D O C T O R A A T S P R O E F S C H R I F T

Faculteit Geneeskunde

Study of T-Cell Parameters in Rheumatoid Arthritis

Studie van T-cel parameters in reumatoïde artritis

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Medische Wetenschappen aan het
Limburgs Universitair Centrum te verdedigen door

ANN VANDERBORGHT

Promotoren : Prof. dr. J. Raus
Prof. dr. P. Stinissen

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Abbreviations

AA	Adjuvant Arthritis
AA	Amino acids
ACR	American College of Rheumatology
Ag	Antigen
APC	Antigen presenting cells
ARA	American Rheumatism Association
AV/BV	variable region of the alpha/beta chain
BC region	Constant region of the beta chain
CD	Cluster of differentiation
CDR3	complementarity determining region
CIA	Collagen Induced Arthritis
CNS	Central nervous system
CSA	cyclosporin A
DC	Dendritic cells
DIG	Digoxigenin
DMARD	Disease modifying antirheumatic drugs
dNTP	deoxynucleotide triphosphate
EAE	Experimental Allergic Encephalomyelitis
EBV	Ebstein-Barr virus
ELISA	Enzyme Linked ImmunoSorbent Assay
FACS	Fluorescence activated cell sorter
FITC	Fluorescein Isothiocyanate
gp	glycoprotein
HLA	Human leucocyte antigen
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
mAb	Monoclonal antibodies
MBP	Myelin Basic Protein
Mϕ	Macrophages
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
MS	Multiple Sclerosis
MTX	Methotrexate
NSAID	Non-steroidal anti-inflammatory
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
RA	Rheumatoid Arthritis
RF	Rheumatoid factor
SAARD	Slow-acting antirheumatic drugs
SF	Synovial fluid
SFL/R	Synovial fluid of the left/right knee
SM	Synovial membrane-tissue
SML/R	Synovial membrane of the left/right knee
TCR	T-cell receptor
TNF	Tumor necrosis factor
TSST-1	Toxic shock syndrome toxin-1

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OVERVIEW OF THE THESIS

Overview of the thesis

In a *general introduction (Chapter 1)*, the pathogenic events in rheumatoid arthritis are chronologically described. The relevance of T-cells in the pathogenesis is discussed and most cell types encountered in the synovium are briefly introduced. An overview is given of new potential therapeutic strategies for rheumatoid arthritis (RA) that are currently tested in clinical studies. The most important results of earlier studies regarding T-cell receptor (TCR) V gene expression in RA are reviewed and the technologies that are used to study TCR repertoires are briefly discussed.

The goals of this thesis are presented at the end of this chapter.

In *chapter 2*, all methods applied in this thesis are described.

In *chapter 3*, the applicability of the semi-quantitative PCR-ELISA to analyze TCR repertoires is evaluated in two test systems: T-cells stimulated with toxic shock syndrome toxin (TSST-1), which selectively express the TCR BV2 gene family, and Jurkat T-cells, an immortalized T-cell line which expresses the TCR BV8 gene family. More evidence is given that the PCR-ELISA technique is a fast, simple and sensitive method to identify overrepresented TCR V genes. Next, the technique was used on peripheral blood, synovial fluid and synovial tissue samples of an RA patient. Overrepresentation of TCR V genes was observed in synovial fluid and tissue samples but not in paired blood samples. The results obtained indicate that PCR-ELISA can be combined efficiently with CDR3 region spectratyping to study the clonal composition of the overrepresented TCR V genes.

In *chapter 4*, TCR V gene expression profiles were compared between blood and synovial lymphocytes from 7 early and 31 chronic RA patients. The TCR V gene profiles were compared to obtain information on the T-cell composition at the diseased site. CDR3 region sequence analysis was performed for some TCR V gene families. In addition, TCR V gene families from left and right knee of an early RA patient with bilateral synovitis and synovial fluid and tissue samples of a chronic RA patient were compared to study whether identical T-cells are present at both sites. To obtain information about the changes in the TCR repertoire at the diseased site over time, peripheral blood and synovial tissue of a chronic RA patient was studied again two years later.

In *chapter 5*, TCR V gene profiles from peripheral blood and synovial tissue biopsies were studied from 12 patients with early RA. Seven patients were treated with Cyclosporin A

(microemulsion) (CSA) (3mg/kg/day), whereas 5 patients received control medication administered. To evaluate the effect of a CSA treatment, blood and tissue biopsies were sampled after 16 weeks of treatment. The clonal characteristics of overrepresented TCR V gene families were determined using CDR3 region spectratyping. The CDR3 region spectratypes provide information about the structural diversity of relevant antigens at different stages in the pathogenesis of the disease. In addition, this study provides information on the effects of CSA on the TCR V gene expression and clonal composition of T-cells in blood and synovium.

In the *chapter 6*, cytokine profiles in blood and synovium of the control subjects and CSA treated patients and controls were evaluated. Quantification of cytokine transcripts was performed using a real-time PCR based system. The data provide information on the cytokine production profile in synovium of early patients, and on the effects of CSA treatment on these cytokine profiles.

Finally, in a *general discussion* all experimental results, discussed in previous chapters are briefly reviewed. The experimental data, combined with information from literature, provide additional evidence for the role of T-cells in the initiation and propagation of the inflammatory response in RA patients.

Chapter 1

GENERAL INTRODUCTION

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population in all parts of the world (1). Despite of many years of intensive investigation, the etiology of this multifactorial disease is still unknown. However, accumulating evidence indicates that RA is a T-cell mediated autoimmune disease (2) in which genetic and environmental factors play a role (1, 3). Rheumatoid arthritis is three times more prevalent in women than in men, mostly begins at an early to middle age, and affects the peripheral joints of hands, feet, knees and ankles, most often in a symmetrical pattern (4). The disease can be quite variable with respect to disease duration and the number, size and severity of the joints affected (5). In some patients the disease lasts for a few months only, and disappears without any noticeable damage. Others have mild or moderate disease with flares and remissions and some patients have severe disease that is active most of the time, lasts for years and causes irreversible joint damage and disability or even premature death if not effectively treated. RA starts as a mono-or oligo-articular arthritis slowly progressing into a poly-articular disease (6).

Rheumatoid arthritis is classified according to criteria published by the American Rheumatism Association (ARA) in 1958 and revised in 1988 (7). These criteria were designed principally for the classification of the disease in the context of epidemiological parameters but can also be used as guidelines for the diagnosis in daily practice (6). The following symptoms are frequently observed in RA patients: morning stiffness, swelling of soft tissues, swelling of the wrist joints, symmetrical arthritis, appearance of rheumatoid nodules, serum factor positiveness and radiographic changes in hands and wrists (8).

2. The role of T-cells in RA

In RA patients, the affected synovium is predominantly infiltrated by mononuclear cells. About 30-50% of the synovial cells are T-cells (9), and the majority of these synovial T-cells are CD4+ (10). Several lines of evidence indicate that T-cells play an important role in the pathogenesis of RA. Synovial T-cells exhibit several features of activation (11,12,13). For instance, they are predominantly CD4 CD45RO positive, express high levels of IL-2 receptors, MHC class II antigens, and increased levels of adhesion molecules (14-16). Some recently developed T-cell directed immunotherapies showed beneficial effects in RA (17-19). In addition, active RA is less severe in AIDS patients who have CD4 cytopenia (20). Partial elimination or inhibition of activation of T-cells using techniques such as total lymphoid irradiation (21) and lymphapheresis (22) lead to an amelioration of the disease. Furthermore, in several experimental

animal models of RA including collagen induced arthritis and adjuvant arthritis the disease can be transferred by autoreactive T-cells. Taken together these findings demonstrate a role for T-cells in RA. However, it is not clear whether synovial T-lymphocytes are responding to an antigen within the joint thus giving rise to the autoimmune response, or whether they infiltrate the synovium as a consequence of an inflammatory process already active within the joint.

Experimental evidence supports the hypothesis that primed memory T-cells migrate from the peripheral circulation into the synovium. The transmembrane trafficking is facilitated by an increased expression of appropriate adhesion molecules induced by cytokines. Subsequently, the T-cells are reactivated in the synovial cavity. This reactivation could be due to cross-reactivity of peripheral antigen-specific T-cells with (auto)antigens expressed in the diseased joint. Synovial reactivation might cause the selective expansion and accumulation of a particular subset of T-cells in the synovial cavity. That is why studying T-cell receptor V gene expression profiles in peripheral blood and synovial samples of RA patients and healthy controls, may be helpful to characterize T-cells that play a pathogenic role in the joints.

3. The tri-molecular complex in RA

Synovial T-cells are most likely reactivated in the joints of RA patients (2). T-cells are activated after recognition of a peptide which is presented in the context of a major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC) (Figure 1.2). In RA, synovial autoantigens can be processed and presented by macrophages, dendritic cells and synoviocytes (23). The characteristics of the MHC (or HLA) complex (genetically determined), the properties of the antigens presented (potential environmental influences) and the composition of the TCR expressed on the T-cell surface (somatic recombination) all determine the final outcome of an antigen driven T-cell response. Here we will discuss the relevance of these elements in RA.

3.1 Synovial T-cells

Synovial T-cells are predominantly CD4 CD45RO positive, express high levels of the IL-2 receptor and MHC class II antigens, and express increased levels of several adhesion molecules (14-16). Naive T-cells leave the thymus, are activated in the peripheral circulation (secondary lymph nodes), express surface activation markers and acquire an increased ability to bind to endothelial cells. Subsequently, the activated or primed T-cells cross the endothelial cell walls, leave the blood vessels, and migrate into the diseased joints (24,25). Without a continued antigenic stimulation, the surface expression of IL-2 receptors decreases and a fraction of the

primed T-cells become memory T-cells (25,26,27). A minority of the memory T-cells will be reactivated in the synovial cavity after recognition of an autoantigen and will secrete IFN-γ (10). Some will become helper cells and stimulate B-cell proliferation. Other memory T-cells in the cavity are innocent bystanders that support the ongoing immune response but they will not be reactivated (25). The release of IFN-γ by reactivated T-cells in the synovial cavity can be a trigger that induces a variety of cytokine-dependent events that recruit non-T-cells (macrophages, dendritic cells) into the diseased joint (26). Moreover, secreted IFN-γ induces the activation of macrophages. Direct contact between reactivated T-cells and synovial macrophages seems to play an important role in rheumatoid synovitis (26,28). A proper and efficient recognition and reactivation of a limited number of antigen-specific T-cells in the synovial cavity is considered to be sufficient to initiate the chronic progressive immune response (25).

3.2 RA: genes and HLA expression

RA is a multifactorial polygenic disease (6,24). Several genetic polymorphisms have been found to be linked to the susceptibility to and/or the severity of the disease (29). Genetic modeling studies suggest that between 55-88% of the RA cases can be accounted for by genetic susceptibility factors (30). The occurrence of familial clustering of rheumatoid arthritis (31,32), the significant association of some T-cell receptor AV and BV genes with the disease (33,34), the association of specific cytokine gene polymorphisms with RA (35-37), the increased concordance rate in monozygotic twins (38-39) and last but not least, the association of RA with an HLA gene cluster support the contribution of genetic factors to the disease. RA has been found to be associated with the human leucocyte antigen (HLA) class II molecules. HLA class II molecules are encoded by genes clustered in three subregions: the HLA-DR, DP and DQ alleles (Figure 1.1). The encoded protein is mainly expressed on the surface of B lymphocytes, activated T-cells, monocytes, macrophages, dendritic cells, epithelial and endothelial cells (40). HLA class II molecules present peptides of about 13-25 AA in length and are dimeric peptides, constituted of a monomorphic α chain and a polymorphic β chain (32).

In a normal population the HLA allele frequency is very diverse, RA patients on the other hand preferentially express some regions of the HLA DRB1 gene cluster (29,41,42). The HLA DR1, different subtypes of the HLA DR4 region and the HLA DR6 group, all part of the HLA DRB1 gene cluster are preferentially expressed in RA patients (12).

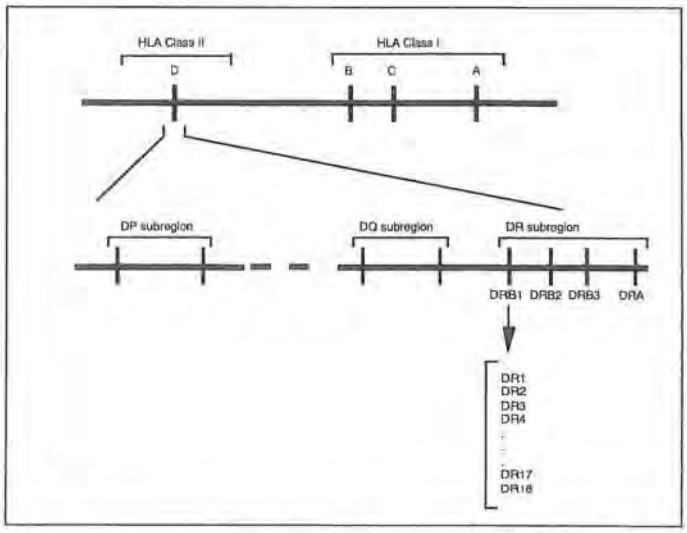

Figure 1.1: Partial schematic representation of the HLA gene cluster on chromosome 6

Sequence comparison between the disease associated HLA DR1/4/6 region genes revealed a conserved region of 5 amino acids: the shared epitope. The amino acid sequence motifs in this conserved region of the HLA DRB1 gene are QKRAA or QRRAA or RRRAA. Moreover, this sequence is the main structural part of the peptide binding-pocket in the HLA-DR heterodimer since it is the contact point between the antigen presenting cell and the antigenic peptide presented to the TCR (Figure 1.2) (43,44).

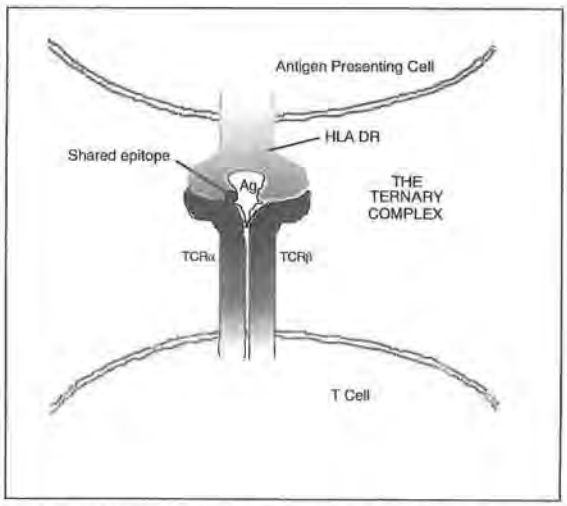

Figure 1.2: The shared epitope and antigen recognition

About 75% of all RA patients in North America and Europe express the QKRRA motif in the antigen binding region (45). On the other hand, some individuals carrying the conserved sequence never develop the disease (10). This illustrates that this 'shared epitope' is not sufficient to develop RA and that other factors and/or genes, alone or in association with the HLA DR alleles, are important for disease development (46).

Some years ago a significantly increased frequency of the HLA DR4 genetic marker in a population with chronic progressive RA was observed (47). This suggests that the HLA DR status probably is better related to RA severity or progression than with disease susceptibility. Especially the amino acid Lysine in position 71 of the conserved sequence motif (70-75) is strongly associated with a chronic progressive disease status (48). However, this association (42) is probably influenced by additional yet undescribed genetic and environmental factors (24). Furthermore, patients inheriting disease associated genes (HLA DR1/4/6) on both haplotypes tend to develop more aggressive disease than patients with a single copy (gene dosage effect) (48). A more aggressive manifestation of the disease is reflected in features such as rheumatoid nodules, rheumatoid factor (RF) positivity and radiological erosions (42).

In summary, HLA genotypes can be used as prognostic markers to predict chronic progressive disease in a subset of patients with an initial diagnosis of RA, rather than as a single susceptibility marker in a population (43). Furthermore, the use of HLA DR susceptibility genes as prognostic markers may justify an early aggressive intervention arresting the disease at an early stage, before irreversible joint damage has occurred (44).

How is RA linked to HLA?

The exact mechanism of the HLA-associated disease induction and/or progression is not fully understood.

There is some evidence that:

- In RA patients HLA DRB1 molecules present specific 'self-antigens' to 'autoreactive T cells' initiating the pathogenic events (29).
  But: Where do these autoreactive T-cells come from ? Which peptides are autoantigens?

- In RA patients the 'shared epitope' HLA DRB1 molecules prevent the activation of protective T-cells, thus, non-RA associated HLA alleles are protective whereas RA associate HLA DRB1 alleles are harmful (49)
  But: What about HLA DRB1 negative RA patients with chronic progressive disease?

However, since the function of HLA molecules is to present antigens to T-cells (49,24), the association of RA with certain HLA subtypes strongly suggests a role for T-cells in the pathogenesis.

3.3 Target antigens in RA

A. Endogenous antigens

Pathogenic T-cells are thought to be autoreactive and recognize self-peptides presented by MHC molecules in the microenvironment of the joint. Connective tissue matrix proteins such as collagen type II (50) and proteoglycans (51,52), and cartilage glycoproteins such as human cartilage glycoprotein-39 (HC gp-39) (53) are potential endogenous arthritogenic stimuli. The isolation of collagen type II and cartilage proteoglycan specific T-cells in the SF of RA patients (51), the differential expression of certain cartilage mRNA's restricted to the joints of RA patients (53) and the results of experimental animal models where disease is induced when genetically susceptible mice strains are injected with specific cartilage constituents (54,52), suggest a potential contribution of these antigens in the pathogenesis.

