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

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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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CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
Apaf-1	Apoptosis protease-activating factor 1
Bcl-2	B cell leukemia/lymphoma-2
CD	Cluster of Differentiation
Cyt c	Cytocrome c
CRP	C-reactive protein
DAS	Disease Activity Score
DMARD	Disease modifying anti rheumatic drug
EULAR	European League against Rheumatism
ESR	Erythrocyte sedimentation rate
FADD	Fas-associated death domain
FasL	Fas ligand
FLICE	Fas ligand-interacting cell effector
FLIP	FLICE inhibitory protein
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
Mcl-1	Myeloid cell leukemia sequence-1
MMP	Matrix metalloproteinase
MTX	Methotrexate
NF-κB	Nuclear factor-κB
NSAID	Nonsteroid antiinflammatory drugs
OPG	Osteoprotegerin
RA	Rheumatoid arthritis
ra	Receptor antagonist
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol-3-OH kinase
RANKL	Receptor activator of the nuclear factor-kB ligand
RIP	Receptor interacting protein
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
STAT	Signal transducer and activator of transcription
SUMO-1	Small ubiquitin-related modifier-1
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitor of the matrix metalloproteinases
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TRADD	Tumor necrosis factor receptor associated death domain
TUNEL	Terminal deoxynucleotidyl-mediated dUTP nick end labelling

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 dyzigotic 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 2. Improvement criteria for treatment evaluation in RA

Improvement criteria	Definition
ACR (19)	"*Response*" - improvement by at least 20% in tender joint count *and* swollen joint count *plus* in at least 3 out of the remaining 5 core set measures (acute phase reactant, physical function, doctor global assessment, patient global assessment, pain)
	"*No response*" - not meeting the mentioned criteria
EULAR (20)	*"Good response":* improvement in DAS >1.2 and final DAS ≤2.4
	"Moderate response" - not meeting criteria for "good response", but improvement in DAS >0.6 and final DAS ≤3.7
	"No response" – not meeting the above-mentioned criteria

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 cel- 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 epiphenomenom 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 eachother 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.

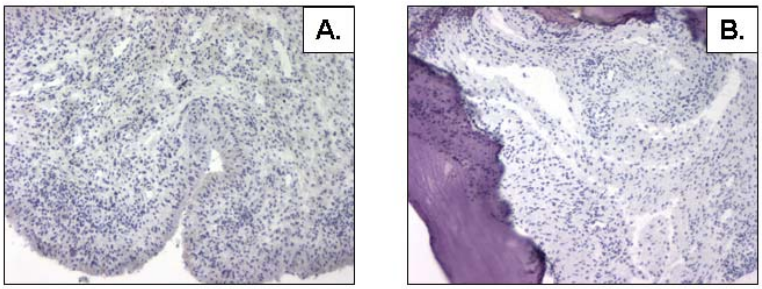

Figure 1. Histological features RA synvoium (A) and RA pannus (B)

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.

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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

(PB) monocytes in patients with RA, the pre-synovial changes are of limited extent (32). Synovial macrophages represent the principal source of synovial cytokines and are important mediators of local inflammation and joint damage (33).

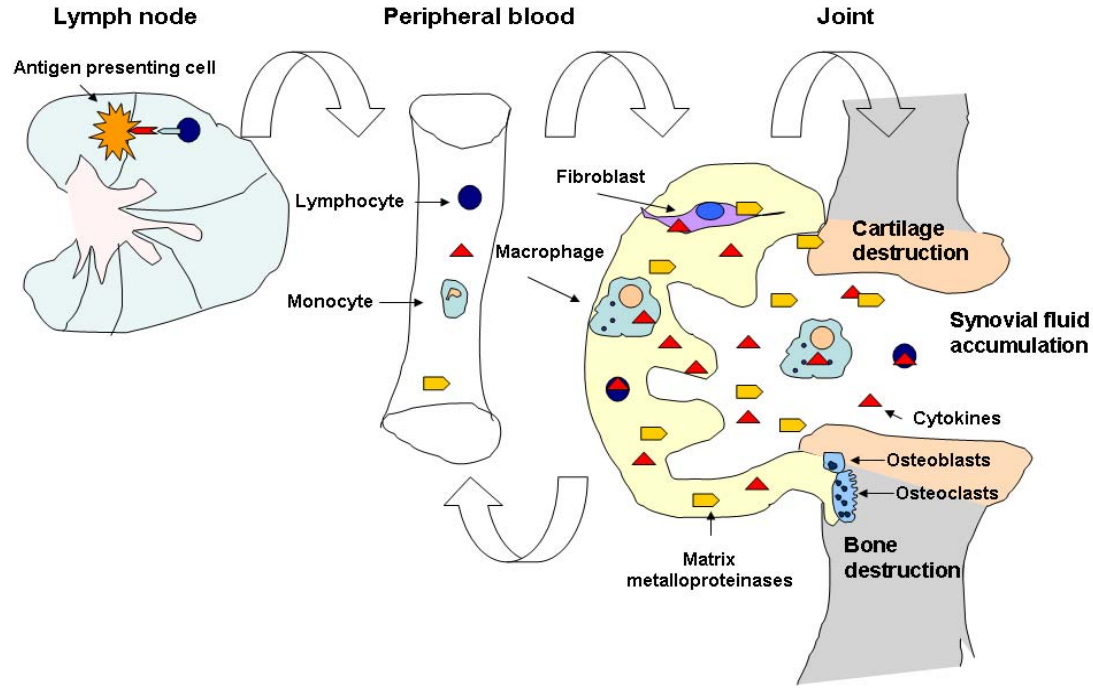

Figure 2. Rheumatoid arthritis – a schematic illustration of the pathogenic mechanism

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 Fc 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 3. Cytokines and their role in RA (40)				
Class	Cytokine	Inhibitor	RA synovial pattern	Effect in RA
Th1 cytokines	IFN-γ		Low amounts in ST and SF	Both pro and anti inflammatory properties
	IL-2	Soluble receptor IL-2R	Low amounts in ST and SF	Proinflammatory
	IL-17		Low levels in the ST	Proinflammatory
Th2 cytokines	IL-4		Low levels in RA	Anti-inflammatory
	IL-13		Little or no expression in RA	Anti-inflammatory
Monokines and fibroblasts-derived cytokines	IL-1	Receptor antagonist IL-1ra Soluble receptors IL-1R	Present both in ST and SF in relatively high amount	Proinflammatory Promotes bone destruction
	TNF-α	Soluble receptors TNF-R	Present both in ST and SF in variable amounts	Proinflammatory Promotes bone destruction
	IL-6		Present both in ST and SF at high levels	Pro and anti inflammatory properties
	IL-8		High levels in SF and ST	Proinflammatory
	IL-15		Present in the ST	Proinflammatory
	IL-18		Present in the ST	Proinflammatory
	GM-CSF		Present both in SF and ST	Proinflammatory
	M-CSF		High SF levels and present in ST	Macrophage proliferation
	IL-10		Present both in ST and SF	Anti- inflammatory
Growth factors	TGF-β		High levels in ST and SF	Pro and anti inflammatory
	PDGF		Present in ST	Increases cell proliferation
	FGF		Present in SF	Increases proliferation and angiogenesis
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 posses 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

