Studies of molecular mechanisms of action of TNF antagonists in rheumatoid arthritis

Anca Irinel Catrina, MD

Stockholm 2004
To my wonderful family
ABSTRACT

Rheumatoid Arthritis (RA) is a common chronic inflammatory disease characterized by progressive bone destruction that leads to joint deformity and physical disability. Even though several therapeutic drugs are available, none have emerged as an ideal RA treatment that delays joint destruction and halts disease progression. A new class of drugs, tumor necrosis factor (TNF) antagonists, has recently been introduced in clinical practice: infliximab (chimeric anti-TNF antibody), etanercept (soluble TNF receptor) and adalimumab (fully human anti-TNF antibody). The exact mechanisms of action of these drugs are still poorly understood, even though the important role played by cytokines in RA pathogenesis is the main rationale behind using them for treatment. This thesis investigates the molecular mechanisms of action for TNF antagonists in RA with a focus on synovial inflammation and bone destruction.

A major feature of RA is synovial inflammation with local accumulation of immune cells through increased cell influx and decreased clearance of resident cells. We demonstrated that early RA, which is characterized by important macrophage infiltration, is associated with low levels of synovial apoptosis. We also identified macrophage infiltration and synovial expression of the anti-apoptotic molecule FLIP (FLICE inhibitory protein) as determinant factors of synovial apoptosis. As apoptosis is a potential relevant mechanism for RA, we investigated if treatment with TNF antagonists modulates this process. We demonstrated that therapy with both infliximab and etanercept induces apoptosis of macrophages but not of lymphocytes in RA joints. Blood-derived macrophages were less susceptible to anti-TNF induced apoptosis, suggesting that induction of apoptosis through TNF blockade is specific for an inflammatory milieu such as the rheumatoid joint.

Synovial inflammation leads to bone destruction that is mediated through either an indirect mechanism induced through cytokine-mediated release of pro-destructive factors such as the matrix metalloproteinases (MMP)-tissue inhibitors of MMPs (TIMPs) system or a direct mechanism mediated through receptor activator of the nuclear factor-κB ligand (RANKL)-osteoprotegerin (OPG) system. We demonstrated that etanercept is able to decrease serum levels of MMPs and the ratio between MMPs and TIMP, which represents a potential mechanism involved in prevention of future development of joint damage. Moreover, baseline MMP-3 serum levels could predict the changes in disease activity during therapy. The RANKL/OPG system is considered to be the final denominator of bone remodeling. We demonstrated that treatment with both etanercept and infliximab increased synovial OPG expression without changes in RANKL expression. The synovial RANKL/OPG ratio thus decreased following therapy, the effect being more pronounced in the responders compared to non-responders to therapy.

In conclusion, we have demonstrated that TNF antagonists modulate important mechanisms implicated in synovial inflammation and bone destruction. We propose that therapies which target synovial TNF independent mechanisms, such as RANKL expression and lymphocyte apoptosis, are also valuable candidates for adjuvant therapy in RA.
PUBLICATIONS

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

I. **Catrina AI**, Ulfgren AK, Grondal L, Lindblad S, Klareskog L.
   Low levels of apoptosis and high FLIP expression in early rheumatoid arthritis.
   *Annals of the Rheumatic Diseases, 2002, 61(10):934-936*

II. **Catrina AI**, Trollmo C, af Klint E, Engstrom M, Lampa J, Hermansson Y, Klareskog L, Ulfgren AK.
   Evidence that anti tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages but not lymphocytes in RA joints.
   *Accepted for publication in Arthritis and Rheumatism*

III. **Catrina AI**, Lampa J, af Klint E, Bratt J, Klareskog L, Ulfgren AK.
    Anti-tumour necrosis factor (TNF)-α therapy (etanercept) down regulates serum matrix metalloproteinase (MMP)-1 and MMP-3 in rheumatoid arthritis.
    *Rheumatology, 2002, 41:484-489*

IV. **Catrina AI**, af Klint E, Ernestam S, Catrina SB, Klareskog L, Ulfgren AK.
    Anti TNF therapy induces synovial osteoprotegerin expression in rheumatoid arthritis.
    *Manuscript*

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease-activating factor 1</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell leukemia/lymphoma-2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>Cyt c</td>
<td>Cytocrome c</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DAS</td>
<td>Disease Activity Score</td>
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<tr>
<td>DMARD</td>
<td>Disease modifying anti rheumatic drug</td>
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<tr>
<td>EULAR</td>
<td>European League against Rheumatism</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythocyte sedimentation rate</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FLICE</td>
<td>Fas ligand-interacting cell effector</td>
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<td>FLIP</td>
<td>FLICE inhibitory protein</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence-1</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>NSAID</td>
<td>Nonsteroid antiinflammatory drugs</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>ra</td>
<td>Receptor antagonist</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of the nuclear factor-kB ligand</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
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<tr>
<td>SF</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>SFMC</td>
<td>Synovial fluid mononuclear cells</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUMO-1</td>
<td>Small ubiquitin-related modifier-1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of the matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNF-R</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>TRADD</td>
<td>Tumor necrosis factor receptor associated death domain</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl-mediated dUTP nick end labelling</td>
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As long as you don’t stop climbing, the stairs won’t end, under your climbing feet they will go on growing upwards.

Franz Kafka
1 RATIONALE
Chronic diseases currently represent the leading cause of death, being among the most prevalent and costly health problems (1). One example is Rheumatoid Arthritis (RA), a chronic inflammatory disease that affects 0.5-1% of the population (2). It is often characterized by progressive joint destruction and deformity, leading to decreased quality of life and work capacity as well as increased mortality (3). Even though several therapeutic strategies for treating RA patients are currently successfully used in clinical practice, none of them has emerged as being an ideal treatment. Thus the main research question in the field is: which drug for which patient? Patient-oriented research is a valuable tool to study the effect of new therapeutic agents and to directly address this issue. Moreover, this type of research offers new insights into the different molecular pathways implicated in disease pathogenesis.
This thesis focuses on the use of TNF antagonists in RA treatment. By describing new mechanisms of action we hope to contribute to a better understanding of RA pathogenesis and of a more rationale use of these drugs, eventually leading to a better treatment for the benefit of the patient.

2 BACKGROUND
RA was described as a distinct disease entity in the early 1800s and the term “rheumatoid arthritis” was introduced by Sir Alfred Baring Garod in 1856 (4). However, a clear classification scheme for the disease was only defined in 1987 (5).

2.1 RA – A SHORT CLINICAL PERSPECTIVE
2.1.1 Epidemiology
RA is one of the most common chronic inflammatory disease with an annual incidence of approximately 0.2% in males and 0.4% in females, and an age peak onset during the sixth decade of life. With few exceptions similar prevalence estimates varying between 0.5-1% of the total population have been obtained from different populations worldwide (6).

2.1.2 Etiology
RA is an inflammatory disease that develops in a genetically susceptible host and is influenced by environmental factors. The genetic influence has been demonstrated by
studies showing a greater concordance in monozygotic as compared to dyzizotic twins (7, 8). Both HLA and non-HLA genes have been identified as potential candidates to explain genetic susceptibility to disease (9). However, it is still debated whether genetic factors are important in determining disease susceptibility or disease evolution. Environmental and lifestyle factors potentially implicated in disease etiology are obesity, smoking, previous blood transfusions and professional exposure to organic solvents. No conclusive epidemiological evidence of a single infectious trigger exists (10).

2.1.3 Classification and natural evolution

RA is defined accordingly to the 1987 American College of Rheumatology criteria (table 1) (5). It is a disease that mainly affects joints with synovial inflammation followed by bone and cartilage destruction, but could also present with extra-articular manifestations. RA in most patients is a progressively disabling disease that is associated with higher-than-normal mortality rates (3). Despite increasingly aggressive treatment and the use of more effective treatment regimens, a substantial percentage of patients still fail to respond adequately to current therapies.

2.1.4 Evaluation and outcome of the patient

RA evaluation consists in estimates of (1) disease course in clinical practice with no single reference comparison and (2) intervention in clinical trials with reference comparison (11). While the first type of estimate offers a picture of the general disease course over time, the second focuses on the change of disease activity during a determined time frame during therapeutic intervention. Disease course is measured through core sets and clinical indices while intervention is evaluated by fulfillment of specific response/improvement criteria.