B. Exogenous antigens

RA is not an infectious disease. However, *Mycobacterial* heat shock proteins (HSP), (HSP-65 (55,52,56) and *E Coli* components such as dnaJ, the binding domain of the *E Coli* dnaK or HSP-70 (57) are believed to play a role in the pathogenesis of the disease. Heat shock proteins are highly conserved during evolution, and have an high degree of sequence homology between eukaryotes and prokaryotes (58). These conserved proteins could play a role in a cross-reactive activation of autoreactive T-cells inducing an autoimmune response in an immunogenetic susceptible host (59). Clear relevance of mycobacterial heat shock proteins in the pathogenesis has been illustrated in Adjuvant Arthritis (AA), an experimental animal model of RA, where T-cells directed against mycobacterial heat shock protein epitopes can induce or suppress the disease (60). In addition viruses such as Ebstein-Barr virus, and more specifically the EBV gp-110 that contains the QKRAA sequence motif (61,62), and retroviruses (63,64) have been suggested to be potential antigens.

How heat shock proteins and viruses may initiate an autoimmune response

The sequence homology between the bacterial or viral antigens and the HLA DRB1 sequence motif QKRAA suggests a molecular mimicry based recognition and subsequent activation.

Albani and Carson described a multistep molecular mimicry hypothesis as a possible link between candidate antigens and the pathogenesis of rheumatoid arthritis (65). It is known that during thymic selection, self-peptides and HLA derived peptides are involved in 'shaping' the maturing T-cell receptor repertoire. In the thymus, immature T-cells that strongly bind to the MHC-peptide complex are negatively selected, whereas a weak T-cell MHC-antigen binding is positively selected and becomes part of the mature T-cell population that can be recruited in an immune response. Autoreactive T-cells are selected for maturation due to the weak interaction between HLA-derived peptides encompassing the 'shared epitope' and the 'self-derived-QKRAA-specific' T-cells. After birth, exposure of the quiescent QKRAA-specific T-cells to bacterial antigens with a high degree of sequence homology to the shared epitope reactivates the QKRAA specific T-cells and causes an increased trafficking of activated T-cells into the joint space.

3.4 Antigen presenting cells

A. Synovial macrophages

Rheumatoid synovial membranes are characterized by an increased infiltration of monocytes. Monocytes leave the bone marrow and end up in the synovial tissues were they are activated and become synovial macrophages (66,24). Cytokine profiles and immunohistochemical studies using monoclonal antibodies against specific surface antigens provide evidence for the presence of monocytes/macrophages in the diseased joint (66,67). On their way to the synovium, monocytes may ingest foreign antigens and transport them into the joint cavity. Homing receptors and adhesion molecules expressed on the surface of the monocytes as well as the endothelial cells guide this transendothelial transport (66,9). In the knee joint, the ingested antigens can be presented to memory T-cells and induce T-cell activation. Next, the activated T-cells produce IFN-γ which subsequently activates monocytes (68,69). These activated monocytes or macrophages secrete pro-inflammatory cytokines such as IL-1 and TNF-α that stimulate fibroblast-like synoviocytes and chondrocytes in the articular cartilage. The latter secrete proteolytic enzymes that degrade proteoglycans and collagen which give rise to an irreversible tissue destruction in the diseased joint (68). In general, the cytokines released in the inflamed knee joints are mainly of macrophage origin, cytokines like TNF-α, IL-1 and IL-6 are found in large amounts whereas T-cell derived cytokines such as IFN-γ and IL-2 are only found in smaller quantities (27). The increased infiltration of macrophages into the cavity sustains the chronic synovitis and causes pain and swelling (67,68).

B. Synoviocytes

The synovial lining is the interface between the intra-articular space and the synovium and consists of a loose association of synoviocytes (69). In normal conditions the synovial lining is 1-3 cells thick but in RA patients the synoviocytes proliferate and irreversibly invade the underlying cartilage and bone. These cells are considered to be most important in pannus formation (70). In general, two different types of synoviocytes are defined: type A like synoviocytes with a macrophage-like appearance and type B like synoviocytes with a fibroblast-like appearance. The macrophage-like type A synoviocytes remove cellular debris in the joint cavity, whereas the fibroblast-like type B synoviocytes synthesize and secrete proteoglycans, hyaluronic acid, cytokines and metalloproteinases in the synovial fluid (71). Immunohistochemical analysis and in situ hybridization demonstrate that the destructive pannus is predominantly composed of fibroblast-like synoviocytes. These type B-like synoviocytes are found at the cartilage pannus junction. They express metalloproteinases and other destructive enzymes. After activation, fibroblast-like synoviocytes proliferate and alter the expression of surface adhesion molecules thus facilitating the migration into the solid tissues. Once activated, RA synovial lining cells express increased amounts of class II histocompatibility antigens and influence the ongoing immune response by presenting newly released antigens to lymphocytes (71).

C. Dendritic cells (DC)

The rheumatoid synovium is enriched with dendritic cells that function as potent antigen presenting cells (APC) (10,72). Dendritic cells differentiate and mature in the synovial tissue under the influence of cytokines (GM-CSF, TNF-α) produced by synovial macrophages (72). During maturation, DC express class II MHC molecules and co-receptors for efficient T-cell stimulation. (73). Since DC are potent antigen presenting cells, even low-affinity self-reactive T-cells, migrated into the knee joint, can effectively be activated (73). It has been suggested that DC present autologous peptides or degraded self-MHC molecules at early stages of the disease. Later on, altered tissue components resulting from the cartilage break-down can be presented to low affinity self reactive T-cells and give rise to the chronic progressive inflammation (69,73).

4. Characterization of disease relevant T-cells through TCR expression studies.

It is thought that autoreactive T-cells are stimulated by antigens, clonally expand and selectively accumulate at the diseased site. Studying TCR V gene expression profiles in peripheral blood and synovial fluid of RA patients may therefore be helpful to characterize the pathogenic T-cells

in RA. Furthermore, CDR3 region clonal composition of overrepresented TCR V gene families may reveal some structural characteristics of initiating autoantigen(s). The characterization of disease related autoreactive T-cells is needed for the development of selective T-cell directed therapies.

4.1 The T-cell receptor (TCR)

T-cells are mononuclear cells that originate in the bone marrow and migrate into the thymus where they proliferate and differentiate into mature functional lymphocytes (74). T-cells recognize antigenic peptides using the heterodimeric transmembrane T-cell receptor (TCR) consisting of two disulphide linked polymorphic chains: the alpha chain and the beta chain (for most mature lymphocytes) or the gamma chain and the delta chain (75). The TCR is non-covalently associated with the CD3-complex, a co-receptor necessary for an efficient signal transduction leading to T-cell activation (76) (Figure 1.3).

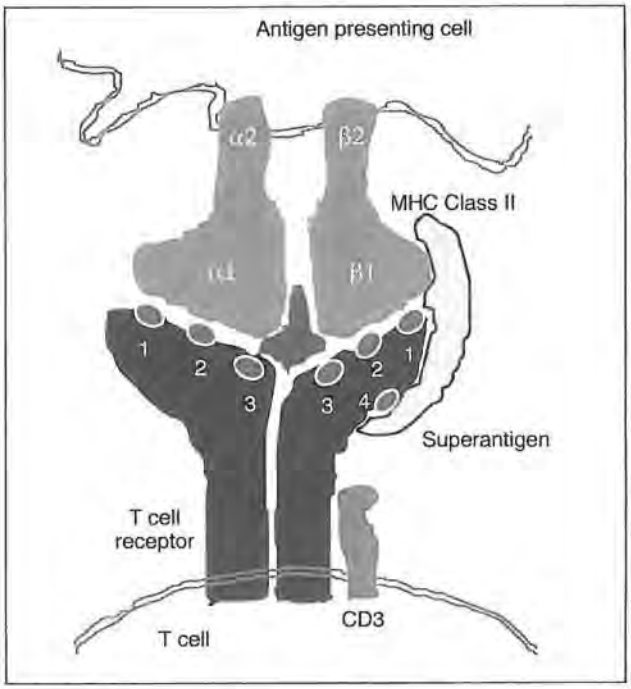

Figure 1.3: Schematic representation of the presentation of an antigen and a superantigen using MHC class II molecules on the surface of an antigen presenting cell (APC) to the T-cell receptor (TCR).

The genes encoding for the TCR protein consist of four gene segments encoding for the variable (V) region, the diversity (D) region (except in the case of the α and γ chain), the joining (J) region segment and the constant (C) region of the TCR protein (76,2). (Figure 1.4).

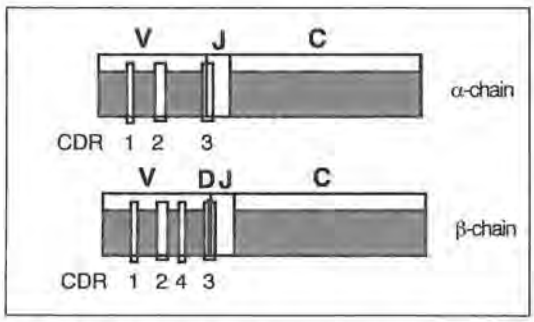

Figure 1.4: Composition of the rearranged variable (V), diversity (D), joining (J) and constant (C) gene regions within the α-chain and the β-chain of the TCR. The complementarity determining regions CDR 1,2,3 and 4 are represented (According to Struyk et al, 1995 (2).

During αβ T-cell development, germline encoded V, (D) and J region gene segments are rearranged, and the rearranged complexes are juxtaposed to one of the C region genes using RNA splicing. TCR α/β diversity is achieved during TCRAV-AJ and BV-BD-BJ recombination by addition of non-germline encoded nucleotides to the V, D and J gene segments and removal of nucleotides on the 3'V, 5' and 3'D and 5'J region gene segments (77). As a result, T-cells with unique receptors for a large number of antigens are generated.

Within the TCR V region gene, three complementarity determining regions can be recognized. The complementarity determining region-1 (CDR1), CDR2 and CDR3 (78). (Figure 1.4). The CDR1 and 2 region of the TCR interact with the α-helices of the MHC molecule on the APC, and do not bind to the peptide presented. The CDR3 region on the other hand, makes direct contact with the peptide bound to the MHC molecule. It is encoded by the V-(D)-J gene junction region and is the most variable region of the TCR. (Figure 1.4). The fourth hypervariable region (CDR4),which is located in between the CDR2 and CDR3 region is highly conserved between particular TCR BV gene families and is supposed to be the target region of superantigen recognition (Figure 1.4).

TCR/peptide/MHC interactions (trimolecular model) are very strict. Only those T-cells that fit into the structure of the MHC/peptide complex are selected for binding. This was illustrated by experiments using single aminoacid (AA) substitutions in the peptides presented to the T-cells. These peptides will bind to a TCR with a different CDR3 region. During T-cell development a central regulatory system determines the fate of maturing T-lymphocytes and thus the peripheral

repertoire. Specific MHC-peptide complexes are able to select the TCR V regions that optimally fit in their structure and thus determine the TCR V gene repertoire.

4.2 Technologies to study TCR V gene expression

Various technologies have been used to study TCR V gene analysis in RA. The technological procedure used will be determined mainly by the aims of the study. TCR V gene analysis can be performed at the genetic level, starting from genomic DNA (79) or RNA (80,81), or at the protein level, by evaluation of the membrane expression of TCR genes (82,83). Peripheral blood, synovial fluid and synovial tissue samples from genetical and clinical heterogeneous study groups can be studied. T-cells can be depleted in specific subpopulations or *in vitro* stimulated with specific antigens prior to analysis.

A. PCR based technologies on RNA/cDNA

TCR repertoire analysis is frequently performed on the transcriptional level using PCR based technologies. TCR AV and BV gene specific primers were designed (47,84) and TCR specific material is amplified with those primers. Next, PCR amplicons are quantified to determine the TCR V gene expression repertoire. The relative expression level of a single TCR V gene against the total TCR V gene repertoire is determined.

Quantification of TCR transcripts can be performed by Southern blot using ^{32}P-labeled TCR $\alpha\beta$ gene family specific or other probes (85). Alternative methods have been described using fluorescently labeled primers (86-88). However, these methods require the use of an automated DNA sequencer or TaqMan equipment (Perkin Elmer). The TCR V gene repertoire can more easily be determined using a semiquantitative PCR-ELISA using a double stranded DNA specific antibody (89). Alternatively, as demonstrated in the present thesis, hybridization of DIG labeled PCR products with an FITC labeled TCR C region specific probe, and binding the PCR products to an anti-FITC coated ELISA plate can be performed. A subsequent quantification is then performed using an anti-DIG conjugated peroxidase (90).

For all PCR based protocols a careful control of PCR cycle numbers is necessary, since quantification should be performed in the linear phase of the amplification reaction. Indeed, at the plateau phase, the amount of PCR amplicons measured is no longer proportional to the amount of starting material, making quantification impossible. Moreover, a comparable amount of T-cell specific messengers can not be guaranteed, even when starting from the same number of cells. This is not only due to differences of the *in vivo* transcription or translation activity, but

also because of possible differences in the transcription efficiency performed *in vitro* in the RT-PCR protocol.

The most important advantage of a PCR based technique is its high sensitivity. Even with a very limited number of starting cells (± 50.000 lymphocytes) TCR V gene analysis can be performed (91,92). Recently, a new PCR based real-time quantification technique, the PCR LightCycler was developed (93). This technique is an alternative for the classical RT-PCR using external or internal mRNA quantification standards. In this technique, real-time instead of end-point detections are performed and quantification is based on the incorporation of the double stranded DNA binding dye SYBR Green. In contrast to previously reported end-point detection systems using TBR and biotin labeled primers (94), the PCR LightCycler system enables simultaneous PCR amplification and quantification. PCR amplification and analysis is performed in the same reaction vessel reducing the risk of PCR product carry-over contamination. A software linked calculation of the measured signals allows quantification in the exponential phase of the PCR reaction, and an on-line quality control of the PCR products allows the discrimination between specific and non-specific PCR amplicons (primer-dimers).

B. Flow-cytometric quantification of TCR surface expression

Quantification of T-cell receptor specific messengers does not always precisely reflect the amount of TCRs expressed on the T-cell surface. Using monoclonal antibodies, the TCR surface expression can be measured with a flow-cytometer (83,95,96). Thus, non-functional messengers that are indistinguishable in PCR based technologies are excluded. However, a limited panel only of monoclonal antibodies is available and more starting material is necessary. Furthermore, only freshly isolated T-cells can be evaluated.

C. Clonal analysis of overrepresented TCR V gene families

Skewed TCR V gene expression may suggest clonal expansion of *in vivo* antigen stimulated T-cells. However, clonal expansion needs to be confirmed by CDR3 region clonal analysis. Some years ago, TCR clonal characteristics were determined on digested genomic DNA using Southern blot analysis. TCR V gene family specific ^{32}P-labeled DNA probes were hybridized on the digested fragments separated on an agarose gel. However, the technique is labor-intensive, uses radioactive labeled probes and the detection of dominant TCR rearrangements is limited to 10% of the rearranged clone against a polyclonal background (97). Furthermore, restriction fragments of identical length visualized after hybridization with TCR specific probes are not necessarily identical and can not be discriminated (2). A more detailed analysis revealing the fine specificity of the T-cells is needed to elucidate structural properties of the initiating autoantigens.

Recently the clonal origin of expanded T-cells has been compared at the CDR3 region sequence level. Only those T-cells with identical CDR3 region sequences represent clonally expanded T-cells. CDR3 region sequence analysis can be performed on T-cell lines and clones generated after *in vitro* selection with a specific antigen (55). However, since the initiating antigen in RA is not known, several groups studied clonal analysis of unstimulated T-cells from peripheral blood and synovial fluid or tissue. Bacterial cloning can be used for this purpose. Overrepresented TCR V gene families are PCR amplified with the appropriate TCR V gene primer sets. PCR amplicons are ligated in specific cloning vectors and recombinant vectors are transformed into bacteria. A representative number of individual colonies are selected for CDR3 region sequence analysis. Based on the number of identical CDR3 region sequences, mono-, oligo-or poly-clonal T-cell populations can be distinguished.
Clonal analysis can also be performed using CDR3 region spectratyping (98). With this technology, fluorescently labeled PCR amplicons of specific TCR BV gene families are separated on a 6% polyacrylamide gel. Fragment length distribution patterns are interpreted and allow recognition of mono, oligo-and poly-clonal T-cell mixtures. CDR3 region spectratyping is a fast and sensitive technique that can be used to determine T-cell clonal characteristics in synovial biopsy samples. These samples can be taken from the centre of the inflammation and may therefore represent the pathogenic T-cell populations. However, T-cells with identical CDR3 region length but different AA composition can not be distinguished using the spectratyping technology.

4.3 TCR V gene expression profiles in RA

Several TCR V gene profile studies in RA are summarized in table 1.1. Parameters such as HLA genotype, disease duration (early versus chronic), technology used and characteristics of the T-cell sub-population screened are important for the interpretation of the data.

It remains unclear whether TCR V gene profiles are restricted in the peripheral circulation of RA patients. In several studies no TCR V genes were found to be overrepresented (80,99,100,101,102), while other studies demonstrate a restricted TCR V gene profile in blood cells. Depending on the technology used, different TCR V genes were shown to be overrepresented. Using flow-cytometry an increased surface expression of TCR BV 17 was reported (82). PCR based technologies on the other hand, revealed overrepresentation of TCR BV 14 in undepleted, unstimulated peripheral blood T-cells (81,102), TCR BV3 in a CD8+ T-cell subset (95) and TCR BV 18, 2, 5.1 in the CD4+ T-cell subset (103).

However, in nearly all studies a restricted TCR V gene usage in synovial fluid was observed. TCR BV 14 (99,102,103), BV3 (102), BV 17 (82), BV6 (99,102,103) and many others (Table

1.1) were reported to be overrepresented in SF samples. Some authors found overrepresentation of particular TCR V genes, which however varied among patients (96,104,105). Other groups did not observe overrepresentation of TCR V genes in the synovial fluid (102,106,107,108).

Comparable conclusions can be drawn from the analysis of synovial tissue samples. Ramanuyan did not find any overrepresented TCR V genes in tissue samples (109). Struyk, Gudmundson and Bucht found no uniform pattern of overrepresented TCR V genes (80,85,96). Other groups showed a clear overrepresentation of TCR BV 3 (100,103), TCR BV 1 (92), BV 14 (103), and BV 2 (81,110) in restricted subsets of an RA population.