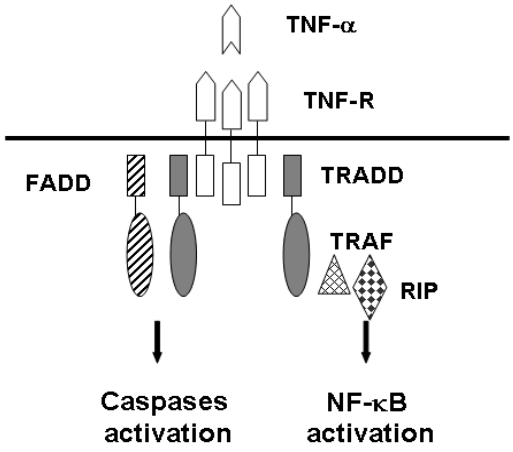

Figure 3. Intracellular signalling pathways of tumor necrosis factor receptors
(*Abbreviations: tumor necrosis factor TNF, TNF receptors TNF-R, TNF receptor associated death domain TRADD, Fas-associating protein with death domain FADD, TNF receptor associated factor TRAF, receptor interacting protein RIP*)

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 cytocrome 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

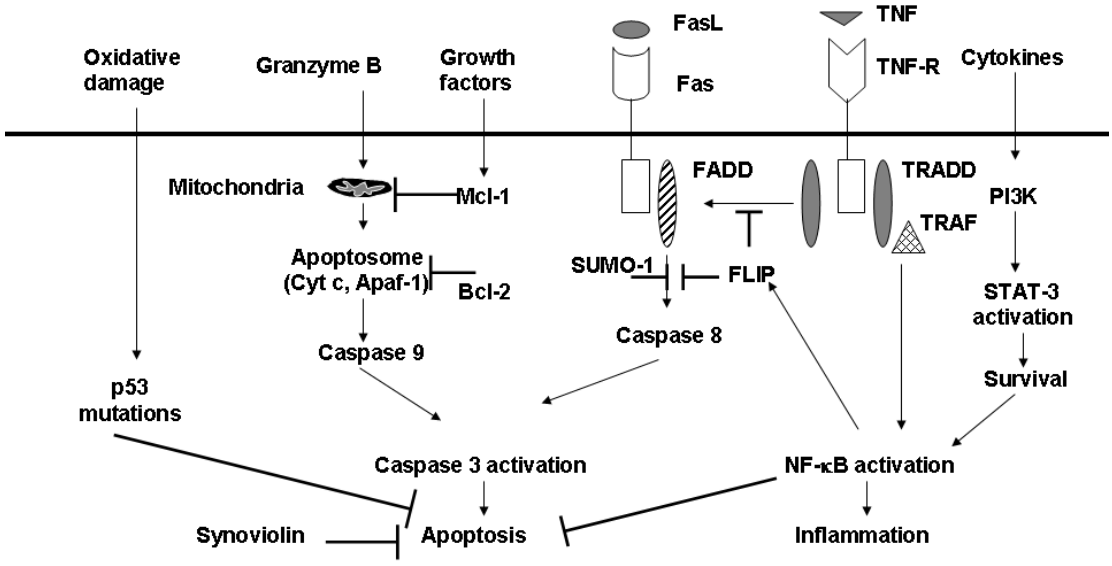

Figure 4. Synovial modulation of apoptosis in rheumatoid arthritis.

(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, cytocrome c cyt c, B-cell leukemia/lymphoma-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-kB,)

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

are easy to study due to their capacity to expand *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 osteclastogenesis 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.

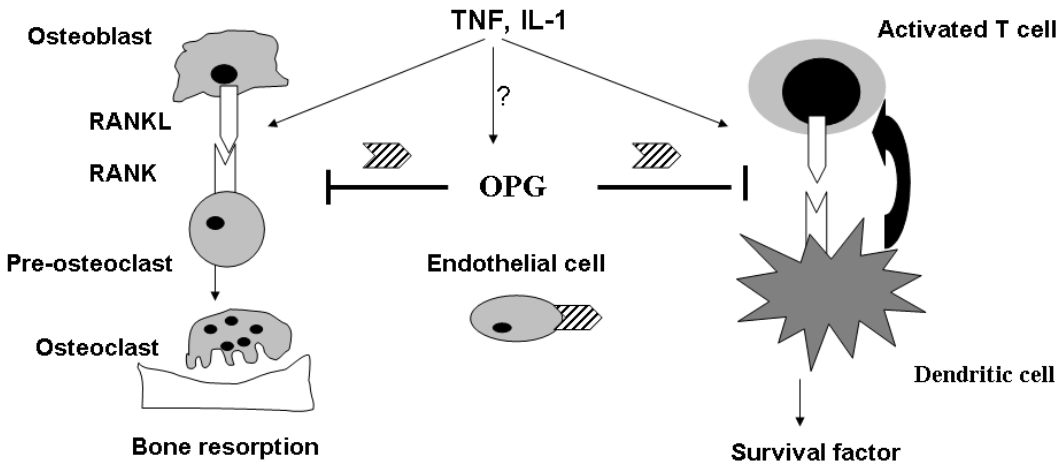

Figure 5. The RANKL/RANK/OPG system in rheumatoid arthritis
(Abbreviations receptor activator of the nuclear factor-κB RANK, receptor activator of the nuclear factor-κB ligand RANKL, osteoprotegerin OPG, tumor necrosis factor TNF, interleukin IL)

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

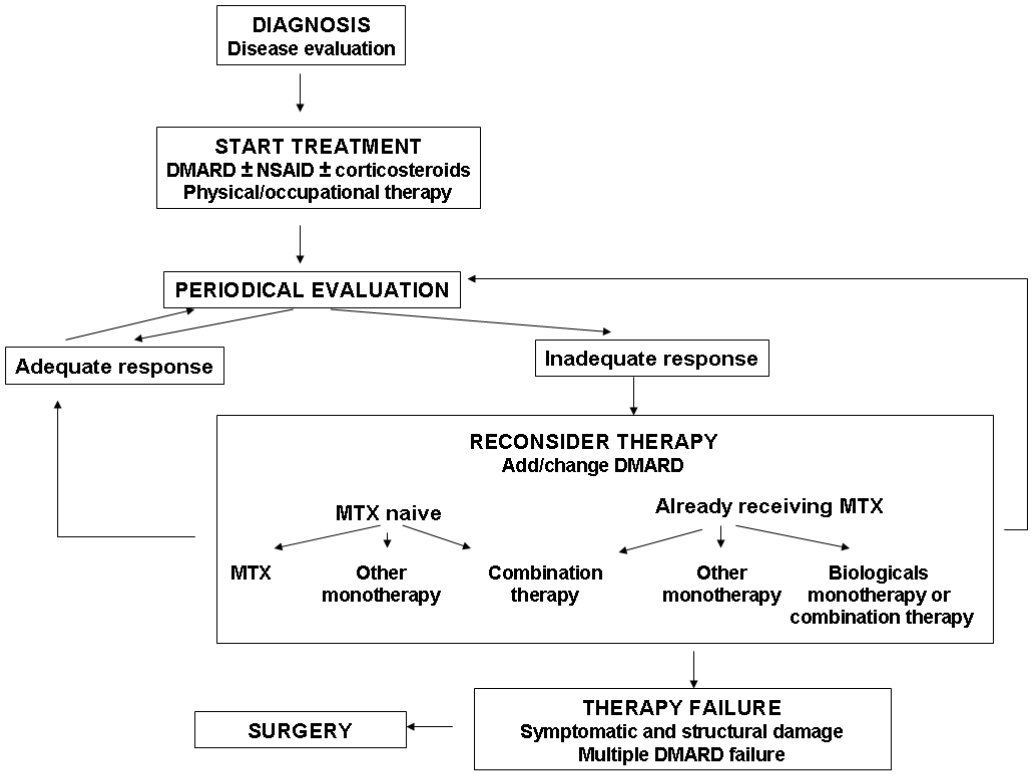

Figure 6. ACR guidelines for the management of rheumatoid arthritis (2002 update), adapted from Arthritis and Rheumatism, 46(2):328-346
(Abbreviations: methotrexate MTX, disease-modifyng antirheumatic drugs DMARD, nonsteroidal antiinflammatory drugs NSAID)

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.

