]. It is reported that neutrophils can secrete cytokines like BAFF and APRIL which are crucial for the cell survival, maturation and differentiation of B cells [63, 64]. In addition, RA SF neutrophils can induce osteoclast formation and bone resorption through the upregulation of functionally active membrane bound RANKL [65].

4.3.5 Dendritic cells (DCs)

DCs present in the synovial sublining layer are mostly located around the lymphoid aggregates, and constitute about 10% of total cell population in SF. They are present in
the blood stream in an immature state, and upon activation they migrate to the lymph nodes where they interact with T and B cells and act as the mediators for innate and adaptive immune system. Activated and mature DCs express high levels of HLA-DR and are referred as professional antigen-presenting cells. They express CD83, a transmembrane glycoprotein of Ig superfamily and are expressed on cultured, follicular and circulating DCs. They also express adhesion molecules CD11a, CD11c, CD54 and co-stimulatory molecules like CD80 that are upregulated during DC activation. DCs are responsible for the activation and perpetuation of immune response for RA pathogenesis and breaking of self-tolerance through the antigen-presentation to autoreactive T cells [66, 67]. In RA, mature DCs secrete pro-inflammatory cytokines IL-1, IL-6 and TNFα, and their production in DCs is mainly influenced by Fcγ receptor dependent pathway [68]. DCs are derived from monocytes in the presence of IL-4 and GM-CSF, and further could differentiate into bone resorbing cells in the presence of M-CSF and RANKL [69].

4.3.6 **Endothelial cells**

Endothelial cells expressing CD31 and CD34 and play an important role in RA. Endothelial cells are active participants in the inflammatory process in recruiting leukocytes and other immune cells. It has been reported that the inflammatory cytokines IL-1 and TNFα upregulate the expression of endothelial cell adhesion molecules ICAM-1, VCAM-1 and E-selectin to recruit leukocyte migration into the synovial joint [70]. Neovascularization is a major feature of the inflamed synovial tissue driven by the hypoxic local environment through up regulation of vascular endothelial growth factor (VEGF) a potent angiogenic stimulator [71].

5. **Pathogenic mechanisms of inflammation and bone destruction in RA**

Once initiated, synovial inflammation is maintained and propagated through contribution of several distinct pathogenic pathways such as increased recruitment of immune cells, defective apoptosis of synovial resident cells, up-regulation of synovial citrullination, profound hypoxia and up-regulation of the RANKL/RANK/OPG pathway.
5.1 Synovial inflammation and apoptosis

Excessive inflammatory cells infiltration in the synovial tissue results from both increased cell recruitment and local synovial defective cell death mechanisms [9, 34]. Apoptosis is a physiological process of programmed cell death, and is a highly conserved multi-step process. Apoptosis is distinguishable from necrosis by formation of apoptotic cell bodies that are cleared by macrophages without extracellular release of intracellular matrix and therefore without concomitant inflammation [72, 73]. Deficient apoptotic machinery has been implicated in the pathogenesis of a wide range of disease.

Apoptosis can be initiated by two major common pathways called extrinsic and intrinsic pathways. The extrinsic apoptotic pathway leads to recruitment of the intracellular adaptor protein FADD following interaction between death ligands (FasL and TNFα) and death receptors (Fas and TNF receptor) to activate pro-caspase 8 [74]. Active caspase 8 initiates a cascade activation of several others caspases leading to effector caspase 3 activation [75]. Among cell death receptor ligands TNF has a dual effect. Dependent on the local tissue environment TNF might either initiate the extrinsic apoptotic pathway or induce NF-κB activation that in turn inhibits the apoptotic machinery. The second intrinsic apoptosis pathway is initiated by various extracellular stimuli such as UV-irradiation and depletion of growth factors, leading to cytochrome c release from mitochondria with recruitment of Apaf-1 and pro-caspase 9 to form the apoptosome. This results in activation of caspase 9 and subsequently activation of caspase 3. Smac/DIABLO acts as antagonist to inhibit the inhibitor of apoptosis protein IAP and further allow activation of caspase 3. Both extrinsic and intrinsic pathways converge with the activation of caspase 3 resulting in subsequent induction of apoptosis. Caspases 3 initiate DNA fragmentation leading to the characteristic appearance of an apoptotic cell with cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [72]. The extrinsic pathway is blocked by FLIP, while the intrinsic pathway associated bcl-2 family is the main regulator of mitochondrial pathway. In which the bcl-2 member of the family being anti-apoptotic while bax members are pro-apoptotic. Figure 3 illustrate the two apoptotic pathways.
A variety of methods could be used to analyze apoptosis in tissues and/or cell cultures. TUNEL technique can be used to label apoptotic fragments of DNA and electron microscopy is used to identify the apoptotic morphology of a cell [76, 77]. Staining with annexin V is used to detect the translocation of phosphatidylinerine molecule from the cytoplasm to the cell surface. In addition, antibodies have been developed recognizing only active but not inactive uncleaved caspases that can be used to detect intracellular active caspases by immunostainings. DNA laddering, ELISA based detection of caspase activity, detection of the mitochondrial membrane potential are other methods that can be used.