For synovial fluid as well as synovial tissue samples overrepresentation but also underrepresentation of some TCR V genes has been reported. TCR BV 1, 4, 5.1, 10 (103), BV 4 (100) were found to be significantly underrepresented in the synovial cavity samples as compared to the peripheral circulation.

Some investigators studied *in vitro* selected T-cell populations and based their selections on antigen specificity (55,48,111) or T-cell activation state (112-115). Some TCR V gene families, including TCR BV 3, 14 and 17 were found to be overrepresented in IL-2R positive synovial tissue T-cells (112). These data are interesting since these BV genes were also reported to be overrepresented in unselected T-cell populations. TCR BV 3, 14 and 17 genes reveal a high degree of sequence homology in the CDR4 region, which is the superantigen recognition site of the TCR sequence. An increased frequency of T-cells with similar CDR4 regions in several RA patients may suggest the involvement of superantigens in the pathogenesis. However, further research on larger study groups is needed to confirm these findings.

In conclusion, no unambiguous over-or under-represented TCR V gene family has been recognized as a disease marker in PB, SF or ST samples of selected or unselected RA derived T-cells. TCR V gene profiles seem to be patient restricted, very diverse and difficult to compare.

Table 1.1: TCR V gene profiles in peripheral blood, synovial fluid and synovial tissue samples of chronic and early RA patients

UNSTIMULATED T-CELLS

RA type	N° patients	DR1/4 restriction	technology used	AV/BV genes overrepresented						
				in blood		in synovial fluid		in synovial tissue		
				type	remarks	type	remarks	type	remarks	Ref.
early	2	yes	PCR	/				no uniform		(80)
chronic	49	no	FACS	BV17		BV17				(82)
chronic	24	no	PCR	/						(99)
chronic	14	no	FACS	/		AV2,BV5.3,8				(83)
chronic	17	no	PCR	/		some skewing				(104)
chronic	5	yes	PCR	BV14		BV14		BV2,6		(81)
chronic	13	no	PCR	/				BV3,17,22↑ BV4↓		(100)
early	3	yes	PCR	/		/				(106)
chronic	32	yes	PCR/FACS	BV3	CD8+					(95)
chronic	12	no	PCR	BV6,13,14		BV6,13,14				(102)
chronic	12	no	FACS	/		/				(102)
chronic	7	no	PCR	BV18,2,5.1						(103)
chronic	35	yes	FACS			no uniform	CD4/CD8	no uniform	CD4/CD8	(96)
Mixed	8	no	PCR			AV10,15,18 BV4,5,19				(116)
early	8	yes	PCR			AV17				(117)
chronic	15	yes	PCR			no uniform CD4+				(92)

Table 1.1 continued

RA type	N° patients	DR1/4 restriction	technology used	AV/BV genes overrepresented					
				in blood		in synovial fluid		in synovial tissue	
				blood	remarks	SF	remarks	ST	remarks	Ref.
chronic	7	no	PCR			BV2,6	CD4+			(79)
chronic	1	no	PCR			/				(107)
chronic	4	yes				AV14,15 BV2				(118)
chronic	1	yes	PCR			BV2.1,3.1				(119)
chronic	3	no	PCR			/	CD4+			(108)
chronic	5	no	PCR			/				(108)
chronic	8	no	PCR					BV1		(92)
Mixed	9	no	PCR					no uniform		(85)
early	3	yes	PCR					/		(109)
chronic	3	yes	PCR					BV2		(110)

STIMULATED T-CELLS

Ag	RA type	N° patients	DR1/4 restriction	technology used	AV/BV genes overrepresented						
					in blood		in synovial fluid		in synovial tissue		
					blood	remarks	SF	remarks	ST	remark	Ref.
HSP-65	chronic	9	no	PCR	restriction	depend	restriction	depend			(55)
Mycobacterium Ag	chronic	1	no	PCR	/		BV6,8,14,18,19				(120)
HLA DR det	chronic		yes	PCR	BV6						(121)
BCG	early	7	no	FACS	BV8						(122)
IL-2	chronic	7	yes	PCR	BV14						(123)
IL-2	chronic	5	no	PCR					BV3,14,17		(112)
IL-2	chronic	10	yes	PCR					BV12,14,17		(113)
IL-2	chronic	11	no	PCR					no pattern		(114)

HSP: Heat Shock protein, HLA: Human Leucocyte antigen, AV/BV: TCR AV /BV genes, FACS: flow-cytometry, PCR: Polymerase chain reaction, IL: Interleukin.
HLA DR1/4 restriction : yes, means that all members of the study group are HLA DR1/4 positive, depletion: CD4/CD8+ means that respectively CD4 or CD8+ T-cells are included, depend: overrepresented TCR V genes are patient dependent, no pattern: no uniform AV/BV gene is represented, no uniform: no uniform pattern can be found.

5. Role of B-cells and autoantibodies

Although T-cells are probably playing a central role in the pathogenesis of RA, autoantibodies may also contribute to the pathogenic cascade. Indeed, B-cells were found to be clonally expanded (124). Most often, B-cells are stimulated to produce antibodies after the presentation of specific antigens in a T-cell dependent manner. In RA, some B-cells produce rheumatoid factor (RF), which is composed of natural autoantibodies against the constant region of immunoglobulin G (IgG) (125). RF can be IgG, IgM and IgA type antibodies. Most often they are associated with a bad prognosis of the disease. It is not known how RF contributes to the diseases progression (54). However, the selective production of RF at the site of tissue inflammation indicates a contribution of RF in synovial tissue injuries (71). Furthermore, there is evidence that the local environment in the rheumatoid joint favors RF production. Recent studies have shown that the presence of type B RA synoviocytes together with IL-10 increases the RF production of SF B-cells (126). Furthermore, the finding of dominant and unique B-cell clones in the RA synovia and not in the peripheral blood, suggests that some synovial B-cells selectively expand in the synovial cavity (127). However, the presence of RF is not specific for RA, and can also be detected in a relative high percentage in other autoimmune and infectious diseases, and even in healthy individuals (128).

Anti-keratin autoantibodies (AKA) and anti-perinuclear factor (APF) autoantibodies have been found to be more specific serological markers for RA (95-100% diagnosis specificity). These autoantibodies appear very early in the disease, even before clinical symptoms arise. They seem to be associated with more active and severe forms of the disease; AKA and APF antibodies, previously considered as two different RA associated autoantibodies, both recognize fillagrin-like structures and are grouped as antifillagrin autoantibodies (AFA) (129). Fillagrin is a protein mainly expressed in epidermal cells. However, a posttranslational modification of fillagrin-like peptides changing arginine residues into citrulline residues generates specific antigenic epitopes recognized by AFA (130). But, how can fillagrin specific autoantibodies be related to RA ? High amounts of AFA have been found in extracts of synovial membranes as compared to synovial fluids or serum samples. Since fillagrin expression has not been observed in articular cartilage, the AFA production in synovial joints of RA patients may result from the cross-reactivity with joint antigens.

Interestingly, citrulline residues have also been found in myelin basic protein (MBP) a candidate autoantigen in MS, an autoimmune disease of the central nervous system. Furthermore, increased amounts of the posttranslationally modified proteins have been found in MS patients as compared to controls (129). Posttranslational modification of MBP therefore may play a role in the pathogenesis of the disease. In conclusion, these observations indicate a possible role of citrulline-containing epitopes in the autoimmune pathogenesis of RA and MS.

6. RA pathogenesis: An hypothetical pathway

In rheumatoid arthritis, some well structured and organized synovial joints are irreversibly damaged by inflammation. The major destructive element of the inflammation is a pannus, that invades joints of wrists, shoulders, ankles, feet and knees (1). A healthy joint consists of a joint space, filled with synovial fluid and surrounded by the synovium or synovial membrane. The synovial membrane is a 1-3 cells thick mono-layer that is located between the joint cavity and the subsynovial layer which is consisting of fibroblasts, collagen fibers and proteoglycans. The synovial cells synthesize and secrete hyalonurate, which is together with other proteins, one of the main components of the synovial fluid in normal subjects. In response to any trauma or inflammation, the amounts of intra-articular fluid increases. Subsequently, Ig secreted by B lymphocytes and plasma cells in the subsynovial lining areas increases the protein concentration in the accumulated fluid. The synovial fluid functions as a lubricant and protects the joint surfaces covered with articular cartilage. After the disease has been initiated, a progressive inflammation gradually destroys the cartilage and underlying bone, activated synovial cells, lymphocytes and neutrophils infiltrate into the joint space (10).

The pathogenic pathways of rheumatoid arthritis can be subdivided in different phases: An initiation phase, with no clinical evidence of disease, followed by an early inflammatory phase which leads to clinical manifestation but is not necessarily accompanied by fully blown RA, a destructive phase, accompanied by erosion and progression of the disease, and a perpetuating phase characterized by irreversible joint destruction (11).

Initiation phase

In RA the self-sustaining synovitis is probably triggered by the presentation of a relevant antigen to a susceptible host. So far, the causative agent remains unknown. Retroviruses (63), superantigens or antigens such as mycobacterial heat shock proteins mimicking host specific proteins (61,50,55) and mycoplasma (29,131,132) are candidate exogenous agents. Connective tissue matrix proteins such as collagen type II (50), and cartilage glycoproteins such as human cartilage glycoprotein-39 (HC gp-39) (53), are potential endogenous arthritogenic stimuli.

Foreign compounds are ingested and processed by macrophages or dendritic cells and finally presented to T-lymphocytes (6,66). However, during these early events of immune recognition and antigen presentation, patients are unlikely to have clinical manifestations (6).

Early inflammatory phase

Following an antigenic stimulation T-cells migrate through the endothelial cell wall of blood vessels and accumulate in the synovium. Activated T-cells produce cytokines such as IFN-γ that

subsequently stimulate monocytes/macrophages to release pro-inflammatory mediators in the synovium. These cytokines (IL-1 and TNF-α) stimulate the proliferation of endothelial cells and induce the expression of adhesion molecules on endothelial cells, thus enhancing the transendothelial migration of lymphocytes and macrophages (133). Moreover, new blood vessels are formed supplying nutrients to the proliferating cells in the progressive pannus (6). Finally, cytokines released by activated monocytes and macrophages, activate synoviocytes and activated synoviocytes can release collagenases and proteases, enzymes that irreversibly can damage the cartilage and bone (134,66,135). Along with this phase of the disease, the first symptoms arise. The accumulation of synovial fluid in the joint cavity causes symptoms like morning stiffness and joint swelling. However, only after a certain threshold value of accumulated macrophages in the joint has been exceeded, clinical manifestations emerge (68).

Irreversible destruction of cartilage

Synovial lining cells (synoviocytes) proliferate and organize in an invasive front of about 5-10 cell layers thick, irreversibly invading the underlying cartilage and bone (6). Cartilage destruction in arthritis is linked to aberrant cytokine and growth factor expression in the affected joints. IL-1 and TNF-α are key destructive mediators in arthritis, since the production of cartilage destructive enzymes is linked to the secretion of these cytokines (136). Furthermore, it becomes clear that the balance of protective and destructive cytokines is more important for the net destruction than the absolute level of these destructive mediators.
In this phase of the disease synoviocytes proliferate, and radiographical changes become visible.

In conclusion, synovial pannus formation and bone destruction is definitely not the result of effects of a single cell type; T-cells, macrophages, dendritic cells and synoviocytes with their respective cytokines turn out to be important in the pathogenesis of the disease. (See Table 1.2, Figure 1.5)

Besides the presence of joint inflammation, approximately 25% of the patients with RA develop extra-articular manifestations, such as the formation of rheumatoid nodules, pleuritis and vasculitic skin lesions. Especially in patients with rapidly progressive disease, subcutaneous nodules are formed at pressure points like the elbow and the back of the forearm. Histologically, rheumatoid nodules have a granuloma like structure composed of three zones: an inner zone of central necrosis, a surrounding cellular palisading zone and an outer area of lymphoid aggregates organized around small vessels. The major proportion of the palisading cells are macrophages, whereas T-cells are found in variable numbers distributed among the macrophages, and more pronounced in the lymphoid aggregates concentrated around the small vessels. The pathogenic mechanism leading to the formation of rheumatoid nodules remains unclear and the exact immunological relation between joint inflammation and subcutaneous nodules is as yet undefined (137).

Table 1.2: Overview of the pathogenic events in RA.

Phase	*Event*	*Cells engaged?*	*Symptoms*
Initiation	Ag presentation	Macrophages/dendritic cells	No symptoms
	Immune response triggering	T-cells	
Early inflammation	Accumulation in synovium	B/T-cells	Morning stiffness
	IFN-γ production	T-cells	Swelling/redness
	IL-1/TNF-α secretion	Monocytes/Macrophages	
	expression of adhesion molecules	Endothelial cells	
	transendothelial migration	T-cells	
	Cell activation	synoviocytes	
	Release of degrading enzymes	synoviocytes	
Irreversible degradation	Pannus formation	synoviocytes	Bone and cartilage
	Destruction	synoviocytes	destruction

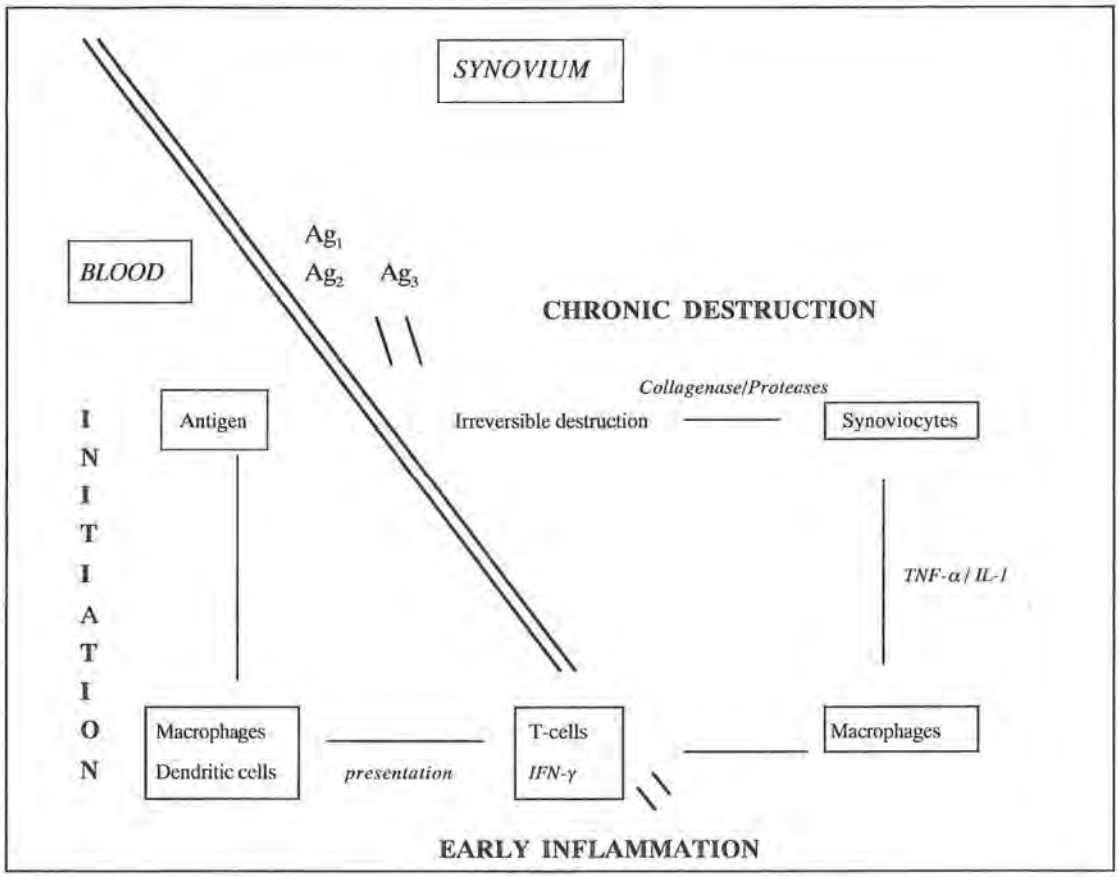

Figure 1.5: Hypothetical scheme of the pathogenic events in RA.

7. Therapies for RA

Standard therapeutic strategies frequently prescribed are based on immunosuppressive drugs. These drugs unselectively suppress not only disease relevant T-cells but also other immune competent cells. Consequently, the use of immunosuppressive drugs can increase the risk of opportunistic infections. On the other hand, recently developed immune-therapies suppress mainly subtypes of T-cells. Some of these new therapies interfere at the tri-molecular complex level by using monoclonal antibodies to lymphocyte surface molecules or by vaccination with T-cells or TCR peptides. For example, new therapeutic strategies using monoclonal antibodies to surface molecules (anti-CD4) or monoclonal antibodies neutralizing secreted cytokines (anti-TNF-α) are currently investigated. In addition therapies directed against specific T-cell subsets, using T-cell vaccination or T-cell receptor peptide vaccination are currently evaluated for future applications. Most of these therapies are still in an experimental phase.

7.1 Anti-rheumatic drugs

Anti-rheumatic drugs can be divided in non-steroidal anti-inflammatory drugs (NSAID's) like aspirin, ibuprofen and salicylates and slow-acting anti-rheumatic drugs (SAARD's) or disease modifying anti-rheumatic drugs (DMARD's). Non-steroidal anti-inflammatory drugs (NSAID's) are first-line drugs, and are used in the early phase of the disease. They provide symptomatic treatment but can not slow down the disease progression. Slow-acting anti-rheumatic drugs (SAARD's) like penicillamine, gold, methotrexate and cyclosporin are second-line drugs that can induce partial remission of the disease but they have serious adverse effects. SAARD's reduce or prevent joint damage and in some way preserve the joint integrity and function. Cyclosporin A (CSA), is a potent immunoregulatory agent that is more specific than most other cytotoxic/immunosuppressive drugs. CSA prevents an antigen or mitogen induced secretion of T-cell related cytokines like IL-2, IL-4, TNF-α and IFN-γ at the transcriptional level (10,138). CSA inhibits T-cell activation events in a very early phase, and induces secondary events on B-cells, macrophages and other cell types that are dependent on T-cell derived cytokines for their activation (139).