Table 4. DMARD treatment in RA

DMARD	Effect
Antimalarials (136)	Stabilize lysosomal membranes and inhibit metabolism of deoxyribonucleotides
Gold compounds (136)	Inhibit NF-κB activation Inhibit osteoclastic activation Inhibit B cell activation Increase IL-1ra production
Penicillamine (136)	Effects on free radical scavenging Catalyzes removal of metals from metalloproteinases
Sulfasalazine (141)	Inhibits monokines production Induces T cell apoptosis Blocks NF-κB activation Suppresses fibroblasts activation
Methotrexate (142)	Adenosine mediated anti-inflammatory effects Induces apoptosis in T cells and synovial cells Decreases production of pro inflammatory molecules
Leflunomide (143)	Blocks T cell proliferation Inhibits tyrosine kinase acitivty and NF-κB activation Decreases production of pro inflammatory cytokines
Immunosupresive drugs (144)	
• Azathioprine	Reduces lymphocyte function and number, cytotoxic
• Chlorambucil	Cross-links DNA and prevents cell replication
• Cyclophosphamide	Cross-links DNA and prevents cell replication
• Cyclosporine	Suppresses IL-2 synthesis and T cell response
• Mycofenolate mofetil	Inhibits T cell proliferation

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.

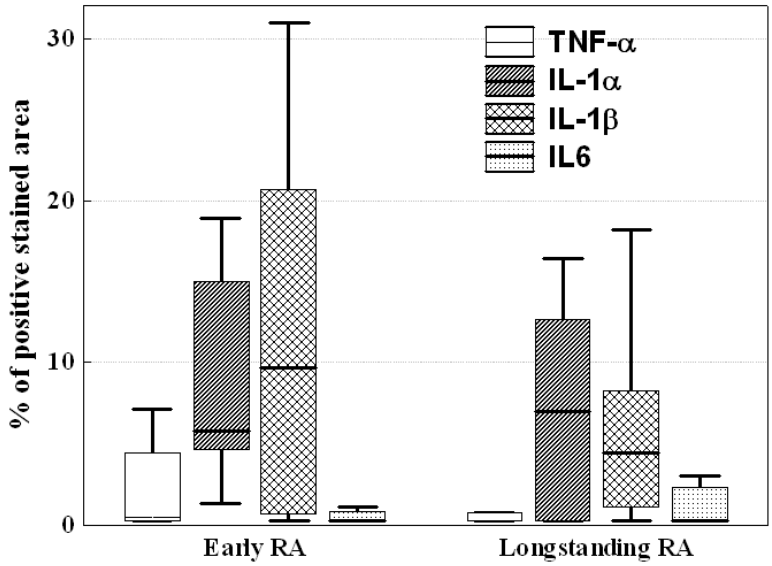

Figure 7. No differences in synovial cytokines expression in early as compared to longstanding end stage RA

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 needled/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

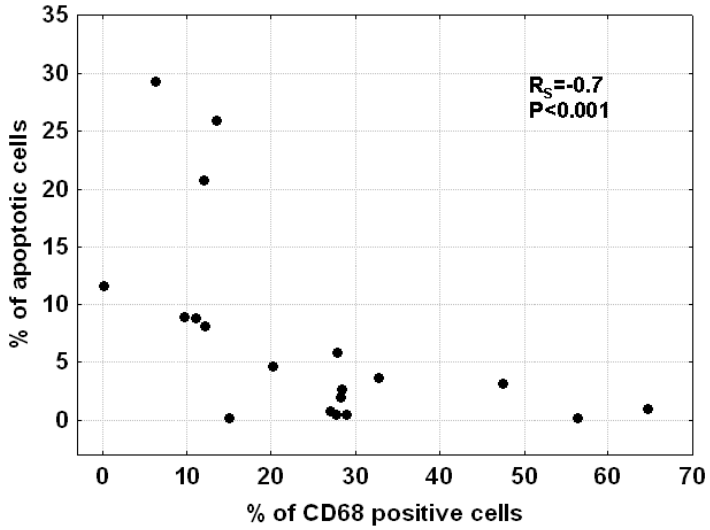

Figure 8. Synovial apoptosis correlates with synovial macrophage number

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, AI 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).

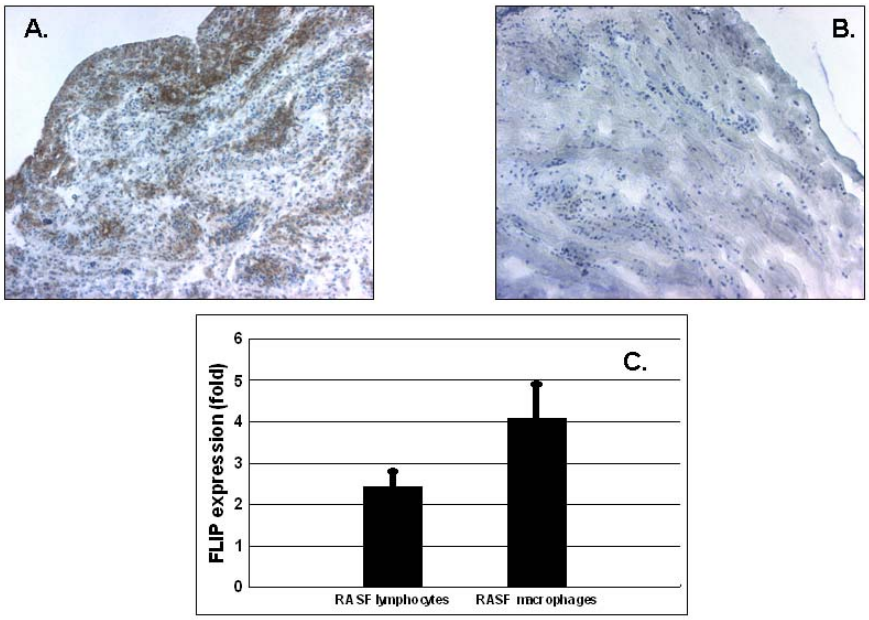

Figure 9. FLIP is present in the rheumatoid arthritis (RA) synovium (A) but not in the normal synovium (B). Synovial fluid (SF) derived macrophages express higher levels of FLIP as compared to synovial fluid derived lymphocytes, as evaluated by flow cytometry (C).