Core sets of valid variables to evaluate disease activity are currently used in clinical practice and comprise tender joint count, swollen joint count, pain on a visual analogue scale, patient’s global disease activity, and assessor’s global disease activity, physical function, radiographic analysis and acute phase reactants (12). Among these, acute phase reactants such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are valuable tools in daily practice. These measurements are nonspecific markers of inflammation, relatively easy to perform and well standardized in different laboratories (13). CRP is less susceptible to confounding factors compared to ESR (14).
It has been demonstrated that they correlate well with other measures of disease activity (15). Moreover, there are some reports suggesting that these two parameters may predict radiological progression (16).

| Table 1. The 1987 American College of Rheumatology classification criteria for RA (5) |
|---------------------------------|------------------------------------------------------------------|
| **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 have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP (proximal interphalangeal), MCP (metacarpophalangeal), wrist, elbow, knee, ankle, and MTP (metatarsophalangeal) 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 acceptable 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 result has 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.

Clinical indices represent a more precise tool for evaluating disease activity compared to clinical core sets, which are useful for a comparative interpretation of distinct clinical
trials. Clinical indices are continuous (such as disease activity score, DAS) or ordinal (such as ARA remission) scales to measure current disease activity. DAS is a composite statistically-derived index including tender joints, swollen joints, ESR and general health (17). It has been validated in different studies and later modified for use with the simple 28 joint count (DAS28 score) (18), being a valuable tool for disease activity assessment in new clinical trials.

Improvement criteria are used to define response to treatment. They consist of evaluation of either changes (such as ACR criteria) or both changes and current disease activity (such as EULAR criteria). The ACR improvement criteria (19) were defined by validation of arbitrarily chosen improvement definitions that maximally discriminated effective treatment from placebo treatment and also minimized placebo response rates (table 2). EULAR response criteria (20) are based upon DAS or DAS28, and define a responder as an individual who has both a significant change in disease activity and a low current disease activity. These criteria define three categories of therapy response: good response, moderate response and no response (table 2).

A golden standard to measure treatment effectiveness is radiographic evaluation. The most frequently used methods are radiographic scoring methods developed by Sharp (21) and Larsen (22). Based on such evaluations a drug could be designated as a DMARD (disease modifying antirheumatic drug) that delays or stops radiographic progression as well as preserving functional capacity (21). Indirect measures of disease progression such as quality of life (23), comorbidity or drug toxicity (24) are also important to evaluate.

<table>
<thead>
<tr>
<th>Table 2. Improvement criteria for treatment evaluation in RA</th>
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<tbody>
<tr>
<td><strong>Improvement criteria</strong></td>
</tr>
<tr>
<td>ACR (19)</td>
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<td></td>
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<td>EULAR (20)</td>
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2.2 PATHOGENESIS - CLUES FOR THE TREATMENT

2.2.1 Pathogenic hypothesis of the disease

RA etiology still represents a challenge. However, insights into pathogenesis offer the basis of a better understanding and treatment. Several candidate pathogenic pathways have been identified and the generally accepted theory today is that immune, inflammatory and genetic mechanisms are involved to different extents in initiation and/or perpetuation of the disease, without being mutually exclusive. To date, both pro- and contra-arguments exist concerning the relevance of these mechanisms in RA pathogenesis, implying careful consideration of their interpretation.

*T cell-mediated antigen-specific immune reactions* have often been postulated in RA. Foreign antigens such as bacteria and viruses could theoretically play an important role in disease initiation, either through a direct mechanism or through molecular mimicry (25). However, none of these proposed mechanisms has yet been proven.

*T cell-mediated autoantigen-driven immune reactions* have received much attention in conjunction with the observation that autoantibodies (such as the rheumatoid factor and anti-citrulline antibody) may represent important diagnostic tools. However, we should always keep in mind that autoantibodies could be either a driving pathogenic mechanism or an epiphenomenon of disease pathogenesis (26).

*T cell-mediated antigen-independent immune reactions* are also implicated in RA pathogenesis. An example is the interaction between T cells and macrophages, partly sustained through IL-15 (27). Further studies to evaluate the relevance of these mechanisms are needed.

*Synovial cell-mediated inflammation* has long been recognized as an important mechanism in disease perpetuation. Synovial resident cells such as fibroblasts and macrophages together with the complex synovial cytokine network are key players in RA in a paracrine or autocrine fashion (28). However, this mechanism could not explain the initiation phase of the disease.

*Genetic mechanisms* that control disease susceptibility are mainly represented by association between RA and the shared epitope (a specific amino acid sequence on DR4) (29). Structural characterization of the interaction between HLA class II and the
T cell receptor gave rise to three potential pathogenic models for RA: (1). positive thymic selection of autoreactive T cells through self-peptide presentation in the context of the shared epitope; (2). peripheral T cell activation through antigen presentation in the context of the shared epitope; (3). tolerance loss through molecular mimicry between the shared epitope and pathogenic agents. Genetic contributions to disease predisposition are not sufficient by themselves to induce disease expression (30).

2.2.2 Normal and rheumatoid synovium

Diarthrodial joints or synovial joints represent the most common type of articulations. They consist of adjoining bones cushioned by hyaline cartilage, being both nourished and lubricated by synovial tissue. The synovium covers all intra-articular structures with exception of the intra-articular cartilage surfaces (31). It consists of two distinct layers, the lining or intimal layer and the sublining or subintimal layer. The lining layer is one-to-three cells deep, mainly containing two ultrastructurally distinct cell populations: macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. These cells interdigitate loosely with each other and are in direct contact with the articular cavity in the absence of a basal membrane. The sublining layer consists of scattered blood vessels, fat cells and fibroblasts residing in a matrix of fibrils and proteoglycans together with occasional mononuclear cells. Synovial membrane and the cartilage are in direct contact with the synovial fluid (SF), a plasma transudate supplemented with saccharide-rich molecules produced by type B synoviocytes.

In RA the synovium undergoes profound changes, resulting in an increased volume and surface on macroscopic evaluation with accumulation of an inflammatory SF in the joint space (28). The lining layer becomes hyperplastic and forms an aggressive front termed “pannus” at the cartilage-bone junction, leading to the characteristic RA bone erosions (figure 1B). Pannus formation is considered a hallmark of the rheumatoid
synovium, being the only specific histological trait of RA as compared with other inflammatory arthritides. Important changes also occur in the sublining layer with massive mononuclear infiltration and blood vessel formation (figure 1A). Thus synovial inflammation with local cell accumulation, as well as bone and cartilage destruction, are the major traits defining RA synovitis.

2.2.3 Rheumatoid synovitis - pathogenesis
It is hard to generate a unified pathogenic hypothesis as the exact sequence of different mechanisms in disease initiation and propagation still represents a research question. A schematic representation of a personal working hypothesis, based on current knowledge regarding synovitis pathogenesis, is presented in figure 2. According to this hypothesis an as yet unknown etiologic agent would initiate the disease in a genetically susceptible host through antigen presentation in the context of the shared epitope. This would result in activation of autoreactive T cells that leave secondary lymphoid organs, enter the circulation and migrate to the target tissue i.e. the synovium. Interaction between immigrated immune cells and resident synovial cells results in local inflammation with production of pro-inflammatory cytokines and growth factors, up-regulation of adhesion molecules and angiogenesis. These factors could create a specific RA synovial milieu that would further promote cell recruitment and cell growth advantages, resulting in synovial hyperplasia. As a consequence of the direct communication between synovial tissue, cartilage and synovial fluid, markers of inflammation and bone destruction accumulate in the joint space. Both inflammation and synovial hyperplasia contribute to pannus formation with consecutive destruction of cartilage and bone.

2.2.4 Rheumatoid synovitis - cellular network
As illustrated in figure 1 the RA synovium is characterized by a complex cell network comprising both migrated and resident cells.

Macrophage-like cells are abundant in RA joints, both in the synovial membrane and the SF. These cells express monocyte/macrophage lineage markers such as CD68, CD14 and CD163 and have an activated phenotype with high levels of HLA-DR expression. It is postulated that they derive from circulating monocytes but it is still debated if the activated phenotype is gained in the synovial compartment or already in the blood. Even though phenotypic changes are demonstrable in the peripheral blood
T cells are present in the RA synovium either in aggregates or diffuse infiltrates in the sublining layer. They express CD3 as a surface marker and belong to either the CD4 or CD8 subtype, with a majority being CD4 positive. They also express markers that identify them mainly as mature memory lymphocytes following chronic immune activation. It is considered today that the synovial imbalance between pro- and anti-inflammatory cytokines favors a polarization of the synovial T cell response toward a T helper (Th) 1 type. An interesting observation is the low proliferation rate of RA-derived T cells in response to recall antigen, mitogen and CD3 ligation. It has been suggested that these defects are acquired through prolonged exposure to proinflammatory cytokines such as TNF (34).