7.2 Biological therapies using monoclonal antibodies

7.2.1 anti-CD4 monoclonal antibody therapy

Anti-CD4 monoclonal antibodies bind to the CD4 molecule expressed on the surface of T-cells inhibiting the T-helper/MHC II interactions. Efficient antigen presentation and subsequent T-cell activation is prevented. Clinical tests in humans revealed both positive and negative results, depending on the type of monoclonal antibody used. Some studies used murine monoclonal Ab whereas others used chimeric Ab consisting of a human Ig constant region and a murine Ig variable region. However, no significant improvement was observed in a double-blind placebo controlled study of murine anti-CD4 monoclonal antibodies with 58 patients in an active stage of the disease (140). In addition, no significant improvement was reported in a study of chimeric anti-CD4 with 60 patients with recent disease onset (141) and 64 patients with chronic progressive disease (142).

7.2.2 Anti-cytokine - cytokine receptor therapy

Cytokines produced by monocyte-macrophages, endothelial cells, synovial lining cells and lymphocytes have a major role in the immune response in RA. Cytokine directed therapies can either inhibit cytokine synthesis, block cytokine release from the secreting cells, inhibit cytokine action on the target T-cell or inhibit intracellular signaling processes activated by cytokines. Anti-

cytokine monoclonal antibodies or soluble cytokine receptors interfere with cytokine functions (66). The pro-inflammatory cytokines IL-1 and TNF-α have been used as targets for anti-cytokine therapy in RA. The clinical effects of the chimeric (mouse/human) anti-TNF-α mAb cA2 (Remicade) have been reported recently. Remicade suppressed synovitis symptoms in a randomized, placebo controlled trial (143). These results were confirmed in a study using the humanized anti-TNF-α Mab CDP571 (144). Recently, multiple infusions with the chimerized monoclonal antibody in combination with Methotrexate (MTX) was reported to increase the tolerability and disease suppressive effect of the anti-TNF-α therapy (145). Alternatively, the activity of the pro-inflammatory cytokine TNF-α can be blocked using a soluble TNF-α receptor, administered as a recombinant TNF-receptor (p75)-Fc fusion protein (Ethanercept). Ethanercept was shown to be safe, well tolerated and efficient in alleviating the inflammatory symptoms either alone (146) or in combination with MTX (147).

7.2.3 Other monoclonal antibodies

Monoclonal antibodies to cell surface adhesion molecules such as ICAM-1 or VCAM-1 can reduce the influx of pathogenic T-cells into the joint cavity. However, more detailed studies on the pathogenesis of RA are needed to find the best target for an efficient inhibition of the disease progression using this approach.

7.3 Biological therapies using T-cell or T-cell peptide vaccination

T-cell vaccination is based on the same principles as a classical immunization, establishing protection by vaccination with a non-pathogenic form of the causative organism (24) The concept of T-cell vaccination was introduced by Cohen and colleagues in animal models of autoimmune diseases such as Experimental Allergic Encephalomyelitis (EAE) and Adjuvant Arthritis (AA). In EAE, an animal model for MS, myelin basic protein (MBP) is a major autoantigen in the disease pathogenesis. T-cell vaccination (TCV) and T-cell receptor peptide vaccination are currently also studied in humans. In TCV, patients are immunized with autologous attenuated activated autoreactive T-cells. An immune response is induced that specifically suppresses the disease-inducing T-cells and further protects the patient (24,148). A T-cell vaccination pilot study with 8 MS patients who were immunized 3 times (2-4 months interval) with irradiated autologous MBP specific T-cell clones has been performed. The study demonstrated that the T-cell vaccine is well tolerated and induces a moderate reduction in the rate of clinical exacerbations (149-151). In RA patients, the immunomodulatory effects in 13 RA patients immunized with a vaccine prepared from synovial fluid and tissue autologous T-cells was studied (17,18). The approach was safe and well tolerated, but nothing was known about the antigen specificity and the relevance for the disease of the cells used for immunization. In addition, better results were expected after immunization with

disease-inducing T-cells isolated from synovial fluid of the patients. However, additional information concerning the CDR3 regions sequences and the antigen specificity is needed (18). Alternatively, instead of immunizing patients with whole T-cells, TCR peptides have been used. A phase I open label, safety and dose ranging study was reported using BV17 TCR peptides for the immunization of RA patients (19). Two years later, a placebo controlled trial using a combination of BV 3,14 and 17 TCR peptides demonstrated clinical improvement and no adverse or toxic effects (152,153).

7.4 Gene therapy for RA

Biological agents such as antibodies to T-cell surface molecules and cytokines are potential therapies for RA. However, in order to establish persistent effects, continuous delivery is essential. In addition, administered peptides have a restricted half life, are degraded in the gut and sometimes can be immunogenic. With the help of gene therapy, biological disease modifiers such as cytokines or cytokine inhibitors can be introduced by direct injection of naked DNA, transfection of synoviocytes or genetically modified autoreactive T-cells (154). Several successful gene therapies in animal models of arthritis have been reported (155,156). Results of a phase I clinical study, evaluating the transplantation of autologous genetically modified synovial cells in the end-stage diseased joints of nine RA patients have been reported (157). Synovial cells were genetically modified to continuously express IL-1Ra in the treated joints. IL-1 is a prototypical proinflammatory cytokine that is actively involved in the progressive joint destruction. IL-1Ra competitively inhibits the binding of IL-1 to the surface IL-1 receptors and thus acts as a endogenous anti-inflammatory mediator. So far, seven patients completed the study, no adverse effects were observed and an expression of IL-1Ra in the transplanted joints was detected using RT-PCR. Thus, the feasibility and safety of delivery of the therapeutic gene into human joints has been demonstrated. Further clinical trials are needed to study the clinical effects of this approach (157).

7.5 Tolerance induction

Furthermore, in addition to the described therapeutic approaches, tolerance induction by oral administration of antigens is currently tested in several laboratories. Recently the efficacy and safety of four different dosages of orally administered collagen type II was tested in a multicentre, double blinded placebo controlled trial. Positive effects were observed with collagen type II at the lowest dosage tested (20 microgram/day) and no side effects were associated with this novel therapeutic agent (158). The results of a phase III clinical trial with Colloral® on the other hand showed that although the Colloral® treatment was safe, no clinical efficacy could be demonstrated (Press release, Autoimmune inc.). In another approach peptide-specific autoreactive T-cells are

inactivated so that HC gp-39 is no longer recognized as an autoantigen by the immune system. Therefore, Anergen's AnergiX technology combines disease-specific autoantigenic peptides and disease associated HLA-derived protein to bind to and deactivate the disease specific T-cells. This AnergiX technology is currently tested in a phase I clinical study.

Table 1.3: Overview of therapies, target T-cells and therapeutic efficiency in humans

Therapy	Target ?	interference with ?	Proven/ Experimental	Efficient ?
Anti-rheumatic drugs				
NSAID's	all immune cells	inflammation	proven	Fast/Short time
DMARD	dependent on type	joint damage	proven	slow/long-time
Biological therapies				
anti-CD4	T-cells	CD4+ T-cell depletion	Phase I + II	±
anti-cytokine	cytokines	cytokine actions	Phase I	+
T-cell vaccination	pathogenic T-cells	excludes T-cells	Phase I	+
peptide vaccination	autoreactive T-cells	excludes T-cells	Phase II	+
Gene therapy				
IL-1Ra	IL-1	destructive enzymes	Phase I	+
Tolerance induction				
Collagen antigens	T/B-cells	immune response	Phase III	no symptoms
HLA/ gp-39-complex	T-cells	immune response	Phase I	pending

Aim of the study

T-cells play an important role in the pathogenesis of rheumatoid arthritis (RA). So far, the contribution of T-lymphocytes to the disease course, as well as the characteristics of the (auto)antigens involved in the activation of these immune-competent cells are not yet completely understood.

This study is aimed to further characterize the pathogenic T-cell response in the autoimmune response observed in the affected joints of RA patients. We will study T-cells in early and chronic RA patients and compare TCR V gene expression profiles in peripheral blood and synovial cavity samples. In addition, TCR V gene profile changes will be studied over time. TCR V gene analysis will thus be the central subject of the thesis.

So far, TCR V gene studies yielded inconclusive results. Genetic and clinical heterogeneity of the study group, the inclusion of depleted or non-depleted *in vivo* stimulated or non-stimulated T-cells and the variability in technological procedures applied, may all explain the observed differences. To exclude *in vitro* manipulations that could affect the *in vivo* situation, we studied unstimulated freshly isolated mononuclear cells from peripheral blood, synovial fluid and/or synovial tissue samples. TCR V gene expression profiles and clonal characteristics were determined.

Goal 1: to optimize a technique that can be used to determine TCR V gene expression profiles in patient samples and to identify expanded TCR V gene elements.

Synovial fluid cells or tissue biopsies taken from RA joints are the most relevant samples to study pathogenic T-cells. However, these samples sometimes only contain as few as 50.000 cells. A PCR based technology is thus required to determine the TCR V gene expression profiles. In this part of the thesis, a simple, fast and sensitive PCR-ELISA will be developed to evaluate the TCR V gene expression. To determine clonal characteristics of overrepresented V gene families PCR-ELISA was combined with CDR3 region spectratyping

Goal 2: to study possible differences in the TCR V gene expression profiles between early and chronic RA patients and to determine the clonal characteristics of overrepresented V gene families in an early and a chronic RA patient.

So far, TCR V gene expression profiles revealed inconclusive results. In the second part of the thesis TCR V gene analysis was performed on undepleted, unstimulated T-cells from blood and synovial samples of early and chronic RA patients using a uniform approach. V gene expression studies on freshly isolated T-cells, may demonstrate *in vivo* antigen driven stimulation. Subsequently, CDR3 region clonal analysis of overrepresented V gene families could reveal some general characteristics of the antigens involved. For example, polyclonally expanded T-cells could suggest a superantigen driven T-cell involvement, whereas oligo-or monoclonal T-cell expansions possibly suggest a 'classical' antigen driven T-cell involvement. Differences in TCR V gene expression in early and chronic RA patients on the other hand, could suggest whether or not T-cell characteristics in early and late disease stages are the same.

Goal 3: to study the TCR V gene profile changes over time in the synovium of early RA patients, and to evaluate the effect of Cyclosporin A (CSA) treatment on TCR V gene expression in blood and tissue samples of early RA patients.

Our previous experiments showed that TCR V gene expression profiles become more diverse along the progression of the disease, and suggest that TCR V gene analysis could best be performed in an early disease stage. To find out whether an antigen driven immune response could be demonstrated at an early disease stage TCR V gene expression profiles in peripheral blood and synovial tissue samples of early untreated RA patients have been studied in the third part of the thesis. Moreover, to evaluate the hypothesis that disease relevant antigens change during disease progression TCR V gene expression has been studied at two timepoints. More detailed information on the diversity of the TCR repertoire has been provided by CDR3 region spectratype analysis. If

similar CDR3 region profiles are observed, this may indicate a persistence of T-cell clones during the disease progression.

CSA is an immune suppressive drug that interferes with T-cell activation at the transcriptional level. Some of the previous findings in this study are compatible with a pathogenic role for T-cells in RA. If T-cell activation is completely blocked with CSA the T-cell driven immune response could be stopped in an early disease stage, and joint destruction prevented. To evaluate the effect of the medication on the V gene expression in the joints, TCR V gene analysis has been performed on blood and tissue samples of CSA treated and untreated early RA patients.

Goal 4: to study the effect of CSA on cytokine mRNA contents in peripheral blood and synovial tissue samples of early RA patients.

Further evidence for the pathogenicity of T-cells in RA could be provided by studying cytokine profiles in the affected joints. In this part of the study, the expression of T-cell related cytokines (IFN-γ, TNF-α, IL-4, IL-10) in blood and tissue samples of early RA patients has been studied. A comparison between the expression of IFN-γ and IL-4 should reveal whether or not a pro or anti-inflammatory cytokine environment is prevalent in the joints. In addition, to find out if CSA affects T-cell and/or non T-cell related cytokine expression, the effect of CSA treatment on the cytokine expression was evaluated at the diseased site.

References

1. Buckley, C.D. Treatment of Rheumatoid Arthritis. Science, medicine and future. *B.M.J.* 315:236-238, 1997.

2. Struyk, L., Hawes, G., Chatila, M., Breedveld, F., Kurnick, J. and van den Elsen, P. T-cell receptors in Rheumatoid Arthritis. *Arthritis Rheum.* 38:577-589, 1995.

3. Klareskog, L., Rönnelid, J. and Holm, G. Immunopathogenesis and immunotherapy in Rheumatoid Arthritis: an area in transition. *J.Int.Med.* 238:191-206, 1995.

4. Gardener, D.L. Problems and paradigms in joint pathology. *J.Anat.* 184:465-476, 1994.

5. Machold, K.P., Eberl, G., Leeb, B.F., Nell, V., Windisch, B. and Smolen, J.S. Early arthritis therapy: Rationale and current approach. *J.Rheumatol.* 25 supplement 53:13-18, 1998.

6. Harris, E.D. Rheumatoid arthritis: Pathophysiology and implications for therapy. *N.Engl.J.Med.* 322(18):1277-1289, 1990.

7. Arnett, F.C., Edworthy, C., Bloch, D.A., et al. The American Rheumatism Association 1987 revised criteria for the classification of Rheumatoid Arthritis. *Arthritis Rheum.* 31:315-324, 1988.

8. McCarthy, DJ. and Koopman, WJ. *Arthritis and allied conditions.* , Philadelphia:Lea & Febiger, 1993. Ed. 12th pp. Volume 1.

9. Kinne, R.W., Palombo-Kinne, E. and Emmrich, F. T-cells in the pathogenesis of Rheumatoid Arthritis. Villains or accomplices? *BBA* 1360:109-141, 1997.

10. Harris, ED.Jr *Rheumatoid Arthritis*, Philadelphia:W.B. Saunders Company, 1997.

11. Smolen, J.S., Tohidast-Akrad, M., Gal, A., et al. The role of T-lymphocytes and cytokines in Rheumatoid Arthritis. *Scand.J.Rheumatol.* 25:1-4, 1996.

12. Auger, I. and Roudier, J. HLA-DR and the development of Rheumatoid Arthritis. *Autoimm.* 26(2):123-128, 1997.

13. Auger, I. and Roudier, J. A function for the QKRAA amino acid motif: Mediating binding of DnaJ to DnaK. Implications for the association of Rheumatoid Arthritis with HLA DR4. *J.Clin.Invest.* 99:1818-1822, 1997.

14. Forre, O., Thoen, J., Lea, T., Dobloug, JH., Melbye, OJ., Natvig, J., Pahle, J., Solheim, BG., In situ characterization of mononuclear cells in rheumatoid arthritis, using monoclonal antibodies. *Scand J. Immunol.* 16: 315-319, 1982.

15. Poulter, LW., Duke, O., Panayi, GS., Hobbs, S., Raftery, MJ., Janossy, G., Activated T-lymphocytes of the synovial membrane in rheumatoid arthritis and other arthropathies. *Scand J Immunol.* 22: 683-692, 1988.

16. Keystone, EC., Snow, KM., Bombardier, C., Chang, C., Nelson, DL., Rubin, LA., Elevated soluble interleukin-2 receptor levels in sera and synovial fluids of patients with rheumatoid arthritis. *Arthritis rheum* 31: 844-849, 1988.

17. van Laar, JM., Miltenburg, AM., Verdonk, MJ., et al. Effects of inoculation with attenuated autologous T-cells in patients with rheumatoid arthritis. *J.Autoimmun.* 6(2):159-167, 1993.

18. Breedveld, F.C., Struyk, L., van Laar, J.M., Miltenburg, A.M., de Vries, R.R. and van den Elsen, P. Therapeutic regulation of T-cells in Rheumatoid Arthritis. *Immunol.Rev.* 144:5-16, 1995.

19. Moreland, L.W., Heck, L.W., Koopman, W.J., et al. Vβ 17 T-cell receptor peptide vaccination in Rheumatoid Arthritis: results of a phase I dose escalation study. *J.Rheumatol.* 23(8):1353-1362, 1996.

20. Solinger, AM., Hess, EV. HIV and arthritis. *J Rheumatol* 17: 562, 1990

21. Field, EH., Engleman, EG., Terrell, CP., Dtrober, S. Reduced in vitro immune responses of purified human Leu-3 (helper/inducer phenotype) cells after total lymphoid irradiation. *J immunol* 132: 1031-1035, 1984.

22. Karsh, J., Klippel, SH., Plotz, PH., Decker, JL., Wright, DR., Flye, MW., Lymphapheresis in rheumatoid arthritis: a randomized trial. Arthritis Rheum 24: 867-873, 1981

23. Thomas, R. Antigen-presenting cells in Rheumatoid Arthritis. *Springer Semin.Immunopathol.* 20:53-72, 1998.

24. Panayi, G.S. The immunopathogenesis of Rheumatoid Arthritis. *Rheumatol.Rev.* 1:63-74, 1992.

25. Panayi, G.S., Lanchbury, J.S. and Kingsley, G.H. The importance of the T-cell in initiating and maintaining the chronic synovitis of Rheumatoid Arthritis. *Arthritis Rheum.* 35(7):729-735, 1992.

26. Firestein, G.S. and Zvaifler, N. How important are T-cells in chronic rheumatoid synovitis? *Arthritis Rheum.* 33:768-773, 1990.

27. Sewell, K.L. and Trentham, D.E. Pathogenesis of Rheumatoid Arthritis. *Lancet* 341:283-286, 1993.

28. Breedveld, F.C. and Verwey, C.L. T-cells in Rheumatoid Arthritis. *Br.J.Rheumatol.* 36:617-621, 1997.

29. Weyand, C.M., Klimiuk, P.A. and Goronzy, J.J Heterogeneity of Rheumatoid Arthritis: from phenotypes to genotypes. *Springer Semin.Immunopathol.* 20(1-2):5-22, 1998.