4.4 BONE DESTRUCTION MARKERS IN RA SYNOVIUM 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

(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 cocordance 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 *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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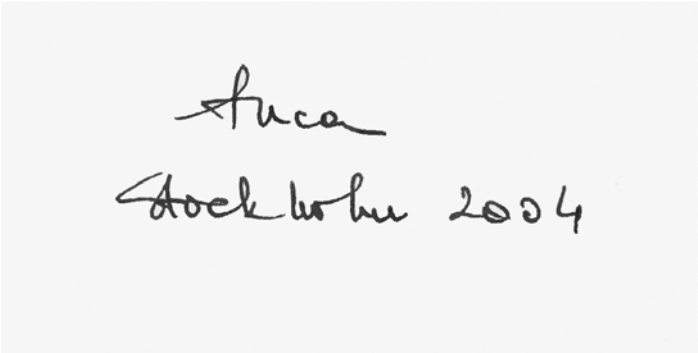

Do you want to get to know something? Look at it from close up! Do you want to get to love it? Look at it from afar!
Ion Luca Caragiale

7 REFERENCES

1. Beaglehole R, Yach D. Globalisation and the prevention and control of non-communicable disease: the neglected chronic diseases of adults. Lancet 2003;362(9387):903-8.

2. Kvien TK. Epidemiology and burden of illness of rheumatoid arthritis. Pharmacoeconomics 2004;22(2 Suppl):1-12.

3. Gabriel SE. The epidemiology of rheumatoid arthritis. Rheum Dis Clin North Am 2001;27(2):269-81.

4. Silman A, Davies P, Currey HL, Evans SJ. Is rheumatoid arthritis becoming less severe? J Chronic Dis 1983;36(12):891-7.

5. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31(3):315-24.

6. Symmons DP. Epidemiology of rheumatoid arthritis: determinants of onset, persistence and outcome. Best Pract Res Clin Rheumatol 2002;16(5):707-22.

7. Aho K, Koskenvuo M, Tuominen J, Kaprio J. Occurrence of rheumatoid arthritis in a nationwide series of twins. J Rheumatol 1986;13(5):899-902.

8. Silman AJ, MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. Br J Rheumatol 1993;32(10):903-7.

9. Gregersen PK. Genetics of rheumatoid arthritis: confronting complexity. Arthritis Res 1999;1(1):37-44.

10. Symmons DP. Environmental factors and the outcome of rheumatoid arthritis. Best Pract Res Clin Rheumatol 2003;17(5):717-27.

11. van Riel PL, van Gestel AM. Clinical outcome measures in rheumatoid arthritis. Ann Rheum Dis 2000;59 Suppl 1:i28-31.

12. van Riel PL, van de Putte LB. Clinical assessment and clinical trials in rheumatoid arthritis. Curr Opin Rheumatol 1994;6(2):132-9.

13. Wollheim FA, Eberhardt KB. The search for laboratory measures of outcome in rheumatoid arthritis. Baillieres Clin Rheumatol 1992;6(1):69-93.

14. van Leeuwen MA, van Rijswijk MH. Acute phase proteins in the monitoring of inflammatory disorders. Baillieres Clin Rheumatol 1994;8(3):531-52.

15. van der Heijde DM, van 't Hof M, van Riel PL, van de Putte LB. Validity of single variables and indices to measure disease activity in rheumatoid arthritis. J Rheumatol 1993;20(3):538-41.

16. van Leeuwen MA, van Rijswijk MH, van der Heijde DM, Te Meerman GJ, van Riel PL, Houtman PM, et al. The acute-phase response in relation to radiographic progression in early rheumatoid arthritis: a prospective study during the first three years of the disease. Br J Rheumatol 1993;32 Suppl 3:9-13.

17. van der Heijde DM, van 't Hof M, van Riel PL, van de Putte LB. Development of a disease activity score based on judgment in clinical practice by rheumatologists. J Rheumatol 1993;20(3):579-81.

18. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38(1):44-8.

19. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology. Preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 1995;38(6):727-35.

20. van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria. Arthritis Rheum 1996;39(1):34-40.

21. Sharp JT, Lidsky MD, Collins LC, Moreland J. Methods of scoring the progression of radiologic changes in rheumatoid arthritis. Correlation of radiologic, clinical and laboratory abnormalities. Arthritis Rheum 1971;14(6):706-20.

22. Larsen A. How to apply Larsen score in evaluating radiographs of rheumatoid arthritis in long-term studies. J Rheumatol 1995;22(10):1974-5.

23. Kosinski M, Zhao SZ, Dedhiya S, Osterhaus JT, Ware JE, Jr. Determining minimally important changes in generic and disease-specific health-related quality of life questionnaires in clinical trials of rheumatoid arthritis. Arthritis Rheum 2000;43(7):1478-87.

24. Mikuls TR. Co-morbidity in rheumatoid arthritis. Best Pract Res Clin Rheumatol 2003;17(5):729-52.

25. Hyrich KL, Inman RD. Infectious agents in chronic rheumatic diseases. Curr Opin Rheumatol 2001;13(4):300-4.

26. Scofield RH. Autoantibodies as predictors of disease. Lancet 2004;363(9420):1544-6.
27. Liew FY, McInnes IB. Role of interleukin 15 and interleukin 18 in inflammatory response. Ann Rheum Dis 2002;61 Suppl 2:ii100-2.
28. Firestein GS. Rheumatoid synovitis and pannus. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 855-884.
29. Huizinga TW. Genetics in rheumatoid arthritis. Best Pract Res Clin Rheumatol 2003;17(5):703-16.
30. Klareskog L, Lorentzen J, Padyukov L, Alfredsson L. Genes and environment in arthritis: can RA be prevented? Arthritis Res 2002;4 Suppl 3:S31-6.
31. Simkin PA. The musculoskeletal system. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 57-68.
32. Highton J, Carlisle B, Palmer DG. Changes in the phenotype of monocytes/macrophages and expression of cytokine mRNA in peripheral blood and synovial fluid of patients with rheumatoid arthritis. Clin Exp Immunol 1995;102(3):541-6.
33. Burmester GR, Stuhlmuller B, Keyszer G, Kinne RW. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? Arthritis Rheum 1997;40(1):5-18.
34. Cope AP. Studies of T-cell activation in chronic inflammation. Arthritis Res 2002;4 Suppl 3:S197-211.
35. Davis LS. A question of transformation: the synovial fibroblast in rheumatoid arthritis. Am J Pathol 2003;162(5):1399-402.
36. Cravens PD, Lipsky PE. Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. Immunol Cell Biol 2002;80(5):497-505.
37. Dorner T, Burmester GR. The role of B cells in rheumatoid arthritis: mechanisms and therapeutic targets. Curr Opin Rheumatol 2003;15(3):246-52.
38. Liu H, Pope RM. Phagocytes: mechanisms of inflammation and tissue destruction. Rheum Dis Clin North Am 2004;30(1):19-39, v.
39. Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci U S A 1994;91(18):8562-6.
40. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397-440.