Fibroblast-like cells are non-phagocytic cells with a stellate morphology lacking HLA-DR expression or macrophage differentiation antigens. These cells share some properties with transformed cells, such as telomerase activity, activation of proto-oncogenes as well as the potential to perpetuate for several passages when cultured in vitro. RA synovial fibroblasts cells are able to secrete large amounts of hyaluronic acid,
cytokines and arachidonic acid metabolites. They invade the cartilage and bone, being responsible for the invasive character of the rheumatoid synovium (35).

**Dendritic cells** are non-phagocytic cells that function as potent antigen presenting cells and which lack expression of macrophage differentiation antigens. These cells are enriched but not specific for RA (36).

**B cells and plasma cells** represent a small proportion of the RA synovial cells but are relevant for disease pathogenesis (37) through their capacity to secrete rheumatoid factor (RF). Rheumatoid factors are autoantibodies directed to the Fe portion of IgG and represent the most established serologic marker for RA.

**Neutrophils** are mainly present in the SF compartment and to a lesser extent in the synovial tissue. SF neutrophils may play an important role in mediating cartilage destruction and local inflammation through release of lysozomal enzymes and generation of destructive oxygen radicals (38).

### 2.2.5 Rheumatoid synovitis - cytokine network

Synovial cells communicate with eachother through either direct cell-cell contact or through soluble mediators, such as cytokines. Cytokines are non-structural proteins or glycoproteins produced by different cells including macrophages, fibroblasts and lymphocytes. The RA synovium is characterized by a complex cytokine network consisting mainly of macrophage- and fibroblast-derived products. Lymphocyte-derived cytokines are present in lower amounts as a result of a particular phenotype of T cells in the RA synovium. According to the type of cytokines produced, a T cell is classified as being either a Th 1 or Th 2 cell. These cells influence eachother, resulting in an equilibrium state corresponding to the physiological levels of immune regulation. It has been proposed that RA synovium T cells are biased towards a Th1 phenotype with almost no Th2 cytokine gene expression (39). Cytokines effects are balanced through inhibitory mechanisms such as receptor-binding antagonists, soluble cytokine receptors, antagonistic cytokines and anti-cytokine antibodies. Interestingly, high levels of different cytokine inhibitors have been described in RA, but it is supposed that these mechanisms are inefficient as a result of the great redundancy of the cytokine loops present in arthritis (28). Table 3 reviews the main cytokines and cytokines inhibitors relevant for RA pathogenesis.
<table>
<thead>
<tr>
<th>Class</th>
<th>Cytokine</th>
<th>Inhibitor</th>
<th>RA synovial pattern</th>
<th>Effect in RA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Th1 cytokines</strong></td>
<td>IFN-γ</td>
<td>Low amounts in ST and SF</td>
<td>Both pro and anti inflammatory properties</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>Soluble receptor IL-2R</td>
<td>Low amounts in ST and SF</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td></td>
<td>IL-17</td>
<td>Low levels in the ST</td>
<td>Proinflammatory</td>
<td></td>
</tr>
<tr>
<td><strong>Th2 cytokines</strong></td>
<td>IL-4</td>
<td>Low levels in RA</td>
<td>Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>Little or no expression in RA</td>
<td>Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td><strong>Monokines and fibroblasts-derived cytokines</strong></td>
<td>IL-1</td>
<td>Receptor antagonist IL-1ra Soluble receptors IL-1R</td>
<td>Present both in ST and SF in relatively high amount</td>
<td>Proinflammatory Promotes bone destruction</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Soluble receptors TNF-R</td>
<td>Present both in ST and SF in variable amounts</td>
<td>Proinflammatory Promotes bone destruction</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>Present both in ST and SF at high levels</td>
<td>Pro and anti inflammatory properties</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-8</td>
<td>High levels in SF and ST</td>
<td>Proinflammatory</td>
<td></td>
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<td></td>
<td>IL-15</td>
<td>Present in the ST</td>
<td>Proinflammatory</td>
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<td></td>
<td>IL-18</td>
<td>Present in the ST</td>
<td>Proinflammatory</td>
<td></td>
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<tr>
<td></td>
<td>GM-CSF</td>
<td>Present both in SF and ST</td>
<td>Proinflammatory</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>High SF levels and present in ST</td>
<td>Macrophage proliferation</td>
<td></td>
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<tr>
<td></td>
<td>IL-10</td>
<td>Present both in ST and SF</td>
<td>Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td>TGF-β</td>
<td>High levels in ST and SF</td>
<td>Pro and anti inflammatory</td>
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<tr>
<td></td>
<td>PDGF</td>
<td>Present in ST</td>
<td>Increases cell proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FGF</td>
<td>Present in SF</td>
<td>Increases proliferation and angiogenesis</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: rheumatoid arthritis RA, interferon IFN, interleukin IL, tumor necrosis factor TNF, receptor R, receptor antagonist ra, synovial tissue ST, synovial fluid SF, tumor growth factor TGF, platelet derived growth factor PDGF, fibroblast derived growth factor FGF
2.2.6 Synovial inflammation and TNF

TNF is a key player of innate immunity and an important modulator of inflammation. It belongs to the TNF superfamily and consists of a 26kDa protein expressed on the cell surface or present in a soluble form following cleavage by a matrix metalloprotease called TACE (TNF-α converting enzyme) (41). Both membrane-bound and soluble forms are biologically active. TNF effects are mediated via two structurally distinct receptors: type I (TNF-RI, p60 or p55) and type II (TNF-RII, p80 or p75) (42). Under different conditions such as inflammation these receptors are shed from cell surfaces and released into the circulation. Soluble receptors possess the ability to bind TNF and it is supposed that they act as either soluble inhibitors or delivery carriers, depending on the relative concentrations of TNF and TNF-R. Both TNF-RI and TNF-RII have high affinity for TNF, but the rate of dissociation is higher for TNF-RII. TNF ligation induces trimerization of the cell surface receptors followed by intracellular signaling. As illustrated in figure 3 there are two main intracellular pathways activated by TNF, resulting in activation of either transcription factors (nuclear factor NF-κB) and expression of survival genes, or intracellular enzymes (caspases) with consecutive apoptosis (43). These two pathways are closely linked as far as inhibition of constitutive NF-κB activation, in the absence of an additional cell death inducing signal, may by itself result in cell apoptosis (44). Although both receptors can transduce the signal for apoptosis and NF-κB activation, TNF-RI is responsible for these signals in most cases (45). An interesting observation is the potential of soluble TNF-RII to induce a so-called retro signaling following ligation of TNF on the cell surface (46). The functional relevance of this particular pathway, also observed for anti-TNF antibodies (47), remains to be explored.

TNF was discovered as a potential key modulator molecule in RA more than a decade ago (48). Subsequent studies confirmed that TNF is present in synovial tissue and SF of RA patients and identified synovial macrophages as the principal source of TNF. Animal studies offered a deeper understanding of the implication of TNF in RA pathogenesis. For example transgenic mice expressing TNF develop spontaneous RA-like disease with synovial inflammation and joint destruction (49). Moreover, TNF administration aggravates disease evolution in arthritis-susceptible mouse strains (50). Finally, maybe the most convincing evidence of all is the high clinical efficacy of different TNF blocking therapeutic approaches in RA patients (51). Even though taken
together these findings indicate a central role of the TNF molecule in disease pathogenesis, other potential TNF-independent associated mechanisms should also be considered, for example macrophage-T cell interaction promoted by IL-15 (52).