30. Schur, P.H. Arthritis and autoimmunity. The fifteenth Sigrid Juselius international symposium. *Arthritis Rheum.* 12:1818-1825, 1994.

31. Koumantaki, Y., Giziaki, E., Linos, A., et al. Family history as a risk factor for Rheumatoid Arthritis: a case control study. *J.Rheumatol.* 24(8):1522-1526, 1997.

32. Toussirot, E. and Wendling, D. Polyarthrite rhumaoïde: ses liens avec les molecules HLA DR. *La Pr.Med.* 24:855-858, 1995.

33. Hall, F.C., Brown, M.A., Weeks, D.E., et al. A linkage study across the T-cell receptor A and T-cell receptor B loci in families with Rheumatoid Arthritis. *Arthritis Rheum.* 40(10):1798-1802, 1997.

34. McDermott, M., Kastner, DL., Holloman, JD., et al. The role of T-cell receptor beta chain genes in susceptibility to rheumatoid arthritis. *Arthritis Rheum.* 38(1):91-95, 1995.

35. Eskdale, J., McNicholl, J., Wordsworth, P., et al. Interleukin-10 microsatellite polymorphisms and IL-10 locus alleles in Rheumatoid Arthritis susceptibility. *Lancet* 352(9136):1282-1283, 1998.

36. Hajeer, A.H., Lazarus, M., Turner, D., et al. IL-10 gene promoter polymorphisms in Rheumatoid Arthritis. *Scand.J.Rheumatol.* 27(2):142-145, 1998.

37. Field, M., Gallagher, G., Eskdale, J., et al. Tumor necrosis factor locus polymorphisms in Rheumatoid Arthritis. *Tissue Ant.* 50(3):303-307, 1997.

38. Ollier, W.E. and MacGregor, A. Genetic epidemiology of Rheumatoid Arthritis. *Br.Med.Bull.* 51(2):267-285, 1995.

39. Jawaheer, D., Thomson, W., MacGregor, A.J., et al. 'Homozygosity' for the HLA DR shared epitope contributes the highest risk for Rheumatoid Arthritis concordance in identical twins. *Arthritis Rheum.* 37(5):681-686, 1994.

40. Mayr, W.R. Recent advances in HLA. *Vox S.* 70 suppl(3):89-94, 1996.

41. Fugger, L. and Svejaard, A. The HLA DQ7 and DQ8 associations in DR4 positive Rheumatoid Arthritis patients. *Tissue Ant.* 50:494-500, 1997.

42. MacGregor, A., Ollier, W., Thomson, W., Jawaheer, D. and Silman, A. HLA-DRB1*0401/0404 genotype and Rheumatoid Arthritis: Increased association in men, young age at onset and disease severity. *J.Rheumatol.* 22:1032-1036, 1995.

43. Nepom, G.T., Gersuk, V. and Nepom, B.S. Prognostic implications of HLA genotyping in early assessment of patients with Rheumatoid Arthritis. *J.Rheumatol.* 23 suppl 44:5-9, 1996.

44. Seidl, C., Koch, U., Buhleier, T., et al. HLA-DRB1*04 subtypes are associated with increased inflammatory activity in early Rheumatoid Arthritis. *Br.J.Rheumatol.* 36:941-944, 1997.

45. Gregersen, PK., Silver, J. and Winchester, RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* 30(11):1205-1213, 1987.

46. Perdiger, A., Giggenbuhl, P., Chales, G., Le Dantec, P. and Yaouanq, J. The role of HLA-DR-DR and HLA-DR-DP interactions in genetic susceptibility to Rheumatoid Arthritis. *Hum.Immunol.* 46:42-48, 1996.

47. Vandevyver, C., Gu, X.X., Geusens, P., et al. HLA class II and T-cell receptor β chain polymorphisms in Belgian patients with Rheumatoid Arthritis: no evidence for disease association with TCR BC2, TCR BV8 and TCR BV11 polymorphisms. *Ann.Rheum.Dis.* 53:580-586, 1994.

48. Weyand, C.M. and Goronzy, J.J Disease mechanisms in Rheumatoid Arthritis: gene dosage effect of HLA-DR haplotypes. *J.Lab.Clin.Med.* 124(3):335-338, 1994.

49. Fox, D.A. The role of T-cells in the immunopathogenesis of Rheumatoid Arthritis: new perspectives. *Arthritis Rheum.* 40:598-609, 1997.

50. Moots, R.J. A fistful of T-cells. *Br.J.Rheumatol.* 37:602-611, 1998.

51. Melchers, I., Jooss-Rudiger, J. and Peter, HH. Reactivity patterns of synovial T-cell lines derived from a patient with rheumatoid arthritis. Reactions defined antigens and auto-antigens suggest the existence of multireactive T-cell clones. *Scand.J.Immunol.* 46(2):187-194, 1997.

52. van den Eden, W., van der Zee, R., Paul, A.G.A., et al. Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases ? *Immunol.Today* 19(7):303-307, 1998.

53. Verheijden, G., Rijnders, A., Bos, E., et al. Human cartilage glycoprotein-39 as a candidate autoantigen in Rheumatoid Arthritis. *Arthritis Rheum.* 40:1115-1125, 1997.

54. Feldmann, M., Brennan, F.M. and Maini, R.N. Rheumatoid Arthritis. *Cell* 85:307-310, 1996.

55. Celis, L., Vandevyver, C., Geusens, P., Dequecker, J., Raus, J. and Zhang, J. Clonal expansion of mycobacterial heat-shock protein reactive T-lymphocytes in the synovial fluid and blood of Rheumatoid Arthritis patients. *Arthritis Rheum.* 40:510-519, 1997.

56. Gaston, J.S. Heat shock proteins and arthritis: new readers start here. *Autoimm.* 26(1):33-42, 1997.

57. Auger, I., Escola, J.M., Gorvel, J.P. and Roudier, J. HLA DR4 and HLA DR10 motifs that carry susceptibility to Rheumatoid Arthritis bind 70kD heat shock proteins. *Nature Med.* 2(3):306-310, 1996.

58. Kaufmann, S.H.E. Heat shock proteins and the immune respons. *Immunol.Today* 11:129-136, 1990.

59. Firestein, G.S. The immunopathogenesis of Rheumatoid Arthritis. *Curr.Op.in Rheumat.* 3:398-406, 1991.

60. Cremer, M.A., Roloniec, E.F. and Kang, A.H. The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. *J.Mol.Med.* 76(3-4):275-288, 1998.

61. Davies, J.M. Molecular mimicry: Can epitope mimicry induce autoimmune disease ? *Immunol.Cell Biol.* 75:113-126, 1997.

62. Roudier, J., Rhodes, G., Petersen, J., Vaughan, J.H. and Carson, D.A. The Epstein-Barr virus glycoprotein gp110, a molecular link between HLA DR4, HLA DR1, and Rheumatoid Arthritis. *Scand.J.Immunol.* 27(4):367-371, 1988.

63. Nakagawa, K., Brusic, V., McColl, G. and Harrison, L.C. Direct evidence for the expression of multiple endogenous retroviruses in the synovial compartment in Rheumatoid Arthritis. *Arthritis Rheum.* 40(4):627-637, 1997.

64. Griffiths, D.J., Cooke, S.P., Herve, C., et al. Detection of retrovirus 5 in patients with arthritis and systemic lupus erythematosus. *Arthritis Rheum.* 42(3):448-454, 1999.

65. Albani, S. and Carson, D.A. A multistep molecular mimicry hypothesis for the pathogenesis of Rheumatoid Arthritis. *Immunol.Today* 17(10):466-470, 1996.

66. Burmester, G.R., Stuhlmüller, B., Keyszer, G. and Kinne, R.W. Mononuclear phagocytes and Rheumatoid Synovitis. Mastermind or Workhorse in Arthritis ? *Arthritis Rheum.* 40(1):5-18, 1997.

67. Tak, P.P., Smeets, T.J.M., Daha, M.R., et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. *Arthritis Rheum.* 40(2):217-225, 1997.

68. Kraan, M.C., Versendaal, H., Jonker, M., et al. Asymptomatic synovitis precedes clinically manifest arthritis. *Arthritis Rheum.* 41(8):1481-1488, 1998.

69. Firestein, G. Invasive fibroblast-like synoviocytes in Rheumatoid Arthritis. Passive responders or transformed aggressors ? *Arthritis Rheum.* 39(11):1781-1790, 1996.

70. Dreher, R. Origin of synovial type A cells during inflammation. An experimental approach. *Immunobiol.* 161(3-4):232-245, 1982.

71. McCarthy, CJ., Eustace, S. and Coughlan, RJ. Simultaneous presentation of rheumatoid arthritis in a sibling pair. *Br.J.Rheumatol.* 33(9):889-890, 1994.

72. Thomas, R. and Lipsky, P.E. Presentation of self peptides by dendritic cells: possible implications for the pathogenesis of Rheumatoid Arthritis. *Arthritis Rheum.* 39(2):183-190, 1996.

73. Thomas, R. and Lipsky, P. Could endogenous self-peptides presented by dendritic cells initiate Rheumatoid Arthritis ? *Immunol.Today* 17(12):559-564, 1996.

74. Mak, TW. *The T-cell receptors*, New York - London:Plenum Press , 1988.

75. Arend, W.P. and Dayer, J.M. Inhibition of the production and affects of interleukin-1 and tumor necrosis factor α in Rheumatoid Athritis. *Arthritis Rheum.* 38(2):151-160, 1995.

76. Davis, M.M. T-cell receptor gene diversity and selection. *Ann.Rev.Biochem.* 59:475-496, 1990.

77. Davies, MM. and Bjorkman, PJ. T-cell antigen receptor genes and T-cell recognition. *Nature* 334(6181):395-402, 1988.

78. Sakkas, LI., Chen, PF. and Platsoucas, CD. T-cell antigen receptors in rheumatoid arthritis. *Immunol.Res.* 13(2-3):117-138, 1994.

79. Cooper, S.M., Roessner, K.D., Naito-Hoopes, M., et al. Unstimulated Rheumatoid synovial T-cells have consistent Vβ gene bias when compared to peripheral blood. *Ann.N.Y.Acad.Sci.* 756:186-189, 1995.

80. Struyk, L., Hawes, G.E., Mekkers, H.M.M., Tak, P.P., Breedveld, F.C. and van den Elsen, P. Molecular analysis of the T-cell β chain repertoire in early Rheumatoid Arthritis: heterogeneous TCR BV gene usage with shared amino acid profiles in CDR3 regions of T-lymphocytes in multiple synovial tissue needle biopsies from the same joint. *Eur.J.Clin.Invest.* 26:1092-1102, 1996.

81. Davey, M.P., Burgoine, G.A and Woody, C.N. TCRB clonotypes are present in CD4+ T-cell populations prepared directly from rheumatoid synovium. *Hum.Immunol.* 55:11-21, 1997.

82. Zagon, G., Tumang, JR., Li, Y., Friedman, SM. and Crow, MK. Increased frequency of Vbeta 17 positive T-cells in patients with rheumatoid arthritis. *Arthritis Rheum.* 37(10):1431-1440, 1994.

83. Bröker, B., Korthäuer, U., Heppt, P., et al. Biased T-cell receptor V-gene usage in Rheumatoid Arthritis. *Arthritis Rheum.* 36:1234-1243, 1993.

84. Breit, T. and Van Dongen, J. Unravelling the human T-cell receptor junctional region sequences. *Thymus* 22(3):177-199, 1994.

85. Bucht, A., Oksenberg, J.R., Lindblad, S., Gronberg, A., Steinman, L. and Klareskog, L. Characterization of T-cell receptor αβ repertoire in synovial tissue from different temporal phases of Rheumatoid Arthritis. *Scand.J.Immunol.* 35:159-165, 1992.

86. Cottrez, F., Auriault, C., Capron, A. and Groux, H. Analysis of the BV specificity of superantigen activation with a rapid and sensitive method using RT-PCR and an automatic DNA analyser. *J.Immunol.Methods* 172(1):85-94, 1994.

87. Lang, R., Pfeffer, K., Wagner, H. and Heeg, K. A rapid method for semiquantitative analysis of the human BV repertoire using TaqMan PCR. *J.Immunol.Methods* 203(2):181-192, 1997.

88. Manfras, B., Rudert, W., Trucco, M. and Boehm, B. Analysis of the α/β T-cell-receptor repertoire by competitive and quantitative family-specific PCR exogenous standards and high resolution fluorescence based CDR3 size imaging. *J.Immunol.Methods* 210(2):235-249, 1997.

89. Bettinardi, A., Imberti, L., Sottini, A. and Primi, D. Analysis of amplified T-cell receptor V beta transcripts by non-isotopic immunoassay. *J.Immunol.Methods* 146(1):71-82, 1992.

90. VanderBorght, A., Van de AA, A., Geusens, P., Vandevyver, C., Raus, J. and Stinissen, P. Identification of overrepresented TCR genes in blood and tissue biopsies by PCR-ELISA. *J.Immunol.Methods* 223:47-61, 1999.

91. Struyk, L., Kunick, J.T., Hawes, G.E., et al. T-cell receptor V-gene usage in synovial fluid lymphocytes of patients with chronic Rheumatoid Arthritis. *Hum.Immunol.* 37:237-251, 1993.

92. Borgato, L., Beri, R., Biasi, D., et al. Analysis of T-cell receptor repertoire in Rheumatoid Arthritis. *Clin.Exp.Rheumatol.* 15(5):475-479, 1997.

93. Rasmussen, R., Morrison, T., Herrman, M. and Wittwer, C. Quantitative PCR by continuous fluorescence monotoring of a double strand DNA specific dye. *Biochemica* 2:4-11, 1998.

94. Motmans, K., Raus, J. and Vandevyver, C. Quantification of cytokine messenger RNA in transfected human T-cells by RT-PCR and an automated electrochemiluminescence-based post-PCR detection system. *J.Immunol.Methods* 190(1):107-116, 1996.

95. Hingorani, R., Monteiro, J., Furie, R., et al. Oligoclonality of Vβ 3 TCR chains in the CD8+ T-cell population of Rheumatoid Arthritis patients. *J.Immunol.* 156:852-858, 1996.

96. Gudmundson, S., Ronnelid, J., Karlsson-Parra, A., T-cell receptor V-gene usage in synovial fluid and synovial tissue from RA patients. *Scand J immunol* 36: 681-688, 1992

97. Savill, C.M., Delves, P.J., Kioussis, D., et al. A minority of patients with Rheumatoid Arthritis show a dominant rearrangement of T-cell receptor β chain genes in synovial lymphocytes. *Scand.J.Immunol.* 25:629-635, 1987.

98. Pannetier, C., Even, J. and Kourilsky, P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol.Today* 16(4):176-181, 1995.

99. Jenkins, R.N., Nikaein, A., Zimmerman, A., Meek, K. and Lipsky, P.E. T-cell receptor Vβ gene bias in Rheumatoid Arthritis. *J.Clin.Invest.* 92(6):2688-2701, 1993.

100. Alam, A., Lulé, J., Coppin, H., et al. T-cell receptor variable region of the β-chain use in peripheral blood and multiple synovial membranes during Rheumatoid Arthritis. *Hum.Immunol.* 42:331-339, 1995.

101. Padula, S.J. and Sampieri, A. T-cell receptor use in early Rheumatoid Arthritis. *Ann.N.Y.Acad.Sci.* 756:147-158, 1995.

102. Huchenq, A., Champagne, E., Sevin, J., et al. Abnormal T-cell receptor BV gene expression in the peripheral blood and synovial fluid of Rheumatoid Arthritis patients. *Clin.Exp.Rheumatol.* 13(1):29-36, 1995.

103. Jenkins, R.N. and McGinnis, D.E. T-cell receptor Vβ gene utilization in Rheumatoid Arthritis. *Ann.N.Y.Acad.Sci.* 756:159-172, 1995.

104. Struyk, L., Kunick, J.T., Hawes, G.E., et al. T-cell receptor V-gene usage in synovial fluid lymphocytes of patients with chronic Rheumatoid Arthritis. *Hum.Immunol.* 37:237-251, 1993.

105. Waase, I., Kayser, C., Carlson, P.J., Goronzy, J.J. and Weyand, C.M. Oligoclonal T-cell proliferation in patients with Rheumatoid Arthritis and their affected siblings. *Arthritis Rheum.* 36(6):904-913, 1996.

106. Padula, S. and Sampieri, A. T-cell receptor use in early Rheumatoid Arthritis. *Ann.N.Y.Acad.Sci.* 756:147-158, 1995.

107. Kato, T., Kurokawa, M., Masuko-Hongo, K., et al. T-cell clonality in synovial fluid of a patient with Rheumatoid Arthritis: persistent but fluctuant oligoclonal T-cell expansions. *J.Immunol.* 159:5143-5149, 1997.

108. Dedeoglu, F., Kaymaz, H., Seaver, N., Schluter, S., Yocum, DE. and Marchalonis, JJ. Lack of preferential Vβ usage in synovial T-cells in rheumatoid arthritis patients. . *Immunol.Res.* 12:12-20, 1993.

109. Ramanujam, T., Luchi, M., Schumacher, H.R., et al. Detection of T-cell receptors in early Rheumatoid Arthritis synovial tissue. *Pathobiol.* 63:100-108, 1995.

110. Pluschke, G., Ginter, A., Taube, H., Melchers, I., Peter, H.H. and Krawinkel, U. Analysis of T-cell receptor Vβ regions expressed by Rheumatoid synovial T-lymphocytes. *Immunobiol.* 188:330-339, 1993.

111. Wilson, D.B., Golding, A.B., Smith, R.A., et al. Results of a phase I clinical trial of a T-cell receptor peptide vaccine in patients with Multiple Sclerosis. I. Analysis of T-cell receptor utilization in CSF cell populations. *J.Neuroimmunol.* 76(1-2):15-28, 1997.