41. Gaur U, Aggarwal BB. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochem Pharmacol 2003;66(8):1403-8.
42. McDermott MF. TNF and TNFR biology in health and disease. Cell Mol Biol (Noisy-le-grand) 2001;47(4):619-35.
43. Aggarwal BB. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB. Ann Rheum Dis 2000;59 Suppl 1:i6-16.
44. Pagliari LJ, Perlman H, Liu H, Pope RM. Macrophages require constitutive NF-kappaB activation to maintain A1 expression and mitochondrial homeostasis. Mol Cell Biol 2000;20(23):8855-65.
45. Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. Trends Cell Biol 1995;5(10):392-9.
46. Watts AD, Hunt NH, Wanigasekara Y, Bloomfield G, Wallach D, Roufogalis BD, et al. A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in 'reverse signalling'. Embo J 1999;18(8):2119-26.
47. Ferran C, Dautry F, Merite S, Sheehan K, Schreiber R, Grau G, et al. Anti-tumor necrosis factor modulates anti-CD3-triggered T cell cytokine gene expression in vivo. J Clin Invest 1994;93(5):2189-96.
48. Feldmann M, Brennan FM, Foxwell BM, Maini RN. The role of TNF alpha and IL-1 in rheumatoid arthritis. Curr Dir Autoimmun 2001;3:188-99.
49. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. Embo J 1991;10(13):4025-31.
50. Cooper WO, Fava RA, Gates CA, Cremer MA, Townes AS. Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta. Clin Exp Immunol 1992;89(2):244-50.
51. Hochberg MC, Tracy JK, Hawkins-Holt M, Flores RH. Comparison of the efficacy of the tumour necrosis factor alpha blocking agents adalimumab, etanercept, and infliximab when added to methotrexate in patients with active rheumatoid arthritis. Ann Rheum Dis 2003;62 Suppl 2:ii13-6.
52. McInnes IB, Gracie JA, Harnett M, Harnett W, Liew FY. New strategies to control inflammatory synovitis: interleukin 15 and beyond. Ann Rheum Dis 2003;62 Suppl 2:ii51-4.

53. Orrenius S. Mitochondrial regulation of apoptotic cell death. Toxicol Lett 2004;149(1-3):19-23.

54. Stadelmann C, Lassmann H. Detection of apoptosis in tissue sections. Cell Tissue Res 2000;301(1):19-31.

55. Fadeel B, Orrenius S, Zhivotovsky B. Apoptosis in human disease: a new skin for the old ceremony? Biochem Biophys Res Commun 1999;266(3):699-717.

56. Perlman H, Pagliari LJ, Volin MV. Regulation of apoptosis and cell cycle activity in rheumatoid arthritis. Curr Mol Med 2001;1(5):597-608.

57. Ceponis A, Hietanen J, Tamulaitiene M, Partsch G, Patiala H, Konttinen YT. A comparative quantitative morphometric study of cell apoptosis in synovial membranes in psoriatic, reactive and rheumatoid arthritis. Rheumatology (Oxford) 1999;38(5):431-40.

58. Matsumoto S, Muller-Ladner U, Gay RE, Nishioka K, Gay S. Ultrastructural demonstration of apoptosis, Fas and Bcl-2 expression of rheumatoid synovial fibroblasts. J Rheumatol 1996;23(8):1345-52.

59. Asahara H, Hasumuna T, Kobata T, Yagita H, Okumura K, Inoue H, et al. Expression of Fas antigen and Fas ligand in the rheumatoid synovial tissue. Clin Immunol Immunopathol 1996;81(1):27-34.

60. Salmon M, Scheel-Toellner D, Huissoon AP, Pilling D, Shamsadeen N, Hyde H, et al. Inhibition of T cell apoptosis in the rheumatoid synovium. J Clin Invest 1997;99(3):439-46.

61. Hasunuma T, Kayagaki N, Asahara H, Motokawa S, Kobata T, Yagita H, et al. Accumulation of soluble Fas in inflamed joints of patients with rheumatoid arthritis. Arthritis Rheum 1997;40(1):80-6.

62. Hashimoto H, Tanaka M, Suda T, Tomita T, Hayashida K, Takeuchi E, et al. Soluble Fas ligand in the joints of patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum 1998;41(4):657-62.

63. Nozawa K, Kayagaki N, Tokano Y, Yagita H, Okumura K, Hasimoto H. Soluble Fas (APO-1, CD95) and soluble Fas ligand in rheumatic diseases. Arthritis Rheum 1997;40(6):1126-9.

64. Handel ML, McMorrow LB, Gravallese EM. Nuclear factor-kappa B in rheumatoid synovium. Localization of p50 and p65. Arthritis Rheum 1995;38(12):1762-70.

65. Marok R, Winyard PG, Coumbe A, Kus ML, Gaffney K, Blades S, et al. Activation of the transcription factor nuclear factor-kappaB in human inflamed synovial tissue. Arthritis Rheum 1996;39(4):583-91.

66. Han Z, Boyle DL, Manning AM, Firestein GS. AP-1 and NF-kappaB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 1998;28(4):197-208.

67. Perlman H, Pagliari LJ, Liu H, Koch AE, Haines GK, 3rd, Pope RM. Rheumatoid arthritis synovial macrophages express the Fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein and are refractory to Fas-mediated apoptosis. Arthritis Rheum 2001;44(1):21-30.

68. Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients. J Immunol 1998;161(2):1018-25.

69. Zhang J, Bardos T, Mikecz K, Finnegan A, Glant TT. Impaired Fas signaling pathway is involved in defective T cell apoptosis in autoimmune murine arthritis. J Immunol 2001;166(8):4981-6.

70. Franz JK, Pap T, Hummel KM, Nawrath M, Aicher WK, Shigeyama Y, et al. Expression of sentrin, a novel antiapoptotic molecule, at sites of synovial invasion in rheumatoid arthritis. Arthritis Rheum 2000;43(3):599-607.

71. Amano T, Yamasaki S, Yagishita N, Tsuchimochi K, Shin H, Kawahara K, et al. Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. Genes Dev 2003;17(19):2436-49.

72. Shouda T, Yoshida T, Hanada T, Wakioka T, Oishi M, Miyoshi K, et al. Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. J Clin Invest 2001;108(12):1781-8.

73. Krause A, Scaletta N, Ji JD, Ivashkiv LB. Rheumatoid arthritis synoviocyte survival is dependent on Stat3. J Immunol 2002;169(11):6610-6.

74. Kim G, Jun JB, Elkon KB. Necessary role of phosphatidylinositol 3-kinase in transforming growth factor beta-mediated activation of Akt in normal and rheumatoid arthritis synovial fibroblasts. Arthritis Rheum 2002;46(6):1504-11.

75. Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358(6381):15-6.

76. Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR. Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. Proc Natl Acad Sci U S A 1997;94(20):10895-900.

77. Alvaro-Gracia JM, Zvaifler NJ, Firestein GS. Cytokines in chronic inflammatory arthritis. V. Mutual antagonism between interferon-gamma and tumor necrosis factor-alpha on HLA-DR expression, proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor production by rheumatoid arthritis synoviocytes. J Clin Invest 1990;86(6):1790-8.

78. Lefebvre V, Peeters-Joris C, Vaes G. Modulation by interleukin 1 and tumor necrosis factor alpha of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. Biochim Biophys Acta 1990;1052(3):366-78.

79. Lorenzo JA, Pilbeam CC, Kalinowski JF, Hibbs MS. Production of both 92- and 72-kDa gelatinases by bone cells. Matrix 1992;12(4):282-90.