In RA synovial tissue, low levels of apoptosis (1-3%) have been reported. It is believed today that high synovial expression of anti-apoptotic factors is responsible for this in macrophages for examples resistance to apoptosis is due to high expression of occurring FLIP associated with low levels of apoptosis in RA synovium [78, 79]. Also, synovial NF-κB activation in the synovium [80] contribute to further inhibition of
macrophage apoptosis [81]. Activated synovial fibroblasts when co-cultured with B cells induce lymphocyte resistance to apoptosis in a cell contact and adhesion molecule dependent mechanism [82]. Enhanced synovial expression of anti-apoptotic molecules Bcl-2 and Bcl-xl, but not pro-apoptotic molecules in RA further contribute to low levels of synovial apoptosis [83]. Synovial RA T cells are also resistant to apoptosis [49, 84]. Chronic exposure to TNFα, IL-2 receptor γ chain signaling cytokines and inhibitory signals received through interaction with stromal cells have been all implicated in generating the apoptotic resistant phenotype of synovial T cells [85]. Infiltrating T cells in the synovium express also high levels of the anti-apoptotic molecules Bcl-2 and Bcl-xl [84].

5.2 Synovial inflammation and citrullination

Citrullination or deimination is a process of post translational modification of charged peptidyl arginine to neutral peptidyl citrulline by peptidylarginine deiminase (PAD) in the presence of calcium [86] (Figure 4). There are five different classes of PAD enzymes identified in humans (PAD-1, PAD-2, PAD-3, PAD-4 and PAD-6) that are expressed in variety of tissues. PAD-2 and PAD-4 are expressed in RA synovium and SF cells [87, 88] Protein citrullination is dependent on the activity of PAD enzymes, which is regulated by calcium concentrations.

Citrullination occurs normally in the hair follicle as an important process for maintaining the structure of the hair fiber [89]. Also, citrullination occurs physiologically in keratin and filaggrin in the cornified layers of epidermis [90] and in the central nervous system [91]. Citrullination also regulates histone arginine methylation at the multiple arginine sites in the histone N-terminal tails [92]. In multiple sclerosis (MS) abundant expression of citrullinated proteins in the brain increases risk of demyelination and subsequent exposure of immunodominant epitopes [93]. Chronic inflammation also associates with excessive citrullination [94]. In RA citrullination of several synovial tissue proteins have been reported including fibrinogen, vimentin and α-enolase [95-97]. Increased citrullination is not a specific trait of the rheumatoid synovium but rather general phenomena in the context of inflammation and occurs in a large array of inflammatory tissues both synovial and extra synovial tissues [98, 99].
Figure 4. Citrullination occurs due to the conversion of amino acid arginine to citrulline by PAD enzyme in the presence of calcium (Klareskog et al, Annu Rev Immunol, 2008).

5.3 Synovial inflammation and the RANKL/RANK/OPG system

In RA, it has been postulated that bone destruction occurs as a direct consequence of inflammation. This has been recently challenged by the observation that bone destruction can occur in RA despite clinical remission [100, 101], while specific anti-rheumatic drugs and/or combination of drugs might protect against destruction even in the absence of clinical remission [102, 103]. Taken together these findings suggest that inflammation and bone destruction are at least partially uncoupled in RA. Bone destruction is a dynamic process and homeostasis of bone metabolism is maintained by balance between bone matrix restoration and resorption [104, 105]. Bone is a rigid and hard tissue that is constantly being reshaped by osteoblasts and osteoclasts, in which osteoblasts are responsible for the production and mineralization of bone matrix to synthesis bone, whereas osteoclasts are multinucleated giant cells responsible for the breakdown of bone by demineralization and subsequent transfer of calcium from bone fluid to the blood. In a healthy individual the function of these two cells maintain the normal physiological bone metabolism and architecture. The underlying key molecular cascade that is responsible for bone resorption and restoration is mediated through RANKL/OPG/RANK pathway system [106, 107].