**2.2.7 Synovial inflammation and apoptosis**

Apoptosis is a particular type of cell death that does not induce bystander cell death, inflammation or tissue scarring. Apoptosis can be induced by a variety of stimuli through different intracellular pathways, the common link being activation of the caspases. The main apoptotic pathways are cell death receptor-mediated apoptosis (43) and mitochondria-mediated apoptosis (53). Cell death receptors (Fas and TNF receptor) activate caspase 8 through intracellular adaptor molecules, resulting in consecutive activation of caspase 3 either directly or via the mitochondrial pathway. Various other stimuli (granzyme B, chemotherapeutics, irradiation and growth factor depletion) induce release of cytochrome c from mitochondria that together with Apaf-1 will induce activation of caspase 9. Both pathways converge with consecutive activation of the effector caspase 3, leading to morphological and biochemical features characteristic for apoptosis. An apoptotic cell is thus characterized by cytoplasmatic activation of caspase 3, shrinkage, DNA fragmentation, nuclei condensation and formation of apoptotic bodies (54). Both impaired and excessive cell death have been associated with several disorders (55). Therefore in normal physiology the apoptotic mechanism is strictly controlled at several levels to avoid inappropriate cell death.
Impaired apoptosis has been implicated as a potential pathogenic mechanism in RA and pro-apoptotic therapies were proven efficient in different disease models (56). Studies using both TUNEL staining, which labels DNA fragments (57) and electron microscopy which identifies the apoptotic morphology (58) showed low apoptotic levels in RA synovial tissue (between 1-3% of cells). However, when the plan for this thesis was elaborated at the beginning of year 2000, little information was available regarding the mechanisms underlying the low levels of RA synovial apoptosis. This was intriguing as both Fas ligand (59) and TNF, frequently designated as classic inducers of apoptosis, were present in the RA joint. Several hints pointed out in the late 1990s to a possible role for anti-apoptotic molecules such as bcl-2 (60), soluble Fas (61-63) and active NF-κB (64-66). Confirmation came later when several other potential apoptotic inhibitor molecules were identified and studied in conjunction with RA. Figure 4 presents a schematic representation of these findings that has been used as a working hypothesis and updated with time. In this context it has been shown that distinct anti-apoptotic pathways are responsible for the typical resistance to apoptosis in different cell types present in the inflamed synovium. Macrophages, for example, are characterized by high levels of FLIP (67) and NF-κB activation (64), while T cells upregulate bcl-2 (60, 68) and FLIP (69). An even more complex anti-apoptotic network has been described in RA-derived synovial fibroblasts, possibly just because these cells

Figure 4. Synovial modulation of apoptosis in rheumatoid arthritis.

(Abbreviations: Fas ligand FasL, tumor necrosis factor TNF, TNF receptors TNF-R, Fas-associated death domain FADD, TNF receptor associated death domain TRADD, TNF receptor associated factor TRAF, cytochrome c cyt c, B-cell leukemialymphoma-2 Bcl-2, myeloid cell leukemia sequence 1 Mcl-1, FLICE inhibitory protein FLIP, apoptosis protease-activating factor 1 Apaf-1, small ubiquitin-related modifier-1 SUMO-1 signal transducer and activator of transcription STAT, phosphatidylinositol-3-OH kinase PI3K, nuclear factor-κB NF-κB.)
are easy to study due to their capacity to expand \textit{in vitro}. Sentrin-1 (SUMO-1) (70) and synoviolin (71), recently identified anti-apoptotic molecules, are present in the RA synovium, especially in fibroblasts. Moreover, these cells are characterized by STAT-3 activation and expression of a dominant negative STAT-3 induces apoptosis in synovial-derived fibroblasts (72, 73). Growth factors such as transforming growth factor (TGF)-β, are able to activate the PI3K pathway that induces Mcl-1 expression and blocks synovial fibroblast apoptosis (74). The p53 protein may trigger apoptosis of a damaged cell if DNA repair processes fail (75). Thus inactivating somatic mutations of p53 that have been described in RA synovial fibroblasts (76) may also play an anti-apoptotic role.

2.2.8 Joint destruction and the MMP/TIMP system

Proinflammatory cytokines such as TNF-α and IL-1 act synergistically to release matrix metalloproteinases (MMPs) from different types of cells such as synovial fibroblasts (77), chondrocytes (78), osteoblasts (79), macrophages (80) and endothelial cells (81). MMPs belong to a family of zinc-dependent endopeptidases participating in extracellular matrix degradation and remodelling. They are synthesized and secreted as latent proenzymes and their activation is due to proteolytic cleavage of a propeptide domain at the N-terminus of the molecule. At present there are at least 19 known human MMPs that can be divided into four groups: the collagenases, the stromelysins, the gelatinases and the membrane-type MMPs (MT-MMPs) (82). In RA, MMPs may act either directly or indirectly through proteolytic release of TNF-α from cell membranes. Stromelysin 1 (MMP-3) and collagenase (MMP-1) have been suggested to play an important role in RA, being able to degrade all the important structural proteins in the extracellular matrix of the joint (83).

MMP-3 (stromelysin 1) degrades a large number of matrix proteins including proteoglycan, fibronectin, laminin and gelatins as well as collagen types III, IV, V and IX (84). MMP-3 levels are increased both in synovial fluid (85) and serum (86) in RA patients, with a highly significant correlation between matched samples (87) suggesting that serum MMP-3 is mainly derived from that synthesized in the synovium. Serum levels of MMP-3 correlate with several disease parameters (CRP, ESR, DAS score) (88-90), as well as with the development of joint damage evaluated with Sharp (91) or Larsen (92, 93) scores. Moreover a specific polymorphism in the MMP-3 gene associated with higher serum levels of proMMP-3 predict a worse RA outcome and
may have an additive effect with the shared epitope on disease severity (94). MMP-1 (fibroblast collagenase) degrades collagen types I, II, III, VII and X (95) and can be detected in RA synovial fluid (96) and serum samples (90) at higher levels compared to in healthy controls. Similar to MMP-3, serum levels of proMMP-1 correlate with disease activity and predicts functional and radiographic outcome in early RA (97).

The main inhibitors of MMPs are the tissue inhibitors of matrix metalloproteinases (TIMPs), a class of low molecular weight proteins that form 1:1 noncovalent high affinity complexes with active MMPs. TIMP-1 is present in RA synovium to a lesser extent compared to MMPs, suggesting an imbalance between the two components (98). Interestingly, TIMP-1 appears to be elevated in RA synovial fluid (99) and serum (87).

### 2.2.9 Joint destruction and the RANKL/OPG system

MMPs are able to degrade non-mineralized cartilage but osteoclasts must be involved in the actual process of bone destruction. Even though bone destruction has long been recognized as a major feature of the rheumatoid joint, the essential players have just recently been identified as new members of the tumor necrosis factor (TNF) ligand and receptor (TNFR) superfamily (100). This new cytokine system consists of a ligand, receptor activator of nuclear factor (NF)κB ligand (RANKL) that exists either in a cell-bound or in a soluble form; a cell-bound receptor, receptor activator of nuclear factor-κB (RANK) and a secreted decoy receptor, osteoprotegerin (OPG). Recently, the RANKL/OPG system has been identified as the essential denominator of bone biology in RA (101-103). TNF, one of the more potent osteoclastogenic cytokines produced during rheumatoid inflammation, is able to induce RANKL expression on the surface of stromal-osteoblastic cells, directly or by promoting IL-1 secretion (104, 105). Thus in RA the RANKL/OPG system may represent the missing link between inflammation and bone destruction.

RANKL, also known as osteoclast differentiation factor (ODF), tumor necrosis factor-related activation-induced cytokine (TRANCE) and osteoprotegerin ligand, is a member of the TNF ligand family (106) produced in osteoblastic lineage cells and activated T cells (107). RANKL binds to its receptor (RANK) mainly present on the surface of osteoclasts, dendritic cells and lymphocytes (108, 109) and induces osteoclastogenesis and dendritic cell maturation (110), mediates T cell proliferation in response to cytokines and contributes to T cell activation (109). Osteoprotegerin
OPG), also known as osteoclast inhibitory factor (OCIF) is a soluble decoy receptor for RANKL (111). OPG is a member of the TNF receptor superfamily and functions as an osteoclastogenesis inhibitor (112). A schematic representation of the RANKL/OPG pathway is presented in figure 6.

![Diagram](image)

**Figure 5. The RANKL/RANK/OPG system in rheumatoid arthritis**

(Abbreviations: receptor activator of the nuclear factor-κB RANK, receptor activator of the nuclear factor-κB ligand RANKL, osteoprotegerin OPG, tumor necrosis factor TNF, interleukin IL)

Even though the RANKL/OPG system is nowadays considered the main player in RA bone destruction, no studies date longer than 4 years ago. The first indications about a possible implication of this system in RA pathogenesis came with the observation that proinflammatory cytokines such as TNF and IL-1 are able to upregulate RANKL expression in human osteoblastic cells *in vitro* (113). It has also been shown both *in vivo* (114) and *in vitro* (115-117) that activated T cells are able to mediate osteoclast formation through RANKL expression, thus describing for the first time a link between the immune and skeletal systems. The first definitive proof of a direct role for RANKL in arthritis pathogenesis came with the observation that RANKL knockout mice are protected from bone erosions in a serum transfer model of arthritis (118). Subsequent studies described the presence of RANKL mRNA at the local site of inflammation in animal models of arthritis (119) and human RA (101-103). Immunohistochemical studies also identified the cellular expression pattern of both RANKL and OPG in the RA synovium. RANKL is present in T cell rich areas and is associated with active synovitis (120), while OPG is mainly expressed by endothelial cells in inactive synovitis (121). RA serum levels of both OPG and RANKL are elevated compared to
in normal controls (122). Interestingly, serum levels of OPG but not RANKL correlate with disease activity and radiographic progression (123). Based on these findings single (124) and combination (125, 126) therapeutic approaches to target the RANKL were studied in different arthritis models, resulting in amelioration of the disease.