80. Saren P, Welgus HG, Kovanen PT. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. J Immunol 1996;157(9):4159-65.

81. Nelimarkka LO, Nikkari ST, Ravanti LS, Kahari VM, Jarvelainen HT. Collagenase-1, stromelysin-1 and 92 kDa gelatinase are associated with tumor necrosis factor-alpha induced morphological change of human endothelial cells in vitro. Matrix Biol 1998;17(4):293-304.

82. Curran S, Murray GI. Matrix metalloproteinases in tumour invasion and metastasis. J Pathol 1999;189(3):300-8.

83. Martel-Pelletier J, Welsch DJ, Pelletier JP. Metalloproteases and inhibitors in arthritic diseases. Best Pract Res Clin Rheumatol 2001;15(5):805-29.

84. Matrisian LM. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 1990;6(4):121-5.

85. Ishiguro N, Ito T, Obata K, Fujimoto N, Iwata H. Determination of stromelysin-1, 72 and 92 kDa type IV collagenase, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 in synovial fluid and serum from patients with rheumatoid arthritis. J Rheumatol 1996;23(9):1599-604.

86. Zucker S, Lysik RM, Zarrabi MH, Greenwald RA, Gruber B, Tickle SP, et al. Elevated plasma stromelysin levels in arthritis [see comments]. J Rheumatol 1994;21(12):2329-33.

87. Yoshihara Y, Obata K, Fujimoto N, Yamashita K, Hayakawa T, Shimmei M. Increased levels of stromelysin-1 and tissue inhibitor of metalloproteinases-1 in sera from patients with rheumatoid arthritis. Arthritis Rheum 1995;38(7):969-75.

88. Ribbens C, Andre B, Jaspar JM, Kaye O, Kaiser MJ, De Groote D, et al. Matrix metalloproteinase-3 serum levels are correlated with disease activity and predict clinical response in rheumatoid arthritis. J Rheumatol 2000;27(4):888-93.

89. Ichikawa Y, Yamada C, Horiki T, Hoshina Y, Uchiyama M. Serum matrix metalloproteinase-3 and fibrin degradation product levels correlate with clinical disease activity in rheumatoid arthritis. Clin Exp Rheumatol 1998;16(5):533-40.

90. Keyszer G, Lambiri I, Nagel R, Keysser C, Keysser M, Gromnica-Ihle E, et al. Circulating levels of matrix metalloproteinases MMP-3 and MMP-1, tissue inhibitor of metalloproteinases 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers [see comments]. J Rheumatol 1999;26(2):251-8.

91. Posthumus MD, Limburg PC, Westra J, Cats HA, Stewart RE, van Leeuwen MA, et al. Serum levels of matrix metalloproteinase-3 in relation to the development of radiological damage in patients with early rheumatoid arthritis. Rheumatology (Oxford) 1999;38(11):1081-7.

92. Cheung NT, Dawes PT, Poulton KV, Ollier WE, Taylor DJ, Mattey DL. High serum levels of pro-matrix metalloproteinase-3 are associated with greater radiographic damage and the presence of the shared epitope in patients with rheumatoid arthritis. J Rheumatol 2000;27(4):882-7.

93. Yamanaka H, Matsuda Y, Tanaka M, Sendo W, Nakajima H, Taniguchi A, et al. Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. Arthritis Rheum 2000;43(4):852-8.

94. Mattey DL, Nixon NB, Dawes PT, Ollier WE, Hajeer AH. Association of matrix metalloproteinase 3 promoter genotype with disease outcome in rheumatoid arthritis. Genes Immun 2004;5(2):147-9.

95. Vincenti MP, Clark IM, Brinckerhoff CE. Using inhibitors of metalloproteinases to treat arthritis. Easier said than done? [see comments]. Arthritis Rheum 1994;37(8):1115-26.

96. Clark IM, Powell LK, Ramsey S, Hazleman BL, Cawston TE. The measurement of collagenase, tissue inhibitor of metalloproteinases (TIMP), and collagenase-TIMP complex in synovial fluids from patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum 1993;36(3):372-9.

97. Green MJ, Gough AK, Devlin J, Smith J, Astin P, Taylor D, et al. Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. Rheumatology (Oxford) 2003;42(1):83-8.

98. Firestein GS, Paine MM, Littman BH. Gene expression (collagenase, tissue inhibitor of metalloproteinases, complement, and HLA-DR) in rheumatoid arthritis and osteoarthritis synovium. Quantitative analysis and effect of intraarticular corticosteroids. Arthritis Rheum 1991;34(9):1094-105.

99. Ishiguro N, Ito T, Miyazaki K, Iwata H. Matrix metalloproteinases, tissue inhibitors of metalloproteinases, and glycosaminoglycans in synovial fluid from patients with rheumatoid arthritis. J Rheumatol 1999;26(1):34-40.

100. Hofbauer LC, Heufelder AE. The role of osteoprotegerin and receptor activator of nuclear factor kappaB ligand in the pathogenesis and treatment of rheumatoid arthritis. Arthritis Rheum 2001;44(2):253-9.

101. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 2000;43(2):250-8.

102. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum 2000;43(2):259-69.

103. Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM. Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. Rheumatology (Oxford) 2001;40(6):623-30.

104. Thomson BM, Mundy GR, Chambers TJ. Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol 1987;138(3):775-9.

105. Kaneyama K, Segami N, Nishimura M, Sato J, Suzuki T, Fujimura K. Osteoclastogenesis inhibitory factor/osteoprotegerin in synovial fluid from patients with temporomandibular disorders. Int J Oral Maxillofac Surg 2003;32(4):404-7.

106. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93(2):165-76.

107. Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, et al. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272(40):25190-4.

108. Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun 1998;253(2):395-400.

109. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390(6656):175-9.

110. Wong BR, Josien R, Choi Y. TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. J Leukoc Biol 1999;65(6):715-24.

111. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997;89(2):309-19.

112. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, et al. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 1998;139(3):1329-37.

113. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 1999;25(3):255-9.

114. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999;402(6759):304-9.

115. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. Biochem Biophys Res Commun 1999;265(1):144-50.

116. Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, et al. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. Arthritis Rheum 2001;44(5):1003-12.

117. Wang R, Zhang L, Zhang X, Moreno J, Celluzzi C, Tondravi M, et al. Regulation of activation-induced receptor activator of NF-kappaB ligand (RANKL) expression in T cells. Eur J Immunol 2002;32(4):1090-8.

118. Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 2001;159(5):1689-99.

119. Romas E, Bakharevski O, Hards DK, Kartsogiannis V, Quinn JM, Ryan PF, et al. Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis. Arthritis Rheum 2000;43(4):821-6.

120. Crotti TN, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M, et al. Receptor activator NF-kappaB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. Ann Rheum Dis 2002;61(12):1047-54.

121. Haynes DR, Barg E, Crotti TN, Holding C, Weedon H, Atkins GJ, et al. Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls. Rheumatology (Oxford) 2003;42(1):123-134.

122. Ziolkowska M, Kurowska M, Radzikowska A, Luszczykiewicz G, Wiland P, Dziewczopolski W, et al. High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment. Arthritis Rheum 2002;46(7):1744-53.