RANKL is also known as osteoclast differentiation factor (ODF), Osteoprotegerin ligand (OPGL), and TNF related activation-induced cytokine (TRANCE) [108] and is a member of the tumor necrosis factor family of transmembrane proteins. Three RANKL isoforms are currently identified where RANKL1 and 2 induces multinucleated osteoclasts while RANKL3 lacking the intracellular and transmembrane domains has no effect on osteoclasts when expressed alone [109]. RANKL is expressed by activated
T-cells, fibroblasts and stromal/osteoblastic lineage cells. OPG is mainly expressed by endothelial cells, osteoblasts and fibroblasts [110, 111]. Receptor activator NF-kappa B (RANK) is a membrane receptor protein that is expressed mainly by osteoclast precursor cells in the synovial joint and is an important factor in the regulation of osteoclastogenesis [112].

RANKL effects are counteracted by Osteoprotegerin (OPG), a secretory active decoy receptor protein also known as Osteoclastogenesis inhibitory factor (OCIF), able to bind directly to both membrane bound and soluble form of RANKL [113]. In the presence of macrophage colony stimulating factor (M-CSF), RANKL acts as a regulatory factor for fusion, activation and differentiation of multinucleated mature osteoclast to promote bone resorption [114]. M-CSF is a homodimeric glycoprotein promoting differentiation of monocytes and osteoclast survival [115, 116]. The binding of RANKL to RANK receptor induces the trimerization of RANK and TRAF6 adaptor molecule, leading to activation of mitogen activated kinase (MAPK) and NF-κB pathway [117]. Activation of RANK receptor also induces the transcriptional factor called activator protein 1 (AP1) through cellular proto-oncogene cFOS induction [118]. RANKL acts as a major stimulator of the Nuclear factor of activated T cells cytoplasmic 1 (NFATc1) through TRAF6, NF-κB and cFOS pathway needed for terminal differentiation of osteoclasts [119]. NFATc1 regulates expression of a set of genes essentials for osteoclasts such as tartrate resistant acid phosphatase (TRAP), cathepsin K, calcitonin, osteoclast associated receptor (OSCAR) and β₃-integrin [119].

RANKL is considered to be the main link between immune mediated inflammation and bone metabolism [120]. Activated macrophages produce pro-inflammatory cytokines such as TNFα, IL-1 and IL6, all able to enhance RANKL mediated osteoclastogenesis [121]. TNFα and IL-1 also up-regulate RANKL expression in osteoblast cells [122]. Activated T cells present at the site of synovial inflammation are able to secrete IL-17 a potent inducer of osteoclastogenesis [123]. This effect is counteracted by T cells producing IFN-γ that can inhibit osteoclasts occur through RANKL signaling and down-regulation of TRAF6. The balance between IL-17 and INF-γ secretion determines the ability of the T cell to mediate osteoclastogenesis [123, 124]. It has also been shown that in adjuvant arthritis model, activated T cells are able to induce osteoclast differentiation directly through RANKL expression [125]. Interaction between RANKL and RANK has a regulatory function on DC activation through T cell-DC interaction [126]. Moreover, RANKL is an important differentiation factor for
cell migration and tissue-specific metastatic behavior of cancer cells and may function as a chemokine [127]. Figure 5 schematic representation of the current view on the link between inflammation and bone destruction and the role of RANKL pathway in destructive arthritis.

**Figure 5.** Bone metabolism is exemplified by perfect balance between RANKL and OPG ratio. During inflammatory conditions and other physiological disorders leads to the excessive RANKL production which results in bone destruction.