2.3 RA TREATMENT - WHAT MORE CAN WE LEARN?
The close relation between pathogenesis and treatment represents the focus of this thesis. On one hand, pathogenic considerations represent the only basis for development of new therapeutic strategies, in that an etiologic agent for RA has not yet been discovered. Conversely, through investigation of the molecular mechanisms of action of different drugs, more can be learned about candidate pathogenic targets and be translated into therapeutic strategies. It has to be stressed, however, that no current available therapy cures RA.

2.3.1 NSAID - old enough to know better
Non-steroidal anti-inflammatory drugs (NSAID) usually represent the initial drug prescribed in RA, both in primary and secondary care settings. These drugs provide
partial relief from pain but do not interfere with disease progression (127). Thus NSAIDs are mainly used in RA as adjuvant therapy in conjunction with DMARDs. NSAIDs block inflammation-induced cyclooxygenase (COX)-2, therefore inducing a decrease of prostaglandin synthesis with consecutive anti-inflammatory effects (128, 129). However, due to the concomitant inhibition of the constitutive COX-1, NSAID treatment may result in serious adverse events (130). Development of specific COX-2 inhibitors was met with great enthusiasm (129), but the recent increase in thrombotic events associated with their use (131) further emphasizes the need for careful consideration in choosing a specific drug. Moreover patients with RA are nearly twice as likely to have a serious complication from NSAID treatment (132).

2.3.2 Corticosteroids - friends or foes?
Corticosteroids represent a yet unsolved controversy in RA treatment. Even though they are the only rheumatology-linked research that led to a Nobel Prize, concerns still exist related to the important adverse reactions associated with high dosage and long-term use. However, from the clinician’s point of view it should be stressed that low dose oral corticosteroids and local injections of corticosteroids are highly effective for relieving symptoms in RA patients (133). Recent evidence also suggests that corticosteroids have a disease-modifying potential, delaying the rate of joint damage (134). Corticosteroids act through specific cytoplasmatic receptors to induce lipocortin production, resulting in changes of lymphocyte functions and downregulation of many proinflammatory enzymes. Potential side-effects such as the increased risk of osteoporosis (135) should be closely monitored.

2.3.3 DMARDS - a friend in need is a friend indeed
By definition a DMARD must demonstrate a sustained improvement in physical function and slowing of structural joint damage (136). The past decade saw a major change in DMARD use in RA, with methotrexate substituting the classic treatment triad (antimalarials, gold and penicillamine) as first therapeutic choice (137). Moreover, other DMARDs (such as sulfasalazine, leflunomide and immunosuppressive drugs) as well as combination therapy received increasing attention (138). A short overview of the currently used DMARDs and their effects in RA is given in table 4.

Different therapeutic approaches for the use of DMARDs have been described: the conventional pyramid approach (use of symptomatic drugs first with subsequent
addition of DMARDs if treatment response is inadequate), step-up approach (starting a first DMARD followed by the addition of a second if there is no adequate response), the step-down approach (initial combination therapy is followed by a reduction of the dose or the abandonment of one or more of the DMARDs) (139), and the sawtooth approach (early, continual and serial use of DMARDs) (140). During the past decade a new class of drugs, the so-called biologicals, fulfilling the DMARD definition and considered revolutionary for RA treatment has emerged.

<p>| Table 4. DMARD treatment in RA |</p>
<table>
<thead>
<tr>
<th>DMARD</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimalarials (136)</td>
<td>Stabilize lysosomal membranes and inhibit metabolism of deoxyribonucleotides</td>
</tr>
<tr>
<td>Gold compounds (136)</td>
<td>Inhibit NF-κB activation&lt;br&gt;Inhibit osteoclastic activation&lt;br&gt;Inhibit B cell activation&lt;br&gt;Increase IL-1ra production</td>
</tr>
<tr>
<td>Penicillamine (136)</td>
<td>Effects on free radical scavenging&lt;br&gt;Catalyzes removal of metals from metalloproteinases</td>
</tr>
<tr>
<td>Sulfasalazine (141)</td>
<td>Inhibits monokines production&lt;br&gt;Induces T cell apoptosis&lt;br&gt;Blocks NF-κB activation&lt;br&gt;Suppresses fibroblasts activation</td>
</tr>
<tr>
<td>Methotrexate (142)</td>
<td>Adenosine mediated anti-inflammatory effects&lt;br&gt;Induces apoptosis in T cells and synovial cells&lt;br&gt;Decreases production of pro inflammatory molecules</td>
</tr>
<tr>
<td>Leflunomide (143)</td>
<td>Blocks T cell proliferation&lt;br&gt;Blocks tyrosine kinase acitivity and NF-κB activation&lt;br&gt;Decreases production of pro inflammatory cytokines</td>
</tr>
<tr>
<td>Immunosuppressive drugs (144)</td>
<td>Reduces lymphocyte function and number, cytotoxic&lt;br&gt;Cross-links DNA and prevents cell replication&lt;br&gt;Cross-links DNA and prevents cell replication&lt;br&gt;Suppresses IL-2 synthesis and T cell response&lt;br&gt;Inhibits T cell proliferation</td>
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2.3.4 Biologicals - a new face of an old story

Biologicals is a general term used to define a new class of therapeutic agents that are similar to endogenous molecules that occur naturally in the body, such as antibodies and soluble receptors (145). Among these, TNF inhibitors are currently used with high efficacy in different clinical settings, including RA. In the current strategy of RA treatment biological treatments are appropriate depending on disease activity and response to other DMARD therapy. However, anti-cytokine therapy should not be
reserved for advanced or DMARD-resistant disease but instead should be used in those with rapidly advancing aggressive disease (146). Three TNF blocking agents are currently available for clinical use: etanercept (a recombinant TNF receptor-Fc fusion protein), infliximab (a chimeric monoclonal antibody) and adalimumab (a fully human anti-TNF antibody) (51).

**Infliximab** was the first TNF antagonist studied in RA patients (147). It is a chimeric anti-TNF human IgG1κ, engrafted to a variable region of a murine anti human TNF-α antibody. It binds with high affinity to soluble and membrane TNF and is able to induce antibody-dependent lysis of cells expressing TNF (148). The terminal half-life is 8-9.5 days, but only 25% patients of those receiving 3mg/kg have undetectable levels of infliximab 8 weeks after administration. Infliximab is approved for use in RA in combination with methotrexate to reduce signs and symptoms and to inhibit progression of structural damage in patients with moderately to severely active disease that has an inadequate response to methotrexate (infliximab package insert).

**Etanercept** is an engineered dimer of p75 TNF receptors linked to the Fc portion of IgG, containing two constant domains CH2 and CH3 as well as the hinge region. Etanercept binds one or two soluble TNF molecules as well as membrane-bound TNF-α and lymphotoxin. In contrast to infliximab it does not mediate antibody-dependent cell lysis (149). The half-life of etanercept is 4.1-12.5 days. Etanercept is indicated for reducing signs and symptoms and inhibiting the progression of structural damage in patients with moderately to severely active RA. It can be used in combination with methotrexate in patients who do not respond adequately to methotrexate alone (etanercept package insert).

**Adalimumab** is a fully human IgG anti TNF monoclonal antibody produced by phage display that is potentially less immunogenic (150). It is approved since 2003 for use in moderate to severe RA unresponsive to an adequate trial of one or more DMARDs (adalimumab package insert).

Even though TNF blocking therapy is considered revolutionary today for RA, safety concerns have been raised, as with all other drugs currently used in clinical practice (151). The most common adverse reactions encountered with TNF antagonists are injection site reactions. In the long-term a potential harmful effect is represented by an
increased risk for infections, autoimmune events and tumor development. Thus prescribing guidelines warn against use in patients with active serious infections and specify that patients should be monitored for signs and symptoms of infection while receiving TNF inhibitors. Autoimmune events with development of anti-DNA antibodies, lupus-like disease and exacerbation of demyelinating disorders have been reported for all three drugs. The potential risk of tumor development is related to the potential loss of immunosurveillance following TNF blockade. This is still an open question as far as high disease activity rather than immunosuppression is linked to development of RA-associated lymphomas (152). Thus a thorough evaluation of the risk:benefit ratio should precede administration of TNF inhibitors in clinical practice.
3 AIMS

3.1 GENERAL AIM
- The general aim of the present thesis was to study the mechanisms of action of TNF antagonists in RA in order to identify potential predictors for therapeutic response and candidate additional therapeutic targets.