112. Howell, M.D., Diveley, J.P., Lundeen, K.A., et al. Limited T-cell receptor β chain heterogeneity among interleukin-2 receptor-positive synovial T-cells suggests a role for superantigen in Rheumatoid Arthritis. *Proc.Natl.Acad.Sci.USA* 88:10921-10925, 1991.

113. Wiliams, W.V., Kieber-Emmons, T., Fang, Q., et al. Conserved motifs in Rheumatoid Arthritis synovial tissue T-cell receptor β chains. *DNA and Cell.Biol.* 12(5):425-434, 1993.

114. Stamenkovic, I., Stegagno, M., Wright, K.A., et al. Clonal dominance among T-lymphocyte infiltrates in Arthritis. *Proc.Natl.Acad.Sci.USA* 85:1179-1183, 1988.

115. Miltenburg, A.M.M., van Laar, J.M., Daha, M.R., De Vries, R.R.P., van den Elsen, P.J. and Breedveld, F.C. Dominant T-cell receptor β chain gene rearrangements indicate clonal expansion in the rheumatoid joint. *Scand.J.Immunol.* 31:121-125, 1990.

116. Lunardi, C., Marguerie, C. and SO, K.O. An altered repertoire of T-cell receptor V-gene expression by rheumatoid synovial fluid T-lymphocytes. *Clin.Exp.Immunol.* 90:440-446, 1992.

117. Fisher, D.K., Opalka, B., Hoffman, A., Mayr, W. and Haubeck, H.D. Limited heterogeneity of rearranged T-cell receptor Vα and Vβ transcripts in synovial fluid T-cells in early stages of Rheumatoid Arthritis. *Arthritis Rheum.* 39:454-462, 1996.

118. Krawinkel, U. and Pluschke, G. T-cell receptor variable region repertoire in lymphocytes from Rheumatoid Arthritis patients. *Immunobiol.* 185:483-491, 1992.

119. Uetmatsu, Y., Wege, H., Stratus, A., et al. The T-cell receptor repertoire in the synovial fluid of a patients with Rheumatoid Arthritis. *Proc.Natl.Acad.Sci.USA* 88:8534-8538, 1991.

120. Sioud, M., Kjeldsen-Kragh, J., Quayle, A.J., et al. Immune responses to 18.6 and 30-kDa mycobacterial antigens in Rheumatoid patients, and V beta usage by specific synovial T-cell lines and fresh T-cells. *Scand.J.Immunol.* 34:803-812, 1991.

121. Weyand, CM., Oppitz, U., Hicok, K. and Goronzy, JJ. Selection of T-cell receptor V beta elements by HLA DR determinants predisposing to rheumatoid arthritis. . *Arthritis Rheum.* 35(9):990-998, 1992.

122. Wilson, KB., Quale, AJ., Suleyman, S., et al. Heterogeneity of TCR repertoire in synovial fluid T-lymphocytes responding to BCG in a patient with early rheumatoid arthritis. *Scand.J.Immunol.* 38:102-112, 1993.

123. Paliard, X., West, S.G., Lafferty, J.A., et al. Evidence for the effect of a superantigen in Rheumatoid Arthritis. *Science* 253:325-329, 1991.

124. Mcgee, B., Small, RE., Singh, R., et al. B lymphytic clonal expansion in rheumatoid arthritis. *J.Rheumatol.* 23(1):36-43, 1996.

125. Atkinson, J.P. Some thoughts on autoimmunity. *Arthritis Rheum.* 38(3):301-305, 1995.

126. Reparon-Schuijt, CC., van Esch, WJ., van Kooten, C., Levarht, EW., Breedveld, FC. and Verweij, CL. Functional analysis of rheumatoid factor-producing B-cells from the synovial fluid of rheumatoid arthritis patients. *Arthritis Rheum.* 41(12):2211-2220, 1998.

127. Mageed, R.A., Moyes, S.P., Vencovsky, J. and Maini, R.N. Somatic mutation and CDR3 length of immunoglobulin lambda variable region genes in the synovium of patients with Rheumatoid Arthritis. *Ann.N.Y.Acad.Sci.* 815:319-323, 1997.

128. Smolen, JS., Autoantibodies in rheumatoid arthritis. In Manual of Biological Markers of Disease. WJ. Van Venrooij and RN Maini, editors. Kluwer Academc Publishers, Dordrecht, The Netherlands. 1996. C11/1-C1.1/18.

129. Girbal-Neuhauser, E., Durieux, J.J., Arnuad, M., et al. The epitopes targeted by rheumatoid arthritis-associated antifillagrin autoantibodies are posttranslationally generated on various sites of (pro)fillagrin by deimination of arginine residues. *J.Immunol.* 162:585-594, 1999.

130. Schellekens, G.A., de Jong, B.A.W., van den Hoogen, F.H.J., van de Putte, L.B.A. and van Venrooij, W.J. Citrulline is an essential constituent of antigenic determinants recognized by Rheumatoid Arthritis-specific autoantibodies. *J.Clin.Invest.* 101:273-281, 1998.

131. Schaeverbeke, T., Bebear, C.M., Clerc, M., Lequen, L., Bebear, C. and Dehais, J. What is the role of mycloplasmas in human inflammatory rheumatic disorders ? *Rev.Rheum.Engl.Ed.* 66(1 Suppl):23S-27S, 1999.

132. Jefferies, W.McK. The etiology of Rheumatoid Arthritis. *Med.Hypoth.* 51:111-114, 1998.

133. Colville-Nash, PR. and Scott, DL. Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. *Ann.Rheum.Dis.* 51(7):919-925, 1992.

134. Zvaifler, N.J. and Firestein, G.S. Pannus and pannocytes. Alternative models of joint destruction in Rheumatoid Arthritis. *Arthritis Rheum.* 6:783-789, 1994.

135. Zvaifler, N.J. Rheumatoid Arthritis: The multiple pathways to chronic synovitis. *Lab.Invest.* 73(3):307-310, 1995.

136. Van den Berg, WB., The role of cytokines and growth factors in cartilage destruction and osteoarthritis and rheumatoid arthritis. *J Rheumatol.*, 58(3): 136-141, 1999.

137. Elewaut, D., De Keyser, F., De Wever, N., Baeten, D., Van Damme, N., Verbruggen, G., Cuvelier, C., Veys, E., A comparative phenotypical analysis of rhuematoid nodules and rheumatoid synovium with special reference to adhesion molecules and activation markers. *Ann Rheum Dis.* 57: 480-486, 1998.

138. Bentin, J. Mechanism of action of cyclosporin in Rheumatoid Arthritis. *Clin.Rheumatol.* 14(2):22-25, 1995.

139. Russel, G., Gravcley, R., Seid, J., Al-Humidan, A.K. and Skjodt, H. Mechanisms of action of cyclosporin and effects on connective tissues. *Semin.Arthritis.Rheum.* 21(suppl 3):16-22, 1992.

140. Wendling, D., Racadot, E., Wijdened, J., et al. A randomized, double blind, placebo controlled multicenter trial of murine anti-CD4 monoclonal antibody therapy in rheumatoid arthritis. *J.Rheumatol.* 25(8):1457-1461, 1998.

141. van der Lubbe, P.A., Dijkmans, B.A.C., Markusse, H.M., Nassander, U. and Breedveld, F. A randomized, double-blind, placebo-controlled study of CD4 monoclonal antibody therapy in early Rheumatoid Arthritis. *Arthritis Rheum.* 38(8):1097-1106, 1995.

142. Moreland, L.W., Pratt, P.W., Mayes, M.D., et al. Double-blind, placebo-controlled multicenter trial using chimeric monoclonal anti-CD4 antibody, cM-T412, in Rheumatoid Arthritis patients receiving concomitant methotrexate. *Arthritis Rheum.* 38(11):1581-1588, 1995.

143. Elliott, M., Maini, R. and Feldmann, M. Repeated therapy with monoclonal antibody to tumor necrosis factor a (cA2) in patients with Rheumatoid Arthritis. *Lancet* 344:1125-1127, 1994.

144. Rankin, E.C.C., Choy, E.H.S. and Kassimos, D. The therapeutic effects of an engeneered human anti-tumour necrosis factor antibody (CDP571) in Rheumatoid Arthritis. *Br.J.Rheumatol.* 34:334-342, 1995.

145. Maini, R.N., Breedveld, F.C., Kalden, J.R., et al. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor-a monoclonal antibody combined with low-dose weekly methotrexate in Rheumatoid Arthritis. *Arthritis Rheum.* 41(9):1552-1563, 1998.

146. Moreland, L.W., Baumgartner, S., Schiff, M., et al. Treatment of Rheumatoid Arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N.Engl.J.Med.* 337:141-147, 1997.

147. Weinblatt, M.E., Kremer, J.M., Bankhurst, A.D., et al. A trial of Ethanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with Rheumatoid Arthritis receiving methotrexate. *N.Engl.J.Med.* 340(4):253-259, 1999.

148. Stinissen, P., Raus, J. and Zhang, J. Autoimmune pathogenesis of multiple sclerosis: role of autoreactive T-lymphocytes and new immunotherapeutic strategies. *Crit.Rev.Immunol.* 17(1):33-75, 1997.

149. Stinissen, P., Medear, R. and Raus, J. Myelin reactive T-cells in the autoimmune pathogenesis of multiple sclerosis. *Mult.Scler.* 4(3):203-211, 1998.

150. Zhang, J.W., Medaer, R., Stinissen, P., Hafler, D. and Raus, J. MHC restricted clonotypic depletion of human myelin basic protein-reactive T-cells by T-cell vaccination. *Science* 261:1451-1454, 1993.

151. Medear, R., Stinissen, P., Truyen, L., Raus, J. and Zhang, J. Depletion of myelin-basic-protein autoreactive T-cells by T-cell vaccination: pilot trial in Multiple Sclerosis. *Lancet* 346(8978):807-808, 1995.

152. Moreland, L.W., Morgan, E.E., Adamson, T.C., et al. T-cell receptor peptide vaccination in Rheumatoid Arthritis. A placebo controlled trial using a combination of BV3, BV14 and BV17 peptides. *Arthritis Rheum.* 41(11):1919-1929, 1998.

153. Bridges, SL.Jr and Moreland, LW. T-cell receptor peptide vaccination in the treatment of rheumatoid arthritis. *Rheum.Dis.Clin.Morth Am.* 24(3):641-650, 1998.

154. Chernajovsky, Y., Feldmann, M. and Maini, R.N. Gene therapy of Rheumatoid Arthritis via cytokine regulation: future and perspectives. *Br.Med.Bull.* 51(2):503-516, 1995.

155. Muller-Ladner, U., Roberts, C.R., Franklin, B.N., et al. Human IL-1Ra gene transfer into human synovial fibroblasts is chondoprotective. *J.Immunol.* 158(7):3492-3498, 1997.

156. Otani, K., Nita, I., Georgescu, H.I., Robbins, P.D. and Evans, C.H. Suppression of antigen-induced arthritis in rabbits by ex vivo gene therapy. *J.Immunol.* 156(9):3558-3562, 1996.

157. Robbins, P.D., Evans, C.H. and Chernajovsky, Y. Gene therapy for Rheumatoid Arthritis. *Springer Semin.Immunopathol.* 20(1-2):197-207, 1998.

158. Barnett, ML., Kremer, JM., St Clair, EW., Clegg, DO., Furst, D., Weisman, M., Fletcher, MJ., Chasan-Taber, S., Finger, E., Morales, A., Le CH Trentham, DE. Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter double-blind placebo-controlled trial. *Arthritis Rheum* 41(2); 290-297, 1998.

Chapter 2

Materials and Methods

In this chapter all the methods that are applied in different parts of the thesis are described, with special emphasis on the methods used to study the TCR V gene expression.

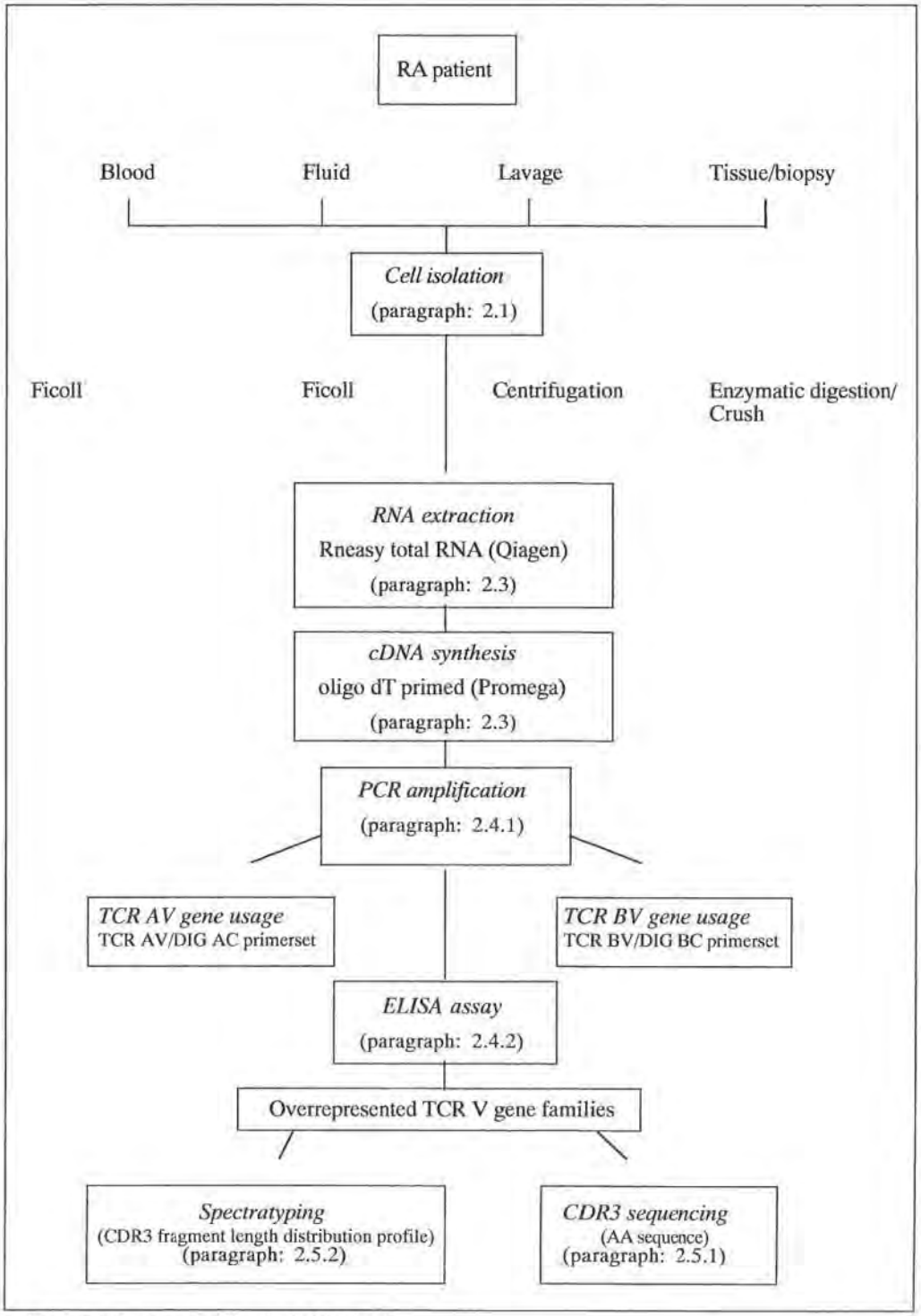

Figure 2.1: Technological flowchart of the thesis.

2.1 Isolation of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by standard Ficoll-Hypaque centrifugation (Pharmacia Biotec, Roosendaal, The Netherlands), washed with RPMI-1640 (Life Technologies, Merelbeke, Belgium) and aliquoted into fractions of $2x10^6$ cells in cold PBS. Ice cold synovial fluid (SF) was diluted in RPMI 1640 (1:15) and centrifuged for 10 minutes at 1800 rpm at 4°C. The cells were resuspended in 5 ml RPMI 1640 and counted. If more than $14x10^6$ cells were obtained, an additional purification of the mononuclear cells was performed using Ficoll-Hypaque as described above. The cells were washed twice with RPMI 1640 and resuspended in PBS in fractions of $2x10^6$ cells. Lavage fluids was transported on ice and the cells were immediately pelleted by centrifugation at 4°C for 15 min at 1500 rpm. The cells were counted and the cell numbers generally varied between 50.000 - $1X10^6$. Fresh synovial tissue specimen were collected in RPMI 1640 at 4°C and manipulated within 3 hours after surgery. The tissue was cut into small fragments and enzymatically digested overnight at RT in RPMI 1640 containing 0.01% (w/v) hyaluronidase, 0.02% (w/v) desoxyribonuclease I (Life Technologies), 0.1% (w/v) collagenase (Sigma, St. Louis, USA), 50 μg/ml gentamycin (Life Technologies) and 250 ng/ml fungizone (Life Technologies) dissolved in RPMI 1640. The single cell suspension was filtered through a sterile coarse wire grid and washed four times in RPMI 1640. These cells were resuspended in cold PBS and aliquoted in fractions of $2x10^6$ cells. Synovial tissue biopsies (5-7 pieces) obtained by fine needle arthroscopy (1) were immediately frozen at -70°C, manually crushed and as such used for RNA extraction.

2.2 Cell culture

Peripheral blood mononuclear cells (PBMC) were cultured in RPMI-1640 culture medium supplemented with 10 % (v/v) heat inactivated fetal calf serum (Life Technologies, Merelbeke, Belgium) at a cell density of $5x10^5$ cells per ml. PBMC were stimulated in a 10 ml culture flask with phytohemagglutinin (PHA) (2 μg/ml) or toxic shock syndrome toxin (TSST-1) (0.1 μg/ml). After 2 days, IL-2 (Boehringer Mannheim, Mannheim, Germany) was added at a final concentration of 1 U/ml. Five to seven days later the cells were pelleted in fractions of $2x10^6$ cells. Cell pellets were washed twice with PBS and immediately frozen at -80°C.
The Jurkat cell line (ATCC TIB 152) was cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum. Jurkat T-cells were also pelleted and frozen in 2 x 10^6 cell fractions. For some experiments, Jurkat T-cells and PHA T-cell blasts were mixed and frozen for PCR. Unstimulated PBMC were pelleted in $2x10^6$, $1x10^6$, $5x10^5$, 10^5 or $5x10^4$ cell fractions.