### 5.4 Synovial inflammation and hypoxia

Local synovial accumulation of immune cells and pro-inflammatory cytokines promotes synovial hypertrophy and results in very low levels of oxygen tension with profound local hypoxia. These changes promote tissue angiogenesis resulting in new vessels of bad quality further contributing to decreased oxygen supply into the tissues. An inflamed joint is characterized by oxygen levels ranging from 2 to 4% (18 to 33 mmHg), as compared to 9 to 12% (69 to 89 mmHg) in patients without RA [128, 129]. The hypoxic environment of the chronically inflamed joint has been also confirmed by synovial identification of different traits associated with the typical anaerobic metabolism with raised carbon dioxide, lactate, and acetate levels, with a consecutive decreased synovial pH level [10]. With the aid of NMR spectroscopy technique, low molecular weight metabolites consistent with hypoxia were identified in the inflamed joints environment [130]. Visualization of tissue hypoxia can be done using intravital pimonidazole staining or immunohistochemical detection of HIF [131, 132]. The central role of hypoxia in the pathogenesis of inflammatory arthritis has been demonstrated when HIF deletion in macrophage and neutrophils resulted in complete protection from arthritis induction [133]. Furthermore, therapeutic inhibition of HIF-1α nuclear translocation suppresses transcription of the VEGF and ameliorates adjuvant-induced arthritis in rats [134]. Both HIF-1 and 2α are expressed in the RA in synovial lining layer and stromal cells [135].
In order to adapt to hypoxia a transcription factor HIF-α (hypoxia inducible factor alpha) is activated, that in turn activates a large set of genes responsible for cell survival despite low levels of oxygen. HIF is a heterodimeric transcription factor consists of β-subunit and an oxygen regulated α-subunit. HIFα and β subunits dimerize and then translocate to nucleus and bind to DNA. HIF-α stability and function is maintained by oxygen-dependent prolyl hydroxylase domain-containing proteins (PHD), targeting critical proline residues. As a consequence, HIF-α subunit becomes recognizable by the von Hippel Lindau (vHL) tumour suppressor protein/E3 ubiquitin ligase, leading to polyubiquitination and proteosomal degradation [136, 137]. HIF-α subunit also contains two transactivation amino-terminal and carboxyl-terminal domains (C-TAD). HIF-α C-TAD interacts with two transcriptional co-activators called Creb binding protein (CBP) and p300 for transcriptional activation [138]. Under hypoxic conditions HIF-α subunit gets stabilized and translocate to the nucleus, and dimerize with β-subunit by basic helix-loop-helix and Per-Arnt-Sim domains and binds to the hypoxia-responsive elements (HRE) to regulate transcription of downstream target genes (Figure 6). Three different HIF isoforms have been reported. HIF-1α is ubiquitously expressed in all tissues. HIF-2α expression is more tissue restricted and mainly present in high-vascularized tissues such as lung, kidney and liver. HIF-3α is expressed only in kidney, brain, heart and thymus [139, 140]. It is believed that HIF-1α and HIF-2α encounter similar responses to hypoxia due to their structural similarities. However, HIF-1α is rapidly and transiently expressed during hypoxia, whereas HIF-2α expression becomes steady after more prolonged hypoxia exposure [141].

Figure 6. Schematic illustration of oxygen dependent HIF-α regulation of downstream target genes.
Apart major regulation by oxygen levels, several other stimuli such as pro-inflammatory cytokines modulate HIF expression under normoxic conditions. TNFα mimics hypoxia by inducing HIF-1α expression and stability via NF-κB pathway [142]. The intracellular IKKb kinase essential for NF-κB activation is also a critical transcriptional activator of HIF-1α in macrophages responding to bacterial infection [143]. Hypoxia induced neutrophil survival is mediated by HIF-1α dependent NF-κB activity [144].

6. Therapeutic targeting of synovial inflammation

Synovial inflammation is targeted today by several distinct drug classes such as NSAID, glucocorticoids, disease modifying anti rheumatic drugs (with methotrexate being the most frequently used) and more newer biological drugs. While biologicals have developed as a direct consequence of the better understanding of the molecular mechanisms responsible for synovial inflammation, older drugs such as DMARDs and glucocorticoids were initially used empirically based on the effects of these drugs on other disease entities. Consequently less is known on the exact mechanism of action of these drugs despite their long use in clinical practice. A better understanding of their clinical and molecular effect is a pre-requisite for a better use of the therapeutic arsenal available today and future development of new therapeutic strategies. We studied therefore not only new potential pathogenic mechanisms important for development of synovial inflammation but also how different distinct anti-rheumatic drugs modulates these processes. We focused our studies on the mechanisms of action of methotrexate and intra-articular glucocorticoids.