3.2 SPECIFIC AIMS
- To describe the synovial apoptosis pattern in the rheumatoid synovium
- To study the effect of TNF antagonists on synovial apoptosis
- To study the effect of TNF antagonists on the MMP-TIMP system in patients with RA
- To study the effect of TNF antagonists on the OPG-RANKL system in patients with RA
4 RESULTS AND DISCUSSION
The present study investigated RA disease evolution in relation to therapeutic intervention. We hope to have contributed to the current knowledge of different pathways implicated in disease pathogenesis.

4.1 CLINICAL STUDIES DESIGN
Our research approach was based on clinical studies involving patients with RA in different stages of the disease and with different therapeutic conditions. Thus we designed either an exploratory comparative study (paper I) or an open label study (papers II, III and IV). In the first study we planned our analyses before the data was collected and all completed analyses were reported. Thus we theoretically decreased the possibility to obtain statistical significance by chance through increasing the number of comparisons following data collection (153). The subsequent studies (papers II, III and IV) investigated the effect of anti-TNF therapy in patients with RA using an open label design. Our design has theoretically some drawbacks such as placebo effect and expectation bias. However, previous studies revealed no changes in serial synovial biopsies from patients receiving placebo or ineffective clinical therapy (154-156). Moreover, we have found similar beneficial clinical responses as previously demonstrated in double-blind, placebo-controlled trials (157, 158) accompanied by significant changes in synovial phenotype. These data, taken together, support the view that the observed synovial changes in serial biopsies are the consequence of a therapeutic biological effect and not a placebo effect. We also took great care in all our studies to blind the histological readings of both therapy and biopsy specimens in order to reduce the risk of expectation bias.

4.2 SYNOVIAL CELLULAR AND CYTOKINE NETWORK
A potential difference between early and late RA has been debated for a long time. Thus we analyzed (paper I) biopsy samples representing two extremes of the disease: early RA biopsies obtained by arthroscopy and longstanding RA biopsies obtained during arthroplasty. As both inflammatory cells and their products are essential traits of the rheumatoid synovium we described the synovial phenotype through immunohistochemistry for both surface CD markers (CD68 for macrophages and CD3 for lymphocytes) and for cytokines (IL-1α, IL-1β, IL-6 and TNF-α).
We demonstrated a distinct pattern of the early inflammatory arthritis characterized by a higher number of macrophages than in longstanding RA. Even though lymphocytes tended to be more numerous in arthroscopic samples the difference did not reach statistical significance. Interestingly we did not observe differences regarding synovial cytokine expression (figure 7). It was beyond the main aim of this study to analyze the effect of different therapeutic strategies due to the low number of cases included. However, we did not find differences between DMARD treated versus DMARD non-treated patients or between corticosteroids versus corticosteroids non-treated patients.

One possible limitation of our study is the lack of available information regarding local and general disease activity in the longstanding RA group. Thus higher local inflammatory activity, usually associated with arthroscopy, might represent a confounding factor. This hypothesis was generated in a subsequent study even without including corrections for the local inflammatory score and disease activity (159). An argument against this hypothesis is our observation that the number of synovial macrophages inversely correlates with disease duration. One common conclusion of both studies is that different pathogenic mechanisms may be responsible for the specific synovial phenotype in early RA biopsies as compared to end stage longstanding arthritis.
The synovial phenotype in early as compared to late RA is still a debated issue, largely depending on different definitions used to classify these two entities and on patient selection. Several studies using only closed needle/arthroscopic biopsies described no differences regarding synovial macrophage infiltration in different stages of the disease (160-162). However, using a similar material one study identified an increased number of macrophages in late as compared to early arthritis (163). The only two studies (159, 164) comparing biopsies obtained by arthroscopy versus joint arthroplasty have found a lower number of macrophages in the later disease entity. An explanation for this discrepancy and a unified hypothesis is still lacking. Studies that will include larger numbers of patients, with biopsies taken by both techniques and corrections for confounding factors (local inflammation score, disease activity and treatment) may solve this problem.

4.3 SYNOVIAL APOPTOSIS AND FLIP EXPRESSION

As we identified a decreased cellularity in longstanding end stage RA synovial biopsies we hypothesized that increased apoptosis may be a potential relevant mechanism associated with this finding. Thus we examined the synovial apoptosis pattern in early as compared to longstanding RA biopsies (paper I). Apoptosis was mainly located in the macrophage-like cells and to a lesser extent fibroblasts. Longstanding RA synovium was characterized by a higher number of apoptotic cells, distributed in

![Graph showing correlation between synovial apoptosis and CD68 positive cells.]

*Figure 8. Synovial apoptosis correlates with synovial macrophage number*
clusters both in lining and sublining layers, while only few apoptotic cells were detected in the early RA biopsies. The same disease duration-dependent pattern of synovial apoptosis has been described in adjuvant arthritis in rats, an animal model of arthritis (165). Moreover, we demonstrated that synovial apoptosis levels negatively correlated with the number of synovial macrophages, suggesting that these cells are the main determinants of synovial apoptosis (figure 8). Thus early RA might be characterized by defective apoptosis with a restoration of the apoptotic mechanisms during natural or drug modified disease progression.

The subsequent step in our investigation was to identify potential mechanisms responsible for the observed synovial apoptosis pattern. As both Fas and TNF, classic apoptosis inducers, were present but apparently inoperative in the RA synovium, we focused our investigations on molecules that were known to block death receptor-mediated apoptosis. Tumor biology offered us a new potential candidate. As this thesis was being designed, FLICE inhibitory proteins, a class of viral (166) and cellular (167) molecules were identified as inhibitors of death receptor mediated apoptosis and promoters of tumor development (168, 169). Thus we examined a potential pathogenic role for FLIP in RA (paper I). We identified FLIP positive cells in early RA cases, both in lining and sublining layers, mainly in the macrophage-like cells and to a lesser extent in fibroblast-like synoviocytes. FLIP expression was low in longstanding RA and inversely correlated with synovial apoptosis. Even though it would have been tempting to speculate a direct link between FLIP expression and synovial apoptosis in RA, we could not exclude at that time that the lower levels of FLIP observed in longstanding RA were only an indirect measure of the decreased number of macrophages. However, several others studies confirmed our hypothesis that FLIP might play an essential role in determining the course of RA synovial apoptosis. It has been shown that RA SF-derived but not PB-derived macrophages express high levels of FLIP and are resistant to apoptosis (67). We have also identified FLIP expression in SF-derived lymphocytes but to a lesser extent compared to in macrophages (figure 9C, Al Catrina, unpublished data). We could not detect FLIP expression in the normal synovium (figure 9B) while high levels of expression were evident in RA synovium (figure 9A). Other investigators have suggested a possible implication of FLIP in inducing the aggressive phenotype of the pannus cells, based on its expression at the pannus-cartilage junction (170). It has also been suggested that FLIP might play an important role in the RA-derived
fibroblast cells, based on increased susceptibility to apoptosis following down-regulation of FLIP (171).

4.4 BONE DESTRUCTION MARKERS IN RA SYNOVIAL AND SERUM

Another focus of our studies was cartilage and bone destruction. As MMP and TIMP are the main molecules implicated in destruction of non-mineralized cartilage we investigated (paper III) the presence of these molecules in synovial biopsies and serum samples from patients with RA. As previously reported we identified an imbalance between MMP and TIMP expression in favor of bone destruction in both synovial tissues and serum samples. We determined a large inter-individual variability in synovial biopsies, as previously described for other synovial markers. MMP-1 was mainly expressed in the sublining layer and around vessels and to a lesser extent in the lining layer. TIMP-1 exhibited a generally low expression with positive signals in isolated areas. Serum levels of MMP-1 and MMP-3 were higher, while TIMP-1 serum levels were in the same range with levels reported previously in normal individuals (90). MMP-3, but not MMP-1, serum levels correlated with inflammatory parameters such as CRP and ESR, suggesting MMP-1 as an inflammation-independent marker of cartilage destruction. We did not find any correlation between synovial expressions and serum levels of the investigated markers in the paired samples available for the study.