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from cells pelleted as mentioned above. After homogenization (Qia Shredder, Qiagen, Leusden, The Netherlands), total RNA was extracted using the Rneasy total RNA extraction kit (Qiagen, Leusden, The Netherlands). Next, RNA was reverse transcribed into single stranded cDNA with AMV reverse transcriptase using an oligo dT primer according to the manufacturer's protocol (Promega, Madison, WI). Finally, cDNA was precipitated with 3M sodium acetate in ice cold ethanol and resuspended in 35 µl of water.

To check the integrity of the isolated cDNA, a control PCR amplification was performed with primers specific for the β2-microglobulin gene. One µl of cDNA was PCR amplified in a total volume of 25µl. The PCR conditions were the same as for the TCR BV gene repertoire analysis (2.4.1). When synovial biopsy samples were studied an additional control amplification was performed with primers specific for the constant region of the TCR β-chain gene to check whether a sufficient amount of T-cell specific mRNA was present in the arthroscopic synovial tissue biopsies. PCR conditions were as described in 2.4.

2.4. Semiquantitative TCR V gene analysis using PCR-ELISA

2.4.1 PCR amplification of TCR V genes

PCR amplification was performed with one out of 19 TCR AV gene specific or one out of 20 TCR BV gene specific primers as the forward primer and a digoxigenin (DIG) labeled TCR AC or BC specific primer as the reverse primer (2). 0.7 µl cDNA was added to an amplification mixture composed of: 2.5 µl 10X PCR buffer (Perkin Elmer, Zaventem, Belgium), 0.25 µl 100X dNTP (dNTP mix, Perkin Elmer, New Yersey, USA), 0.175 µl *Taq* polymerase (5 U/µl AmpliTaq DNA polymerase, Perkin Elmer), 0.2 pmol DIG labeled TCR AC or BC primer and 0.2 pmol of a TCR AV or BV gene specific primer in a total volume of 25 µl. PCR was performed in microtiter plates using the GeneAmp system 9600 (Perkin Elmer). The PCR amplification cycle consisted of 20 sec at 94°C for denaturation, 20 sec at 55° C for primer annealing and 40 sec at 72°C for primer extension, repeated for 35 cycles. For low cell numbers ($< 5x10^5$) the sensitivity of the technique was increased by using DIG labeled dNTP's (Boehringer Mannheim) at different concentrations depending on the cell number.

2.4.2 Enzyme Linked ImmunoSorbent Assay

PCR amplicons were quantified using an ELISA based method. Twenty-five µl PCR mixture was hybridized with 3.75 pmol (50 µl) fluorescein isothiocyanate (FITC) labeled TCR AC (5'FITC-GAA CCC TGA CCC TGC CGT GTA CC) or TCR BC (5'FITC-CCG AGG TCG CTG TGT TTG AGC CAT) probes by heating at 95°C for 3 min followed by annealing for 30 min at 55°C in the PCR cycler. Subsequently, 30 µl of the DNA hybrids were transferred to a flat bottom immunoplate (Life Technologies) which was pre-coated with anti-FITC mAB and blocked with 3% (w/v) BSA (Sigma) in PBS. Coating was performed using a 1/500 dilution of anti-FITC mAB (1 mg/ml) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3). The anti-FITC mouse monoclonal antibody (IgG1) was made in our Institute by immunization with FITC-labeled rabbit IgG. The antibody is commercially available from Eurogenetics (Tessenderlo, Belgium). The hybridization was performed in hybridization buffer (0.15 M NaCl, 15 mM sodium citrate, 0.04% (w/v) ficoll, 0.02 % (w/v) polyvinyl-pyrolidone, 0.04 % (w/v) BSA, 1 mM EDTA, 0.014 M trihydroximethyl-aminomethane, pH 7.5) for 2 h at RT. ELISA plates were then washed four times with washing buffer (1.5 mM KH_2PO_4, 4.5 mM Na_2HPO_4, 0.13 M NaCl, 0.5% Tween 20). Captured DNA hybrids were visualized by staining with 100 µl anti-DIG peroxidase conjugate (150 U/ml, Boehringer Mannheim) which was diluted 1/5000 in conjugate buffer (1.5 mM KH_2PO_4, 4.5 mM Na_2HPO_4, 0.13 M NaCl, 0.05% Tween 20). After four washings, substrate solution containing color A (0.1 M Na_2HPO_4, 0.05 M citric acid, 0.002 %(w/v) gentamycine (Life Technologies), 0.05 % (v/v) H_2O_2, pH 5.0) and color B (0.05 M citric acid, 0.01 M tetramethylbenzidine, 4 % (v/v) dimethylsulfoxide, pH 2.4) mixed at 1 : 1 was added. DIG labeled amplicons were incubated for 30 min at 37° C with the substrate mixture and the reaction was terminated with 100 µl 1M H_2SO_4. The A_{450} was measured using an automated ELISA reader (Titertek plus MS212, Biomedicals NV, Brussels, Belgium).

The expression levels of each V gene were calculated as follows. First the mean A_{450} values of triplicate wells in ELISA were calculated. For each V gene specific PCR amplification one control amplification with water was performed. The mean A_{450} values of these control wells (in triplicate) were subtracted from the mean A_{450} values of the cDNA. Next, the expression level of each V gene was calculated as the percentage of total BV or AV gene expression with the formula $\% BV_x = (A_{450}(BV_x) \times 100) / \Sigma A_{450}(BV_n)$

To enable semiquantitative analysis of the TCR AV and BV PCR amplicons, it was important to perform PCR amplification with comparable amounts of starting cDNA copies. The amount of TCR specific cDNA was estimated by PCR amplification of the TCR C region. The PCR products were serially diluted from 1/25 to 1/400 and quantified by ELISA as described earlier. Equal amounts of cDNA from the PB and SF were then used in the TCR V gene repertoire analysis with the same PCR conditions.

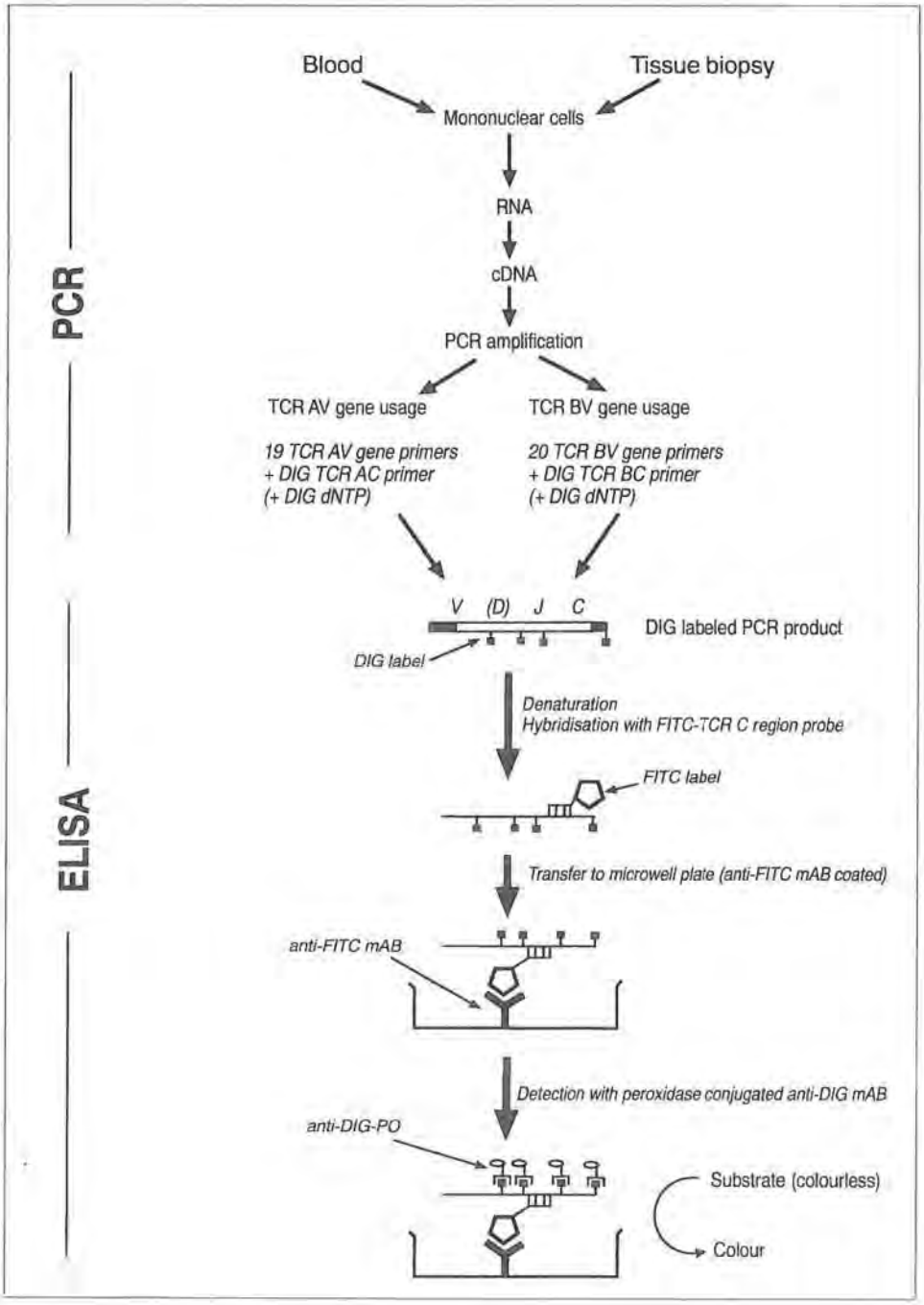

Figure 2.2: Schematic overview of the PCR-ELISA

2.5. T-cell receptor clonal analysis

2.5.1 Bacterial cloning and CDR3 region sequencing

CDR3 region sequences were determined by subcloning the TCR BV gene amplification products into a TA cloning vector, following the manufacturer's instructions (Invitrogen, Leek, The Netherlands). cDNA was amplified using the TCR BV region specific primer and a TCR C region specific primer using the conditions as described in 2.4.1. PCR amplicons were ligated in the pCR2.1 cloning vector and transformed by heat shock in *E. Coli* cells. Subsequently, plasmid DNA was prepared of 10 to 15 recombinant plasmids and inserts were amplified by PCR using BV and BC region specific primers as described before. The amplicons were sequenced using the dye terminator cycle sequencing reaction mix (Perkin Elmer) with a TCR C region specific primer. The PCR conditions were: 30 sec 96°C followed by 10 sec 96°C, 5 sec 50°C and 4 min 60°C for 25 cycles. Fluorescently labeled PCR amplicons were purified on a sephadex-G50 M column, vacuum dried and resuspended in 5 µl 1:50 25mM EDTA / formamide. The DNA sequences were evaluated on a 6% polyacrylamide gel using the 373 sequencing software.

2.5.2. CDR3 fragment size analysis

PCR amplicons to be analyzed for CDR3 fragment sizes were reamplified for 25 cycles in a nested PCR using the V gene family specific primer as forward primer and a FAM labeled TCR BC region specific primer (5'FAM-GT GGC CAG GCA CAC CAG TGT GGC C) (Perkin Elmer) as reverse primer. Fluorescently labeled nested PCR products were diluted 1/30 in loading buffer (24:1 formamide / 25 mM EDTA) and separated on a 6% polyacrylamide, 8 M urea gel in 90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3 on the 373 ABI DNA sequencer (Perkin Elmer). Fragment sizes of TCR V gene products were calculated using the 672 Genescan software (Perkin Elmer). The GeneScan-1000 ROX labeled internal standard (Perkin Elmer) was used for fragment length analysis.

2.6 Analysis of cytokine production

2.6.1 Quantification of cytokine secretion by ELISA

To analyze the cytokine production of T-cell clones, T-cells were stimulated with relevant antigens. Cell supernatants were harvested at 72 hours of culture, and the production of

TNF-α, IFN-γ, IL-4, IL-10 and IL-2 was determined in the supernatants using commercially available ELISA kits according to manufacturer's instructions (Biosource, Camarillo, CA).

2.6.2 Quantification of cytokine mRNA level

Using the real-time PCR LightCycler following cytokine messengers were quantified: TNF-α, IL-4, IL-10 and IFN-γ. The PCR amplification of the β_2 microglobulin gene was used for intersample normalization. Briefly, 2 µl cDNA was PCR amplified in glass capillaries (final volume: 10µl) using following reagents: 1µl 5 pmol/µl cytokine specific forward and reverse primer (sequences in table 2.1), 1 µl PCR LightCycler master mix (*Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR® Green I dye and 10mM $MgCl_2$ (Roche, Boehringer mannheim, Brussels, Belgium), 2.5 µl $MgCl_2$ (final concentration in 10 µl respectively: 5mM for β_2 microglobulin, IFN-γ PCR and 4 mM for TNF-α, IL-4 and IL-10 amplification) and an additional volume of 2.5 µl water. The following PCR amplification profile was used: predenaturation for 1 min. at 95°C followed by 50 cycles of 5 sec at 95°C, 5 sec at 60°C for primer annealing and 11 sec at 72°C for elongation. Serial dilutions of the pQA (for β_2-microglobulin, TNF-α, IL-4, IFN-γ) and pQB (for IL-10) vector cDNA (SANOFI Elf Bio Researches, Labege, France (3) were used as an external quantification standard (4). The PCR standard RNA's (st-RNA) were obtained from two nonhomologous, cloned synthetic constructs, pQA and pQB, comprising the cytokine primer pairs (5). The pQA and pQB plasmids were linearized with EcoRI, and 2 µg was transcribed into RNA using the T7 polymerase transcription kit (Boehringer Mannheim) followed by an RNA-free DNase treatment to remove the DNA template. The obtained standard RNA's were extracted with water-saturated phenol-chloroform (24:1; vol/vol) and precipitated with ethanol. Poly(A) RNA was purified using the mRNA separator kit (Clontech, Palo Alto, CA) and quantified by absorbance at 260 nm. The RNA was used as an external standard in the LightCycler quantification assay.

Quantification of PCR amplicons was performed by continuously monitoring of the fluorescence intensity of the double strand DNA binding dye SYBR® GREEN (Boehringer Mannheim). The data were analyzed using the LightCycler quantification software (6). Since melting characteristics of specific PCR amplicons and PCR byproducts such as primer-dimers are different, incorporated fluorescence of byproducts and specific PCR amplicons can be discriminated by measuring fluorescence at a temperature in-between the meltpoint of the specific and aspecific PCR amplicons (78°C for β_2 microglobulin, 80°C for IFN-γ, 83°C for TNF-α, 75°C for IL-4 and 84°C for IL-10). In addition, the real-time analysis allows to choose a fluorescence level where all curves of individual amplifications are in the log-linear portion,

allowing for accurate quantification. As shown in Fig.2.3, a line (the so called background noise band) is drawn at a specific fluorescence level, and this line crosses the log-linear portion of each amplification curve. The crossing points of the amplification curves of the cytokine standards are used to draw a standard curve, and this curve is used to determine the cytokine levels of the tested samples (Fig. 2.3). In this way, all samples are analyzed in their log-linear phase allowing for accurate measurement of their cytokine content.

To control for possible differences in cDNA content of individual samples, individual cytokine contents (pg/μl) were normalized using the β_2 microglobulin levels (pg/μl) allowing comparison of cytokine levels between samples. Therefore, arbitrarily units were calculated as: (cytokine content of PBMC (pg/μl cDNA) / β_2 microglobulin levels (pg/μl cDNA)).

Table 2.1: Nucleotide sequences of the PCR primers.