123. Skoumal M, Kolarz G, Woloszczuk W, Hawa G, Klingler A. Serum osteoprotegerin but not receptor activator of NF-kappaB ligand correlates with Larsen score in rheumatoid arthritis. Ann Rheum Dis 2004;63(2):216-7.

124. Romas E, Sims NA, Hards DK, Lindsay M, Quinn JW, Ryan PF, et al. Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. Am J Pathol 2002;161(4):1419-27.

125. Zwerina J, Hayer S, Tohidast-Akrad M, Bergmeister H, Redlich K, Feige U, et al. Single and combined inhibition of tumor necrosis factor, interleukin-1, and RANKL pathways in tumor necrosis factor-induced arthritis: effects on synovial inflammation, bone erosion, and cartilage destruction. Arthritis Rheum 2004;50(1):277-90.

126. Redlich K, Gortz B, Hayer S, Zwerina J, Doerr N, Kostenuik P, et al. Repair of local bone erosions and reversal of systemic bone loss upon therapy with anti-tumor necrosis factor in combination with osteoprotegerin or parathyroid hormone in tumor necrosis factor-mediated arthritis. Am J Pathol 2004;164(2):543-55.

127. Guidelines for the management of rheumatoid arthritis: 2002 Update. Arthritis Rheum 2002;46(2):328-46.

128. Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin Arthritis Rheum 2003;33(3):155-67.

129. Crofford LJ, Lipsky PE, Brooks P, Abramson SB, Simon LS, van de Putte LB. Basic biology and clinical application of specific cyclooxygenase-2 inhibitors. Arthritis Rheum 2000;43(1):4-13.

130. Brooks P. Use and benefits of nonsteroidal anti-inflammatory drugs. Am J Med 1998;104(3A):9S-13S; discussion 21S-22S.

131. Topol EJ. Failing the Public Health -- Rofecoxib, Merck, and the FDA. N Engl J Med 2004.

132. Singh G, Triadafilopoulos G. Epidemiology of NSAID induced gastrointestinal complications. J Rheumatol Suppl 1999;56:18-24.

133. Lundberg IE, Grundtman C, Larsson E, Klareskog L. Corticosteroids--from an idea to clinical use. Best Pract Res Clin Rheumatol 2004;18(1):7-19.

134. Kirwan JR. The effect of glucocorticoids on joint destruction in rheumatoid arthritis. The Arthritis and Rheumatism Council Low-Dose Glucocorticoid Study Group. N Engl J Med 1995;333(3):142-6.

135. Strand V, Simon LS. Low dose glucocorticoids in early rheumatoid arthritis. Clin Exp Rheumatol 2003;21(5 Suppl 31):S186-90.

136. Sturrock RD. Disease-modyfing antitheumatic drugs 1: antimalarials, gold and penicillamine. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 399-403.

137. Riise T, Jacobsen BK, Gran JT. Changes in therapy of rheumatoid arthritis during the period 1979 to 1996. Scand J Rheumatol 2001;30(4):199-202.

138. O'Dell JR. Therapeutic strategies for rheumatoid arthritis. N Engl J Med 2004;350(25):2591-602.

139. Wilske KR, Healey LA. Remodeling the pyramid--a concept whose time has come. J Rheumatol 1989;16(5):565-7.

140. Fries JF. Reevaluating the therapeutic approach to rheumatoid arthritis: the "sawtooth" strategy. J Rheumatol Suppl 1990;22:12-5.

141. Capell H, Madhok R. Disease-modifying antirheumatic drugs 2: sulfasalzine and dapsone. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 405-416.

142. Battistone MJ, Williams HJ. Disease-modifying antirheumatic drugs 3: methotrexate. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 417-429.
143. Keystone E, Haraoui B. Disease-modifying antirheumatic drugs 4: leflunomide. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 431-438.
144. Furst DE, Clements PJ. Disease-modifying antirheumatic drugs 5: immunosuppressives. In: Hochberg MC, Silman A, Smolen J, Weinblatt ME, Weisman M, editors. Rheumatology. 3rd ed: Mosby, Elsevier Limited; 2003. p. 439-448.
145. Andreakos ET, Foxwell BM, Brennan FM, Maini RN, Feldmann M. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. Cytokine Growth Factor Rev 2002;13(4-5):299-313.
146. Wolfe F, Cush JJ, O'Dell JR, Kavanaugh A, Kremer JM, Lane NE, et al. Consensus recommendations for the assessment and treatment of rheumatoid arthritis. J Rheumatol 2001;28(6):1423-30.
147. Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Bijl H, et al. Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. Lancet 1994;344(8930):1125-7.
148. Markham A, Lamb HM. Infliximab: a review of its use in the management of rheumatoid arthritis. Drugs 2000;59(6):1341-59.
149. Jarvis B, Faulds D. Etanercept: a review of its use in rheumatoid arthritis. Drugs 1999;57(6):945-66.
150. Kempeni J. Update on D2E7: a fully human anti-tumour necrosis factor alpha monoclonal antibody. Ann Rheum Dis 2000;59 Suppl 1:i44-5.
151. Scheinfeld N. A comprehensive review and evaluation of the side effects of the tumor necrosis factor alpha blockers etanercept, infliximab and adalimumab. J Dermatolog Treat 2004;15(5):280-294.
152. Baecklund E, Sundstrom C, Ekbom A, Catrina AI, Biberfeld P, Feltelius N, et al. Lymphoma subtypes in patients with rheumatoid arthritis: increased proportion of diffuse large B cell lymphoma. Arthritis Rheum 2003;48(6):1543-50.
153. Motulsky H. Multiple comparisons. In: Intuitive statistics: Oxford University Press Inc; 1995.
154. Ruderman EM, Weinblatt ME, Thurmond LM, Pinkus GS, Gravallese EM. Synovial tissue response to treatment with Campath-1H. Arthritis Rheum 1995;38(2):254-8.

155. Smeets TJ, Kraan MC, Versendaal J, Breedveld FC, Tak PP. Analysis of serial synovial biopsies in patients with rheumatoid arthritis: description of a control group without clinical improvement after treatment with interleukin 10 or placebo. J Rheumatol 1999;26(10):2089-93.

156. Cunnane G, Madigan A, Murphy E, FitzGerald O, Bresnihan B. The effects of treatment with interleukin-1 receptor antagonist on the inflamed synovial membrane in rheumatoid arthritis. Rheumatology (Oxford) 2001;40(1):62-9.

157. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, et al. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999;340(4):253-9.

158. Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. Lancet 1994;344(8930):1105-10.

159. Smeets TJ, Barg EC, Kraan MC, Smith MD, Breedveld FC, Tak PP. Analysis of the cell infiltrate and expression of proinflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis. Ann Rheum Dis 2003;62(7):635-8.

160. Tak PP, Kummer JA, Hack CE, Daha MR, Smeets TJ, Erkelens GW, et al. Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue. Arthritis Rheum 1994;37(12):1735-43.

161. Tak PP, Smeets TJ, Daha MR, Kluin PM, Meijers KA, Brand R, et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum 1997;40(2):217-25.

162. Baeten D, Demetter P, Cuvelier C, Van Den Bosch F, Kruithof E, Van Damme N, et al. Comparative study of the synovial histology in rheumatoid arthritis, spondyloarthropathy, and osteoarthritis: influence of disease duration and activity. Ann Rheum Dis 2000;59(12):945-53.