Figure 9. FLIP is present in the rheumatoid arthritis (RA) synovium (A) but not in the normal synovium (B). Synovial fluid (SF) derived macrophages express higher levels of FLIP as compared to synovial fluid derived lymphocytes, as evaluated by flow cytometry (C).
n=9), even though biopsies were obtained from the cartilage-pannus junction, which is the main source for MMPs in the rheumatoid synovium. Lack of correlation between serum and synovial levels of MMP-1 and MMP-3 was later confirmed by others (172).

Bone destruction, beside cartilage destruction, is a characteristic of RA and implies activated osteoclasts. Thus we investigated (paper IV) synovial expression of OPG and RANKL, considered as main denominators of osteoclast activity. When these studies began little information regarding expression of these two proteins in RA synovial biopsies was available. We have identified OPG expression in synovial tissue, with a characteristic pattern mainly restricted to endothelial cells and few mononuclear cells. We did not detect OPG expression in the T cell rich areas. In contrast, RANKL was mainly present in T cells areas, modestly expressed in isolated endothelial cells, but absent in other mononuclear cells.

4.5 ANTI-TNF THERAPY INDUCES SYNOVIAL APOPTOSIS

We used another approach to investigate the potential relevant pathological pathways in RA. This consisted of studies of the cellular mechanisms of action of efficient therapeutic strategies. Since TNF is considered a key molecule in RA and anti-TNF therapy represents the most recent advance in RA treatment, we focused our studies on the two available TNF antagonists at the time this thesis started: etanercept and infliximab.

We have first investigated the effect of both drugs on synovial cellularity (paper II). We reported for the first time that etanercept, similarly to infliximab, induces a decrease in the number of synovial macrophages. However, no changes in the lymphocyte population were observed following etanercept treatment, suggesting a cell type-specific effect. We observed the same significant decrease in macrophage number in the infliximab-treated group. However, we did not detect a decrease in the number of lymphocytes as reported in an early study that used a higher dose of infliximab than us (173). Thus drug dosage may be important for the effect of the drug on target cells as another study using the same dose as us observed the same lack of effect on the synovial lymphocyte population (174).

As the decrease in synovial cellularity may either be due to decreased cell recruitment at the site of inflammation or to increased clearance of resident cells we further
investigated if TNF antagonists were able to modulate synovial apoptosis in RA (paper II). We demonstrated that treatment with both etanercept and infliximab increased synovial apoptosis levels as evaluated by both single TUNEL and active caspase 3 staining. To our knowledge this is the first study to describe synovial expression of active caspase 3, thus offering an option for apoptosis detection in synovial biopsies besides the classical TUNEL method. Caspase 3 activity was detected in mononuclear cells as well as in some isolated fibroblasts-like cells and endothelial cells. The implication of the apoptotic cell death as a potential mechanism of action of anti-TNF drugs is emphasized by the tendency towards higher levels of increase in the apoptotic index in the ACR responders versus ACR non-responders, although larger numbers of observations are needed to substantiate the possible use of apoptotic markers to discriminate between responders and non-responders to TNF-blockade. In concordance with our previous observations synovial apoptosis correlated with the macrophage number but not with individual macrophage-derived cytokines, suggesting a concerted action of these molecules in defining cell susceptibility to apoptosis.

Our findings are partially different from a previous study that reported a decreased synovial cellularity following treatment with infliximab without changes in the number of apoptotic cells (174). At least for the early time point (48h) in this study, it is possible that the time point for arthroscopy might explain the differences. Even though apoptosis induction is a rapid event, altering the status of an anti-apoptotic milieu such as the rheumatoid synovium requires complex changes. Effects of such complex events may thus become evident as a change of apoptotic cells only at later time points, including the 8 weeks of treatment (with 2 weeks after the last infliximab injection), as in our study. This approach is sustained by a recent study in Crohn’s disease demonstrating an increased level of apoptosis following 10 weeks of infliximab therapy compared to baseline (175). It also appears that in the study conducted by Smeets et al, following 48h of infliximab treatment the number of apoptotic cells corrected per number of total synovial cells may have increased (even though non significantly), as they describe a global decrease in cellularity (with the exception of CD22) with the same number of TUNEL positive cells per tissue area (174). However, we could neither exclude a possible type 1 error in our study (due to multiple comparisons, even though we corrected for such multiple comparisons), nor a type II error in the previous study (due to low number of patients). In order to address these issues further investigations including a greater number of patients and different time points are needed.
4.6 ANTI-TNF APOPTOSIS - A SYNOVIAL RESTRICTED EFFECT

To further evaluate the eventual pro-apoptotic effect of the two TNF-blocking drugs, we evaluated the *in vitro* effect of etanercept and infliximab on RA SF and PB mononuclear cells (MC) (paper II). Treatment with both drugs induced apoptotic cell death of SF-derived macrophages, but not of lymphocytes. The pro-apoptotic effect of infliximab had been previously reported for monocytes derived from patients with Crohn’s disease (176), while no studies investigating the effect of etanercept on the monocyte population are currently available. An interesting finding of our study is the lack of effect of the two tested drugs on lymphocyte apoptosis. Previous studies in Crohn’s disease described a pro-apoptotic effect of infliximab, but not etanercept, on activated but not resting lymphocytes (177, 178). A potential explanation for this difference is the phenotype of joint-derived lymphocytes of patients with RA, known to differ substantially from gut-derived lymphocytes of patients with Crohn’s disease. Gut-derived lymphocytes from Crohn’s patients are highly activated and able to produce high levels of cytokines (179). We and others (180-182) have demonstrated that lymphocytes derived from both synovial tissue and synovial fluid cells express low levels of cytokines and show signs of anergy. Moreover, repeated *in vitro* treatment with TNF, a setting that mimics the RA synovial chronic exposure to TNF, suppresses T cells activity (34).

Even though anti-TNF therapy is able to some extent to reverse peripheral T cell reactivity (183), it might be so that the complex arthritic milieu influences synovial T cell reactivity that might not fully recover at the site of inflammation. One other possible explanation is that T cell survival may be independent of TNF and other cytokines such as IL-15 may be responsible for the apoptosis resistant phenotype observed for RA-derived lymphocytes (184). In accordance with this hypothesis we found that IL-15 synovial modulation is independent of TNF, in as much as treatment with infliximab does not modify synovial expression of this cytokine (185). Low doses of both etanercept and infliximab (1 and 10µg/ml) were more effective in inducing apoptosis compared to higher doses (100µg/ml). The lack of effect of high concentrations of the two drugs has been previously observed for etanercept (effect on cytokine suppression (186, 187)) and for infliximab (induction of apoptosis in the presence of minute amounts of TNF (188)). These findings suggest an escape mechanism at high concentrations of the anti-TNF agents, possibly mediated through redundant proinflammatory pathways.
To test if the anti-TNF proapoptotic effect is a synovial restricted event we evaluated the effect of both etanercept and infliximab on PBMCs obtained from RA patients. Both infliximab and etanercept treatment increased the proportion of apoptotic cells in the monocyte population of PBMC, but the effect was less potent in comparison with SFMC, suggesting that the particular milieu of the rheumatoid joint influences monocyte susceptibility to apoptosis.

Keeping in mind the differences between monocyte populations in SF versus PB we propose several potential mechanisms to explain the proapoptotic effect of the anti-TNF therapy. Firstly, as previously demonstrated, both anti-TNF antibodies (47) and soluble TNF receptors (46) can bind membrane TNF and transmit an intracellular signal, and it has been speculated that apoptosis of the target cell could be a result of this pathway (189). Monocytes in synovial tissue and fluid have an activated phenotype with sustained cytokine secretion, while just scarce translation could be detected at the peripheral level (190). This particular behavior correlates with the lower potency of the drugs to induce apoptosis in PBMC. Secondly, it has been shown that TNF protects monocytes from death receptor-mediated apoptosis through upregulation of FLIP (191). FLIP is abundantly expressed in RA monocytes derived from synovial tissue and synovial fluid and to a lesser extent in peripheral monocytes (67). This is in agreement with our preliminary data which suggest a decrease in synovial FLIP expression following treatment with both etanercept and infliximab, suggesting a possible FLIP-mediated apoptotic mechanism (Catrina AI, unpublished data). Thirdly, the activation of NF-κB, a common feature in the RA synovium, protects monocyte-derived macrophages against apoptosis. Both etanercept and infliximab are able to inhibit NF-κB, which could be followed by macrophage apoptosis (44).