6.1 Anti-inflammatory effects of methotrexate (MTX) in RA

MTX is a folate antagonist that acts by inhibiting dihydrofolate reductase and blocks the conversion of dihydrofolate to tetrahydrofolate in the folic acid metabolism, essential for purine and pyrimidine synthesis. MTX enters the cell and exert its effect through the reduced folate carrier (RFC1) [145]. After absorption MTX is converted into 7-hydroxymethotrexate in the liver and shows low to medium protein binding and high tissue distribution [146]. After administration MTX diminishes its presence in 24 hours and its half-life is 6 to 8 hours. MTX and hydroxymethotrexate are metabolized to polyglutamate derivatives (MTXGlu) stored in tissues as well as in liver and erythrocytes for long time [147]. MTXGlu inhibit 5-aminomidazole-4-
carboxyamideribonucleotide (AICAR) transformylase leading to accumulation of intracellular AICAR and AICAR metabolites, which in turn directly inhibit adenosine and AMP deaminases. As a consequence adenosine and adenine nucleotides accumulate intracellularly [146]. In parallel an increase in the extracellular adenosine has also been reported but the exact mechanism leading from intra to extra-cellular accumulation of adenosine is still incompletely understood. Adenosine is a purine nucleoside which can binds to adenosine receptors such as A1, A2a, A2b and A3 present on different cells and exerts either immunostimulatory or immunosuppressive effects. It has been shown that adenosine acts as an endogenous regulator of innate immunity and activation of A1-receptor stimulate neutrophils, while activation of A2a and A3 receptor has immunosuppressive effects [148]. It is believed that MTX partly exerts its anti-inflammatory effects through an A2a or A3 receptor mediated effects [149].

Other suggested mechanisms of action for MTX are suppression of T cell proliferation, apoptosis induction [150, 151] and modulation of cytokine production including IL-4, IL-6, IL-13, TNFα, IFN-γ and GM-CSF [152-154]. MTX also inhibits the cell contact-dependent interaction between fibroblast and T cells [155]. Other studies have demonstrated that MTX decreases IgG FcγR expression on monocytes and thus prevent activation of immune complex formation [156]. MTX also has an inhibitory effect on cellular adhesion molecules including E-selectin and ICAM-1, which result in the reduction of tissue cellular recruitment [157, 158].

6.2 Anti-inflammatory effects of intra-articular glucocorticoids (GCs) in RA

GCs are potent anti-inflammatory drugs, widely used as an adjuvant for the treatment of RA to dampen the inflammation in accessible joints[159]. Among intra-articular GCs, triamcinolone hexacetonide and methylprednisolone acetate are the most commonly used. Treatment with intra-articular GCs significantly reduces synovial infiltration of T-cells [160] Fcγ receptor expression [161] and production of pro-inflammatory cytokines and adhesion molecules such as TNFα, IL-1β, HMGB-1, ICAM-1 and VEGF [33]. Treatment with intra-articular GCs appears not only to modulate synovial inflammation but possibly also cartilage and bone destruction as demonstrated by decreased serum levels of markers for cartilage turnover [162] and decreased
synovial RANKL expression following intra-articular administration of GC [160]. *In vitro* exposure of T cells to GCs induces apoptosis, primarily mediated through the mitochondrial cell death pathway [163].
7. GENERAL AIM OF THESIS
The general aim of the current thesis is to identify and characterize new molecular mechanisms responsible for synovial inflammation and bone destruction in RA.

7.1. Specific aims of the thesis

- To characterize sensitivity of RA synovial T cells to GC-induced apoptosis \textit{in vivo} and \textit{in vitro}.
- To characterize synovial citrullination patterns in relation to local inflammation and anti-rheumatic treatment in RA.
- To characterize synovial expression of bone remodeling markers (RANKL, RANK and OPG) in early untreated RA and their modulation by methotrexate.
- To study the interaction between inflammation and hypoxia in promoting inflammation associated bone loss in RA.
8. RESULTS AND DISCUSSION

The present work focused on the investigation of alternative molecular mechanisms that might be relevant for and contribute to perpetuation of chronic synovial inflammation in RA.

**Low levels of apoptosis in RA synovial T cell population is independent of local levels of inflammation.**

Others and we have previously showed that low levels of synovial apoptosis characterize RA monocyte/macrophage population [79, 164]. We aimed in the first study (paper 1) to extend our findings to characterize synovial T cells apoptosis. We first confirmed low levels of synovial RA apoptosis evaluated as both TUNEL positive (5.2±1.3, mean±SEM) and active caspase 3 positive (3±0.4, mean±SEM) cells. Furthermore, we identify the synovial CD3+ cells as a highly apoptosis-resistant subpopulation, with less than 2% of them showing morphological signs of apoptosis. Administration of intra-articular GC failed to induce synovial apoptosis despite a general reduction of the CD3 subpopulation and good clinical response in all treated patients as evaluated by arthroscopy (figure 7).