163. Katrib A, Tak PP, Bertouch JV, Cuello C, McNeil HP, Smeets TJ, et al. Expression of chemokines and matrix metalloproteinases in early rheumatoid arthritis. Rheumatology (Oxford) 2001;40(9):988-94.

164. Catrina AI, Ulfgren AK, Lindblad S, Grondal L, Klareskog L. Low levels of apoptosis and high FLIP expression in early rheumatoid arthritis synovium. Ann Rheum Dis 2002;61(10):934-6.

165. Tak PP, Klapwijk MS, Broersen SF, van de Geest DA, Overbeek M, Firestein GS. Apoptosis and p53 expression in rat adjuvant arthritis. Arthritis Res 2000;2(3):229-35.

166. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 1997;386(6624):517-21.

167. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, et al. Inhibition of death receptor signals by cellular FLIP. Nature 1997;388(6638):190-5.

168. Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P, Grandien A. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J Exp Med 1999;190(7):1025-32.

169. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. Immune escape of tumors in vivo by expression of cellular FLICE- inhibitory protein. J Exp Med 1999;190(7):1033-8.

170. Schedel J, Gay RE, Kuenzler P, Seemayer C, Simmen B, Michel BA, et al. FLICE-inhibitory protein expression in synovial fibroblasts and at sites of cartilage and bone erosion in rheumatoid arthritis. Arthritis Rheum 2002;46(6):1512-8.

171. Palao G, Santiago B, Galindo M, Paya M, Ramirez JC, Pablos JL. Down-regulation of FLIP sensitizes rheumatoid synovial fibroblasts to Fas-mediated apoptosis. Arthritis Rheum 2004;50(9):2803-10.

172. Tchetverikov I, Ronday HK, Van El B, Kiers GH, Verzijl N, TeKoppele JM, et al. MMP profile in paired serum and synovial fluid samples of patients with rheumatoid arthritis. Ann Rheum Dis 2004;63(7):881-3.

173. Tak PP, Taylor PC, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, et al. Decrease in cellularity and expression of adhesion molecules by anti- tumor necrosis factor alpha monoclonal antibody treatment in patients with rheumatoid arthritis. Arthritis Rheum 1996;39(7):1077-81.

174. Smeets TJ, Kraan MC, van Loon ME, Tak PP. Tumor necrosis factor alpha blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue. Arthritis Rheum 2003;48(8):2155-62.

175. Di Sabatino A, Ciccocioppo R, Cinque B, Millimaggi D, Morera R, Ricevuti L, et al. Defective mucosal T cell death is sustainably reverted by infliximab in a caspase dependent pathway in Crohn's disease. Gut 2004;53(1):70-7.

176. Lugering A, Schmidt M, Lugering N, Pauels HG, Domschke W, Kucharzik T. Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 2001;121(5):1145-57.

177. ten Hove T, van Montfrans C, Peppelenbosch MP, van Deventer SJ. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50(2):206-11.

178. Van den Brande JM, Braat H, van den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003;124(7):1774-85.

179. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol 2003;3(7):521-33.

180. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41(9):1669-76.

181. Berg L, Ronnelid J, Klareskog L, Bucht A. Down-regulation of the T cell receptor CD3 zeta chain in rheumatoid arthritis (RA) and its influence on T cell responsiveness. Clin Exp Immunol 2000;120(1):174-82.

182. Ulfgren AK, Lindblad S, Klareskog L, Andersson J, Andersson U. Detection of cytokine producing cells in the synovial membrane from patients with rheumatoid arthritis. Ann Rheum Dis 1995;54(8):654-61.

183. Berg L, Lampa J, Rogberg S, van Vollenhoven R, Klareskog L. Increased peripheral T cell reactivity to microbial antigens and collagen type II in rheumatoid arthritis after treatment with soluble TNFalpha receptors. Ann Rheum Dis 2001;60(2):133-9.

184. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity 2001;14(2):105-10.

185. Ernestam S, Catrina AI, af Klint E, Sundberg E, Grundtman C, Engstrom M, et al. Effect of infliximab on synovial expression of IL15. In: EULAR conference; 2004; Berlin; 2004.

186. Frishman JI, Edwards CK, 3rd, Sonnenberg MG, Kohno T, Cohen AM, Dinarello CA. Tumor necrosis factor (TNF)-alpha-induced interleukin-8 in human blood cultures discriminates neutralization by the p55 and p75 TNF soluble receptors. J Infect Dis 2000;182(6):1722-30.

187. Suffredini AF, Reda D, Banks SM, Tropea M, Agosti JM, Miller R. Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. J Immunol 1995;155(10):5038-45.

188. D'Auria F, Rovere-Querini P, Giazzon M, Ajello P, Baldissera E, Manfredi AA, et al. Accumulation of plasma nucleosomes upon treatment with anti-tumour necrosis factor-alpha antibodies. J Intern Med 2004;255(3):409-18.

189. van Deventer SJ. Transmembrane TNF-alpha, induction of apoptosis, and the efficacy of TNF-targeting therapies in Crohn's disease. Gastroenterology 2001;121(5):1242-6.

190. Vazquez-Del Mercado M, Delgado-Rizo V, Munoz-Valle JF, Orozco-Alcala J, Volk HD, Armendariz-Borunda J. Expression of interleukin-1 beta, tumor necrosis factor alpha, interleukins-6, -10 and -4, and metalloproteases by freshly isolated mononuclear cells from early never-treated and non-acute treated rheumatoid arthritis patients. Clin Exp Rheumatol 1999;17(5):575-83.

191. Perlman H, Pagliari LJ, Nguyen N, Bradley K, Liu H, Pope RM. The Fas-FasL death receptor and PI3K pathways independently regulate monocyte homeostasis. Eur J Immunol 2001;31(8):2421-30.

192. den Broeder AA, Joosten LA, Saxne T, Heinegard D, Fenner H, Miltenburg AM, et al. Long term anti-tumour necrosis factor alpha monotherapy in rheumatoid arthritis: effect on radiological course and prognostic value of markers of cartilage turnover and endothelial activation. Ann Rheum Dis 2002;61(4):311-8.

193. Brennan FM, Browne KA, Green PA, Jaspar JM, Maini RN, Feldmann M. Reduction of serum matrix metalloproteinase 1 and matrix metalloproteinase 3 in rheumatoid arthritis patients following anti- tumour necrosis factor-alpha (cA2) therapy. Br J Rheumatol 1997;36(6):643-50.

194. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 2000;343(22):1594-602.

195. Crnkic M, Mansson B, Larsson L, Geborek P, Heinegard D, Saxne T. Serum cartilage oligomeric matrix protein (COMP) decreases in rheumatoid arthritis patients treated with infliximab or etanercept. Arthritis Res Ther 2003;5(4):R181-5.
196. Larsson E, Erlandsson Harris H, Lorentzen JC, Larsson A, Mansson B, Klareskog L, et al. Serum concentrations of cartilage oligomeric matrix protein, fibrinogen and hyaluronan distinguish inflammation and cartilage destruction in experimental arthritis in rats. Rheumatology (Oxford) 2002;41(9):996-1000.
197. Yeung RS. The osteoprotegerin/osteoprotegerin ligand family: role in inflammation and bone loss. J Rheumatol 2004;31(5):844-6.