4.7 ANTI-TNF THERAPY DECREASES MMP/TIMP SERUM RATIO

As MMP-3 and MMP-1 serum levels have been identified as potential predictors for joint damage in RA (91, 97), we aimed to investigate (paper III) if an efficient anti-inflammatory therapy, such as anti TNF antagonists, may also modulate bone destruction parameters. We demonstrated that etanercept therapy significantly decreases serum levels of MMP-3 as well as the ratio of MMP-3 to TIMP-1. We also observed a decrease in MMP-1 serum levels during etanercept therapy with reduction of the MMP-1/TIMP-1 ratio. Etanercept therapy did not consistently influence serum levels of TIMP-1 despite a general decrease in all inflammatory markers investigated.
This finding suggests that TIMP-1 is mainly influenced by other pro- and/or anti-inflammatory mechanisms than TNF. The ability to decrease MMP serum levels is not unique for etanercept, but also for other TNF antagonists (192, 193), suggesting a more class-specific than drug-specific effect. Interestingly we did not observe an effect of etanercept on the synovial expression of the studied markers. This could be due to the low number of patients subjected to arthroscopy and/or lack of specificity between the recognition of active and inactive forms of MMPs.

One interesting finding of this study was the capacity of baseline MMP-3 serum levels to predict changes in disease activity induced by etanercept treatment. About 30% of the observations were explained by a regression model that included all studied parameters, suggesting serum MMP-3 as a potential candidate marker for further evaluation in larger clinical studies.

4.8 ANTI-TNF THERAPY INCREASES SYNOVIAL OPG EXPRESSION

As the OPG/RANKL system has been identified as the main determinant of RA bone biology, but no studies regarding therapeutic modulation of these parameters at the site of inflammation are currently available, we investigated (paper IV) the effect of etanercept and infliximab on synovial expression of OPG and RANKL. Treatment with infliximab induced a significant increase in synovial OPG expression, while the increase observed following etanercept treatment did not reach statistical significance. No changes regarding synovial OPGL expression were observed following treatment with either infliximab or etanercept. Inactive RA, irrespective of treatment, has been associated with high synovial expression of OPG and low expression of RANKL resulting in a high OPG/RANKL ratio (103). Thus the observed increase in synovial OPG levels could be either due to a direct effect of the TNF blockade or to an indirect effect mediated through decreased local inflammation. We did not observe a parallel change in RANKL expression, suggesting a selective effect of anti-TNF treatment on OPG. Moreover, treatment with corticosteroids, while increasing the OPG/RANKL ratio, does not change OPG expression but decreases RANKL expression (AI Catrina et al, non published data). These data taken together demonstrate a direct specific effect of different therapeutic agents on the OPG/RANKL system in RA synovium.

In apparent contradiction with our results a previous study observed that in RA infliximab treatment decreases serum levels of both RANKL and OPG without
changing the OPG/RANKL ratio (122). However, to date there is no information as to whether serum and synovial fluid levels reflect their local levels in the synovium. The synovial OPG/RANKL ratio also appears to be inversely correlated with local inflammation and disease activity in RA (103). We also demonstrate an increase in synovial OPG/RANKL ratio following anti-TNF treatment that parallels a decrease in disease activity. These data taken together suggest an independent regulation of the OPG/RANKL system at the site of inflammation versus serum in patients with RA and strengthens the importance of synovial biopsy evaluation when investigating mechanisms of action of different therapeutic agents.

As RANKL/OPG ratio is the main determinant in the process of bone modelling we sought to investigate a possible influence of anti-TNF therapy on this parameter. Therapy with both etanercept and infliximab decreased the RANKL/OPG ratio. To determine if the observed changes are clinically relevant we analysed the differences between responders and non-responders to therapy. Responders to therapy in both groups showed a tendency towards a more pronounced decrease in the RANKL/OPG ratio as compared to non-responders (statistically significant in the infliximab treated group, non-significant in the etanercept treated group). This finding appears to be in contradiction with the delay in radiological damage apparent in previous clinical studies in both groups (194). In this respect, treatment with either infliximab or etanercept was demonstrated to reduce serum COMP levels in both responders and non-responders to therapy (195). However, COMP is considered to be a marker reflecting processes not directly linked to inflammation (196), while the OPG/RANKL system also modulates immune pathogenic mechanisms potentially important in RA such as T cell activation, dendritic cell survival and monocyte activation (197). Thus it is possible that the observed difference between responders and non-responders is linked to the complex influences of the OPG/RANKL system on synovial biology.

4.9 POINTS OF PERSPECTIVE
The present thesis has been designed with the aim to better understand the mechanisms of action of a highly efficient treatment of RA, treatment with TNF antagonists. We hoped that such an approach would offer more insights into disease pathogenesis and potentially identify new therapeutic targets. When we started the work of this thesis in 2000 even though anti-TNF therapy was considered the main discovery of the past decade in the field of rheumatology, and particular RA, little information regarding the
precise mechanism of action was available. Moreover, no direct studies to investigate in parallel the effect of the two drugs belonging to same class but essentially different chemically was available at that time. Our investigation started from two intriguing generally accepted findings. First that TNF, a classic apoptosis inducer is present in the RA synovium. Second, RA is characterized by synovial inflammation and low levels of apoptosis. Following our studies we proposed that expression of FLIP by synovial macrophages may be a link between the two mentioned axioms.

We continued to follow apparent discrepancies in well-established notions regarding RA. For example, it was known that \textit{in vitro} TNF is able to induce apoptosis and thus to reduce the number of cells in different cell systems. However, one main finding in the first pathological studies of synovial biopsies from patients treated with infliximab, a TNF antagonist, revealed an impressive decrease in synovial cellularity. It has been shown that this effect is related to decreased cell recruitment at the site of inflammation. However, another convenient explanation for a decrease in the cell number is a higher rate of cell clearance through increased apoptosis. In trying to confirm this hypothesis we succeeded in demonstrating that both infliximab and etanercept are able to induce cell-type specific apoptosis in RA. An important concern in publishing our work was related to studies in Crohn’s disease in which infliximab, but not etanercept, was able to induce apoptosis of activated lymphocytes, suggesting a potential theory for the differences of efficiency of the two drugs in Crohn disease versus RA. However we propose an alternative/complementary hypothesis. The difference may be due not to a difference in effect but to a difference in the target cell. According to this hypothesis both drugs are able to induce apoptosis in activated macrophages (present in both RA and Crohn’s disease) but not in hyporesponsive lymphocytes (present in RA but not in Crohn’s disease). Further studies to unify these findings in a common hypothesis are currently needed.

Cartilage and bone destruction occurs in RA as a consequence of ongoing synovial inflammation. Thus we investigated if treatment with an efficient anti-inflammatory treatment such as TNF antagonists may also exert joint protective effects. We demonstrated a significant downmodulation of the main pathways implicated in bone and cartilage destruction in RA: the MMP/TIMP system and the OPG/RANKL system. Thus it is plausible that the bone protective effect of anti-TNF antagonists evident in patients treated with either etanercept or infliximab is at least partly mediated through these pathways.
The findings presented in this thesis open several perspectives. First, it may be that pathways identified during this thesis work as potential TNF-independent mechanisms (synovial RANKL expression, synovial expression of MMPS, synovial expression of IL15, lymphocyte apoptosis) may represent future therapeutic targets for RA patients. Second, study of the potential treatment-relevant pathogenic pathways identified by this work (MMP, OPG, RANKL) in large cohorts of patients may help to identify treatment predictors. Third, the mechanisms described offer a rationale for the use of TNF antagonists in specific clinical settings.

Even though this thesis work has added to the knowledge of the therapeutic use of TNF antagonists in RA, further studies are still needed to identify new mechanisms of action and to better characterize those presented here in the hope of a more rationale use of these drugs and a future development of superior agents for the benefit of the RA patients.
5 CONCLUDING REMARKS

- Inflammatory and bone destruction pathways are important pathogenic features of the rheumatoid synovium
  - RA synovial macrophages express high levels of the anti-apoptotic molecule FLIP and are the main determinants of RA synovial apoptotic pattern.
  - Synovial membrane is characterized by an imbalance between MMP and TIMP expression in favor of bone destruction
  - Inappropriate expression of osteoprotegerin in the rheumatoid synovium may contribute to bone destruction in RA.

- Therapy with TNF antagonists such as etanercept and infliximab is highly active in patients with RA through downmodulation of both synovial inflammation and cartilage and bone destruction.
  - TNF antagonists induce cell type-specific apoptosis and decrease synovial cellularity.
  - TNF antagonists are joint protective through complex mechanisms implicating both a decrease of pro-destructive factors as well as upregulation of protective mechanisms.
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Do you want to get to know something?
Look at it from close up! Do you want to
get to love it? Look at it from afar!

Ion Luca Caragiale
7 REFERENCES


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