![Figure 7](image.png)

*Figure 7. Knee arthroscopic images of RA joint before (A) and after (B) intra-articular GCs.*

Also confirming our previous data no changes in the number of macrophages evaluated as both CD68 and CD163+ cells were observed following intra-articular administration of GC (figure 8).
Figure 8. Synovial biopsy tissues showing surface markers CD68 (A) and CD163 (B) before and after intra-articular GC treatment.

Taken together our biopsy data suggest that in vivo intra-articular GC reduces synovial inflammation through reduction of the number of CD3+ lymphocytes without influencing the monocyte/macrophage population. The mechanism responsible for the reduction of the CD3+ cell number is decrease local recruitment [33] rather than increased apoptosis.

RA apoptosis-resistant T cell phenotype is due to soluble factor(s) cross talk between monocytes and lymphocytes.

To further investigate the mechanisms rendering synovial T cells resistant to apoptosis, we first examined the capacity of different synthetic glucocorticoid preparations to mediate apoptosis of T cells derived from the SF of RA patients. None of the investigated compounds were able to induce CD3+ cell apoptosis when SF derived mononuclear cells containing a mixture of both lymphocytes and monocytes were cultured together. A positive control for apoptosis induction in our cell systems showed that GC were able to induce apoptosis of CD3+ cells derived from the SF of patients with another type of inflammatory joint disease (psoriasis disease). Separate cell culture of RA-derived isolated CD3+ cells in the presence of GC resulted in apoptosis induction suggesting that interaction between monocytes and lymphocytes is essential for maintaining an apoptosis-resistant phenotype in RA lymphocytes. Interestingly, when isolated monocytes and lymphocytes were co-cultured in transwell experiments, allowing inter-cellular communication via soluble factors but not physical cell-cell
contact, CD3+ cells were rendered again resistant to GC mediated apoptosis. We conclude that T cell resistance to apoptosis seems to be an RA specific trait and mediated through the soluble cross talk with monocytes. The exact mediator(s) remains to be determined.

**Synovial citrullination is readily detected in the RA synovium and dependent on local inflammation.**

Others and we have previously shown that citrullination occurs in different contexts of inflammation and in a variety of tissues [98]. In (paper 2) we aimed to further characterize the pattern of synovial citrullination in relation to local inflammation. At the time of this study was initiated technical difficulties in detecting the presence of citrullinated proteins in tissues and Western blots appeared, due to the disappearance form the market of the well validated modification method originally described by Senshu [165]. To circumvent this we chose to use a mouse IgM monoclonal antibody (F95) raised against a deca-citrullinated peptide that we have previously validated by comparison with the Senshu method [166]. As expected citrullination was readily detected in the inflamed RA synovium and virtual absent from the healthy non-inflamed synovial tissue. We further analyzed expression patterns of PAD-2 and PAD-4 the only peptidyl deiminases present in synovial tissue. PAD-2 and PAD-4 expression was detected in all baseline RA samples and a vast majority of the healthy synovial biopsies. Correlations between semi-quantitative scores for synovial citrullination and PAD enzymes expression on one hand and inflammation on the other hand further confirmed the closed interrelationship between the two phenomena.

**Synovial citrullination is specifically down regulated by GC but not MTX.**

Following our observations showing a high degree of correlation between synovial citrullination and inflammation we hypothesized that successful anti-rheumatic treatment would result in a significant decrease of the levels of synovial citrullination. Our hypothesis was partially confirmed when synovial biopsies obtained following intra-articular GC showed significant lower levels of both citrullinated proteins and PAD-4 expression. In contrast no such changes were observed for MTX despite clinical good response in a majority of the patients. To investigate a potential direct effect of GC on cellular citrullination and PAD expression independent of inflammation we further tested the *in vitro* effect of both GC and MTX in two *in vitro* assays: synovial explants and cultured SFMC. In both systems GC significantly down regulated both
expression of citrullinated proteins and PAD-4 expression. One possible explanation of the differential effect is a direct NF-κB dependent down regulation of PAD-4 expression by GC but not MTX. The exact molecular mechanisms behind our observations as well as the effects of other anti-rheumatic drugs remain to be determined.

**Disconnection between synovial inflammation and bone destruction in early RA.**

Resident and inflammatory cells in the rheumatoid synovium are a source for local joint production of both RANKL and OPG. Expression of these molecules in RA appears to be independent of local inflammation. However, all previous studies were conducted in established RA where several confounding factors, and especially concomitant anti-rheumatic drugs, preclude a clear conclusion. Therefore in (paper 3) we investigated expression of RANK, RANKL and OPG in early-untreated RA synovium in relation to local synovial inflammation and radiographic evaluation of bone destruction. As previously showed, early-untreated RA synovium has architecture indistinguishable of established RA, with high number of macrophages and lymphocytes and increased vascularity [167]. Also, a relative high RANKL in relation to OPG expression was observed accompanied by a general high level of expression of RANK. No correlation between synovial expression of RANKL and local synovial inflammation were observed.

**Targeting synovial inflammation and bone destruction in early untreated RA.**

To further analyze the interplay between inflammation and bone destruction in early-untreated RA, we followed up newly diagnosed RA patients with a new biopsy after 8 weeks of MTX treatment. As expected MTX reduced synovial inflammation, as evaluated by immunophenotyping of the cells. A reduction of the RANK and RANKL/OPG ratio synovial expression was also observed. In accordance with baseline data however no correlations were observed between markers of inflammation and markers of bone turnover in the biopsies obtained after 8 weeks of MTX treatment. Also, changes in the RANKL/OPG ratio were observed both in responders and non-responders to therapy and mainly limited to those not showing radiographic progression. Taken together our biopsy findings give further support to a partial uncoupling between inflammation and bone destruction in RA and suggest synovial
expression of RANKL and OPG as a potential synovial biomarker for patients that will eventually show progression of their erosions despite treatment.

**In vitro studies of the molecular mechanisms responsible for inflammation-independent bone protective effect of MTX.**

To confirm the potential direct effect of MTX on bone metabolism we first investigated the effect of *in vitro* treatment with MTX on SFMC, osteoblast like cells and fibroblasts cells. Using either FACS, quantitative PCR, Western blots or ELISA we were able to confirm a direct effect of MTX to reduce RANKL expression both at the RNA and protein level. Incubation of CD14+ osteoclast precursors with conditioned medium from MTX treated osteoblasts reduced pre-osteoclast formation as compared to conditioned medium form control treated osteoblasts even in the absence of exogenous RANKL. These findings suggest that the MTX induced changes in the RANKL/OPG ratio in osteoblasts supernatants might have functional relevance. We were however not able to detect many mature osteoclasts, probably due to very low levels of RANKL in culture supernatants. We further examined the direct effect of MTX on osteoclast differentiation and maturation in a system where enough exogenous RANKL was provided. MTX was able to decrease the number of mature osteoclasts formed from CD14+ precursors. This effect was not mediated through induction of apoptosis as evaluated by FACS staining with annexin V and 7-AAD (figure 9).

![Figure 9. Flow cytometric analysis showing no changes in annexin V (A) and 7AAD (B) gated CD14+ cells after MTX treatment.](image-url)
Taken together our *in vitro* results demonstrate a dual mechanism by which MTX might influence bone destruction independent of inflammation: modulation of the RANKL expression and direct cellular effect on osteoclast precursors. A schematic representation of our view on the effect of MTX on bone metabolism in RA generated by our study is given in (figure 10).

**Figure 10.** Schematic illustration of the current studies on the effects of methotrexate on bone metabolism in RA.

**Hypoxia directly induces RANKL expression in osteoblasts and promotes osteoclastogenesis**

To further characterize inflammation-independent mechanisms we investigated in (paper 4) if hypoxia a central trait or the rheumatoid joint can modulate bone destruction. The inflamed RA synovium is characterized by profound hypoxia as low as 1-2% oxygen and local expression of both HIF-1α and HIF-2α [135] (figure 11).

**Figure 11.** Immunohistochemistry showing HIF-1α (A) and HIF-2α (B) expression in RA synovial tissue.
Exposure of osteoblasts to hypoxia increased RANKL and decreased OPG mRNA expression. As a result RANKL cellular protein expression was increased and soluble OPG decreased. Transiently transfected siRNA against pVHL under normoxic conditions induced increase in RANKL mRNA expression in osteoblast cells suggesting that the modulation of RANKL through a HIF mediated mechanism. HIF-2α but not HIF-1α silencing was able to abolish the effect of hypoxia on RANKL expression and ChIP confirmed the direct interaction between HIF-2α with at least one hypoxia responsive element (HRE) in the RANKL promoter. This is the first report to demonstrate HIF-2α recruitment to the RANKL promoter.

**Hypoxia acts synergistically with TNF to promote osteoclastogenesis**

As our original hypothesis was that hypoxia modulates bone destruction independent of inflammation we tested the effect of these two conditions on RANKL/OPG pathway and osteoclastogenesis either alone or in combination. Simultaneous exposure of osteoblasts to hypoxia and TNF resulted in an additive induction of the RANKL/OPG ratio as compared to each condition alone. Culture of PB derived CD14+ cells in the presence of conditioned media from osteoblasts exposed to either hypoxia or TNF alone without exogenous RANKL added resulted in minimal non significant fold increase in the number of both pre-osteoclasts (mono and binuclear TRAP positive cells) and mature osteoclasts (multinucleated TRAP positive cells). When same cells were cultured in the presence of conditioned medium from osteoblasts exposed to both hypoxia and TNF, a significant fold increase in the number of both pre-osteoclasts and mature osteoclasts was observed. Taken together our findings suggest that hypoxia increases the RANKL/OPG ratio in osteoblasts promoting their osteoclastogenetic potential. We further demonstrated that also in the presence of excess exogenous RANKL chemical hypoxia (DMOG) acts additively with TNF to promote differentiation and maturation of osteoclasts through a direct cellular mechanism. We propose that hypoxia acts through dual mechanisms and synergistically with inflammation to promote bone destruction in RA (figure 12).
Figure 12. Schematic representation of the complex interaction between inflammation and hypoxia leading to bone destruction in RA.
9. CONCLUSIONS

The current thesis provides new insights in the molecular mechanisms responsible for chronic inflammation and bone destruction in rheumatoid arthritis:

1. Chronic synovial inflammation is partially dependent on defective T cell apoptosis dependent on the soluble cross talk with monocytes.

2. Synovial citrullination is dependent on local inflammation and targeted by local administration of glucocorticoids but not by systemic treatment with methotrexate.

3. Bone destruction is partly uncoupled from synovial inflammation in early RA and specific targeted by methotrexate.

4. Synovial expression of RANKL and OPG is a potential synovial biomarker for future radiographic progression.

5. Hypoxia induces RANKL expression through a HIF-2α mechanism and promotes osteoclastogenesis.

6. Hypoxia and inflammation additively promotes bone destruction.
10. FUTURE PERSPECTIVES

The present thesis aimed to identify and study new molecular pathways contributing to chronic inflammation and bone destruction in RA. We started from our previous observations showing that defective apoptosis is an important feature of the rheumatoid joint that can be reversed by targeting synovial macrophages with TNF antagonists. Continuing the same line of research we investigated synovial derived T cells and demonstrated that these cells are highly resistant to apoptosis when present in the specific inflammatory joint milieu. However upon isolation, T cells were rendered sensitive to apoptosis suggesting that soluble factors secreted by monocytes are essential in inducing the apoptosis resistance phenotype. It is therefore most probably that targeting of monocytes and/or the interaction between monocytes and T cells is more effective than selective target of T cells in RA. Also, future directions of research opened by this observation are investigation of the interaction between T cell subsets and monocytes as well as characterization of the soluble factor(s) responsible for this. Once inflammation develops in a certain tissue, local citrullination increases. Our hypothesis was that, despite the fact that citrullination is not a RA specific phenomena, it still can contribute to local propagation of synovial inflammation. We also thought that effective anti-rheumatic treatment would result in a decrease of the levels of synovial citrullination. To our surprise we observed lower levels of citrullination following administration of intra-articular glucocorticoids but not following methotrexate treatment suggesting a more fine regulation of synovial citrullination. Future extension of our studies to other anti-rheumatic drugs and identification of the exact molecular mechanisms responsible for this effect are on the future research agenda.

Observations coming from clinical studies have suggested that synovial inflammation and bone destruction are at least partly uncoupled. For example, there are patients not having any clinical detectable inflammation of the joints that still progress to bone destruction and the other way around, patients with ongoing joint inflammation despite anti-rheumatic treatment not necessarily progress to bone destruction. We wanted to better understand this intriguing and challenging observation, so we focused in the second part of the thesis on the interaction between inflammation and bone destruction. We were able to demonstrate that markers of increased bone destruction are present in the early-untreated RA synovium and their expression is independent of local
inflammation but direct modulated by methotrexate treatment. We also identified hypoxia as a new molecular mechanism, modulating bone destruction independently but additively with inflammation. More in depth studies on the modulation of the RANKL pathway and hypoxia in the context of chronic inflammation will contribute to better understanding of RA pathogenesis and potential identify new therapeutic targets.

Even though the current thesis has added to the knowledge on disease pathogenesis in RA, further research is needed in order for this knowledge to be translated in clinical practice and used for the better of RA patients.
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12. REFERENCES


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