Cytokine	Nucleotide sequence (5'-3')	
	Forward primer	Reverse primer
IFN-γ	GCA GAG CCG GGT TGT CTC CT	ATG CTC TTC GAC CTC GAA AC
TNF-α	ACA AGC CTG TAG CCC ATG TT	AAA GTA GAC CTG CCC AGA CT
IL-4	TGC CTC CAA GAA CAC AAC TG	AAC GTA CTC TGG TTG GCT TC
IL-10	GAG TACCAGG GGC ATG ATA TC	AAA TTT GGT TCT AGG CCG GG
β_2 M	CCA GCA GAG AAT GGA AAG TC	GAT GCT GCT TAC ATG TCT CG

β_2 M: β_2 Microglobulin

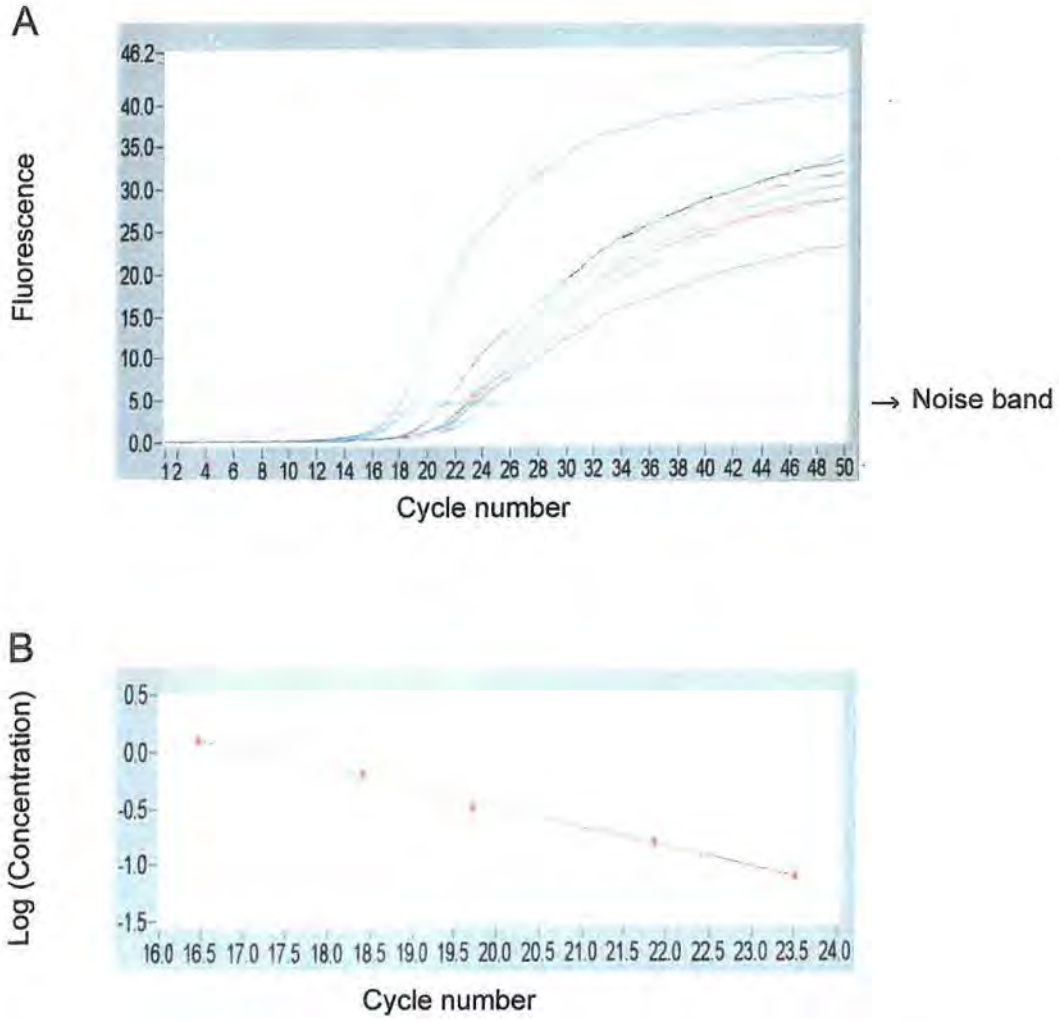

Figure 2.3: Cytokine mRNA quantification by LightCycler (Roche) technology.
Panel A: cDNA is PCR amplified in the presence of the DNA binding dye SYBR Green. Real-time fluorescence is measured at each PCR cycle and plotted versus the respective cycle number. The background noise level (horizontal green line) crosses the amplification curves at the log-linear portion of each PCR curve.
Panel B: Standard curve for quantification. Serial dilutions of known cytokine quantification standards (pQA and pQB vectors) are PCR amplified and the SYBR Green fluorescence is measured continuously. The crossing points of the amplification curves with the background noise level (see panel A) are plotted versus the logarithm of the concentration of the diluted standards. The linear standard curve is used for quantification of the tested samples.

3. References

1. Elewaut, D., De Keyser, F., Van Den Bosch, F., et al. Enrichment of T-cells carrying ß7 integrins in inflamed synoviam tissue from patients with early spondylarthropathy, compared to Rheumatoid Arthritis. *J.Rheumatol.* 25:1932-1937, 1998.

2. Vandevyver, C., Mertens, N., van den Elsen, P., Medaer, R., Raus, J. and Zhang, J. Clonal expansion of myelin basic protein-reactive T-cells in patients with multiple sclerosis: restricted T-cell receptor V-gene rearrangements and CDR3 sequence. *Eur.J.Immunol.* 25:958-968, 1995.

3. Legoux, P., Minty, C., Delpech, B., Minty, AJ., Shire, D. Simultaneous quantification of cytokine mRNAs in interleukin-1b stimulated U373 human astrocytoma cells by a polymerisation chain reaction involving co-amplification with an internal multi-specific control. *Eur. Cytokine Netw.* 3: 553-563, 1992.

4. Vandevyver, C., Motmans, K., Stinissen, P., Zhang, J. and Raus, J. Cytokine mRNA profile of Myelin Basic Protein reactive T-cell clones in patients with Multiple Sclerosis. *Autoimm.* 28:77-89, 1998.

5. Vandevyver, C., Motmans, K., Raus, J., Quantification of cytokine mRNA expression by RT-PCR aand electrochemiluminescence. *Genome Research* 5: 195-201, 1995.

6. Rasmussen, R., Morrison, T., Herrmann, M., Wittwer, C. Quantitative PCR by continuous fluorescence monitoring of a double strand DNA specific binding dye. *Biochemica* 2:4-11, 1998.

Chapter 3

Identification of Overrepresented T-cell Receptor Genes in Blood and Tissue Biopsies by PCR-ELISA

Based on:

Identification of overrepresented T-cell receptor genes in blood and tissue biopsies by PCR-ELISA.

Ann VanderBorght, Annegret Van der Aa, Piet Geusens, Caroline Vandevyver, Jef Raus and Piet Stinissen.

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Abstract

The analysis of T-cell receptor variable (TCR V) gene repertoires in blood or tissues may provide important information when studying immunopathological mechanisms. The overexpression of a TCR gene may indicate the expansion of the corresponding T-cell subset. In autoimmune diseases, clonally expanded T-cell subsets in the affected organs may represent pathogenic lymphocytes. We describe a simple, rapid and sensitive method to determine the TCR AV and BV gene repertoire using a PCR-ELISA method. RNA is extracted from lymphocytes, transcribed to cDNA, which is then used as a template for PCR with 19 different TCR AV gene and 20 BV gene specific primers as the forward primer, and a digoxigenin (DIG) labeled AC/BC primer as the reverse primer. The DIG labeled PCR amplicons are hybridized with a fluorescein isothiocyanate (FITC) labeled TCR C region specific probe. Finally, the amplicons are quantified by ELISA using anti-FITC coated microtiter plates, and an anti-DIG conjugated peroxidase. Although PCR-ELISA cannot accurately quantify the expression level of a given TCR gene, overrepresented TCR V genes are easily identified by comparing the relative expression levels of each individual V gene in the total V gene repertoire. We demonstrate that this technique can be used to determine TCR profiles in blood and tissue samples containing as few as 50,000 T-cells. In combination with CDR3 fragment size analysis, this method is an efficient tool to identify clonally expanded T-cell subsets in the synovial biopsies of rheumatoid arthritis patients.

Introduction

T-cells recognize an antigenic peptide presented in the context of MHC class I or II molecules on the surface of an antigen presenting cell by their specific T-cell receptor (TCR) (1). The TCR is a dimeric glycoprotein consisting of an alpha and beta chain. Each chain is the product of a rearrangement process in the thymus where TCR variable (V), diversity (D) and junctional (J) region segments are coupled to a constant (C) gene domain (2). Due to the immense diversity created by this random combination of gene segments and other processes such as random nucleotide insertion, the V-D-J region is a hypervariable sequence which is unique for a given T-cell clone. This hypervariable region or CDR3 region can therefore be used as a selective marker to identify a T-cell clone in a T-cell population (3, 4).

In the blood of healthy subjects the circulating T lymphocytes express a random combination of T-cell receptor variable region of the TCR alpha chain (TCR AV) and T-cell receptor variable region of the TCR beta chain (TCR BV) genes. However, in some conditions the TCR repertoire can be highly skewed towards the expression of a single or limited number of TCR V genes. For instance, tumor infiltrating T-cells in breast tumors express a restricted BV gene repertoire, while malignant T-cell expansions in a T-cell lymphoma will lead to the overrepresentation of the corresponding TCR V gene (5,6). Furthermore, TCR V gene repertoire analysis can be an efficient tool to identify clonally expanded T-cell subsets in the affected organs of patients suffering from an autoimmune disease such as multiple sclerosis (MS) (7) or rheumatoid arthritis (RA) (8). Because of their accumulation in the affected organs, it can be argued that these T-cells probably play a role in the disease process.

Several methods have been reported to determine the TCR V gene repertoire of T-cell populations. TCR V gene expression can be studied by flow-cytometry using fluorescently labeled monoclonal antibodies recognizing specific TCR V genes expressed on the T-cell surface (9,10). However, this method requires relatively large amounts of cells and is hampered by the lack of antibodies specific for some V gene segments. Because of these limitations PCR based methods became very popular in the study of TCR V gene repertoires. Primers were designed to amplify all TCR AV and BV gene segments (2, 11). RNA isolated from T-cells can be amplified using reverse transcriptase PCR (RT-PCR). After measuring the relative amounts of each PCR product it is possible to determine the (semi)quantitative expression levels of each V gene segment in the T-cell population studied. The quantification of the amplified TCR transcripts can be performed by Southern blotting using ^{32}P-labeled probes specific for the TCR amplicons (12, 13). Alternative methods were described to quantify the amplified V gene segments using fluorescent primers (14, 15, 16) or double stranded DNA specific antibodies (17). Unfortunately, these methods require sophisticated and expensive instruments such as an automated DNA sequence analyzer or TaqMan equipment (Perkin Elmer). However, PCR

products can also be identified by relatively simple ELISA based methods as shown by Kohsaka et al. (18) who used anchored PCR to amplify all TCR BV genes in a single PCR, and quantified the expression levels of individual V gene segments by specific DNA probes and ELISA.

We have optimized an ELISA technique to quantify the expression levels of individually amplified TCR BV and AV genes in a total T-cell population. We have validated this technique by testing whether TCR BV2 expressing cells were easily identified among T-cells stimulated with the superantigen toxic shock syndrome toxin-1 (TSST-1). In addition, we tested whether Jurkat cells expressing the TCR BV8 gene were identified among mixtures of Jurkat cells and PHA stimulated T-cells. The sensitivity of PCR-ELISA was significantly increased by using DIG labeled dNTP's in the reaction mixture. In our hands, the PCR-ELISA is a powerful and sensitive technique for semiquantitative analysis of the TCR V gene repertoire without the need for radioactive labeled probes. Finally, we demonstrate that PCR-ELISA can be used to determine the TCR V gene repertoire of blood T-cells and of lymphocytes isolated from the synovial fluid or synovial tissue of rheumatoid arthritis (RA) patients. Autoimmune processes involving T-cells may play an important role in the pathogenesis of this disease (19, 20). The identification of overrepresented TCR V gene segments in the synovial samples can be helpful when studying autoreactive T-cells in these patients. The expansion of a TCR BV or AV gene segment, however, does not necessarily imply the clonal expansion of one particular T-cell clone. Indeed, when T-cells are stimulated by superantigens, T-cells with a particular BV gene segment will proliferate, but these T-cells will express heterogeneous CDR3 sequences of polyclonal origin. To study the clonality of an overexpressed TCR BV gene family, the CDR3 length distribution patterns can be evaluated with the immunoscope technique as described by Pannetier and coworkers (21,22). Here we demonstrate that PCR-ELISA in combination with immunoscope is an efficient and rapid approach for identifying overexpressed TCR V genes and studying their poly-, oligo- or monoclonal origin.

Materials and methods

Isolation and culture of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll-Hypaque (Sigma, St-Louis, MO) density gradient centrifugation as described in 2.1 Freshly isolated PBMC were stimulated with PHA (2μg/ml) or TSST-1 (0.1μg/ml) and Jurkat cells were cultured as described in 2.2.

Mononuclear cells were isolated from synovial fluid and synovial tissue as described in 2.1

TCR V gene analysis and CDR3 region spectratyping

RNA extraction (2.3), cDNA synthesis (2.3), semiquantitative TCR V gene analysis (2.4) and CDR3 fragment length determination (2.5.2) were performed as described earlier.

Results

Amplification kinetics of TCR V gene specific PCR primers

Reliable quantification of PCR amplicons can be performed in the exponential phase of the PCR amplification reaction only. When the reaction enters the plateau phase, the amount of PCR product is no longer proportional to the starting amount of the target molecules (23,24). To study the optimal number of PCR cycles for semiquantitative PCR-ELISA we amplified equal amounts of cDNA from $2x10^6$ PHA stimulated T lymphocytes with primers specific for TCR BV9, BV12 and BV13.2 as forward primer in combination with the DIG labeled TCR BC region specific primer as the reverse primer. Samples were taken every two cycles between amplification cycle 20 and 50 and the PCR amplicons were quantified in triplicate by ELISA. As shown in figure 3.1, the amplification reactions all level off after approximately 40 cycles. Therefore, when starting from this amount of T-cells ($2x10^6$), we can assume that it is possible to perform a semiquantitative analysis at 35 to 38 cycles of PCR.

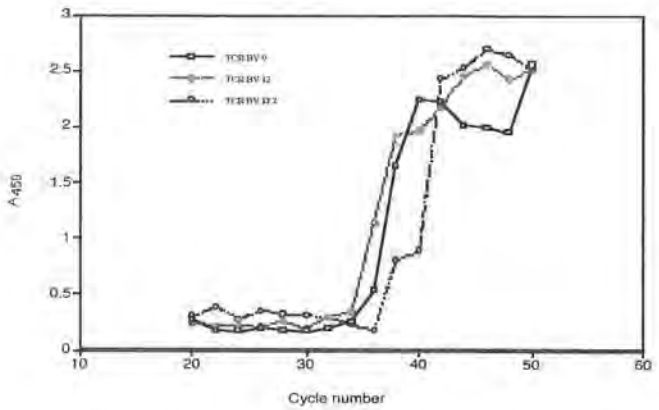


Figure 3.1: Kinetics of PCR amplification reactions. cDNA was amplified with three randomly chosen TCR BV gene specific primers. Every two cycles between cycle 20 and 50 one PCR amplification reaction was terminated, and quantified using the ELISA system. The A_{450} is expressed as a function of the cycle number.

Inter- and intra-assay variation

To evaluate the inter-assay variation of the PCR-ELISA system, the PCR amplicons of one complete TCR BV gene analysis were quantified in three independent ELISA experiments. For this experiment RNA was isolated from lymphocytes isolated from the synovial fluid of an RA patient. Each ELISA experiment was performed with independently coated and blocked ELISA plates. As shown in figure 3.2, the TCR V gene profiles obtained in the three independent experiments were similar. The relative differences in % TCR BV values never exceeded more than 2%.

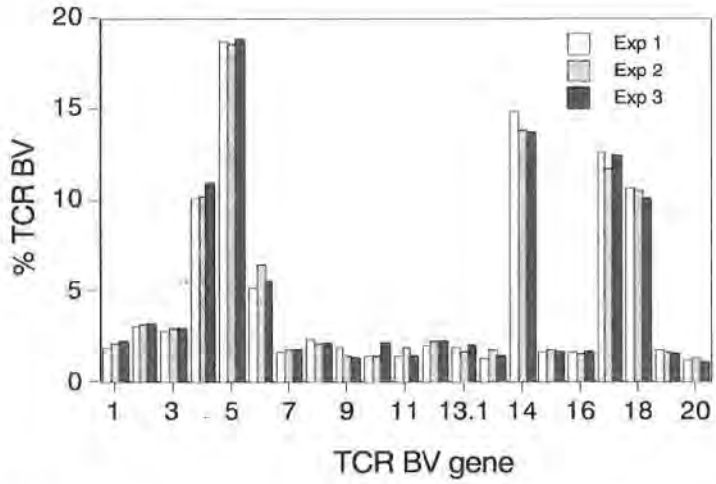


Figure 3.2: Inter assay variation of the PCR-ELISA system. A PCR-ELISA TCR repertoire was performed using RNA isolated from the synovial fluid cells of an RA patient. Three independent ELISA measurements of the same PCR reaction were performed to evaluate the inter assay variation.

On the other hand, the intra-assay variation was evaluated by comparing the TCR V gene usage of two independent PCR amplifications, each followed by an ELISA in duplicate. For this experiment lymphocytes were isolated from the synovial fluid of an RA patient. As illustrated in Figure 3.3 it is clear that the variations between these experiments were mostly introduced during the PCR experiments and not during the ELISA analysis. All experiments indicated preferential expression of TCR BV2, BV7 and BV13.1.

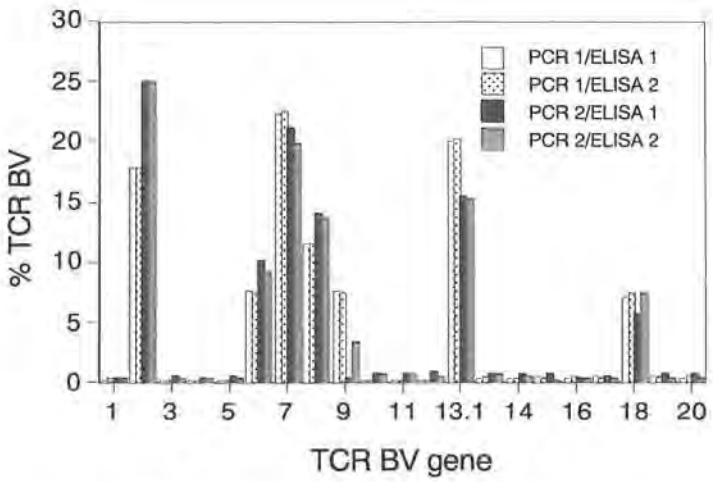


Figure 3.3: Intra assay variation of the PCR-ELISA system. Two independent PCR-ELISA analysis were performed using RNA isolated from the synovial fluid cells of an RA patient. The ELISA was carried out in duplicate on the two independently performed PCR amplification reactions. The r^2 value for the two independent PCR amplifications is 0.95.

TCR BV gene repertoire analysis of TSST-1 stimulated T-cells

Superantigens stimulate T-cells in a TCR BV gene specific way. For instance, TSST-1 specifically stimulates TCR Vβ2 expressing T-cells (25). We studied whether these expanded $V\beta2^+$ T-cells can be easily detected by PCR-ELISA. PBMC of a healthy subject were stimulated with TSST-1 (0.1 μg/ml) or PHA (2 μg/ml) as a nonspecific control, and activated T-cells were expanded during a 7 days culture period. A fraction of the T-cells was restimulated with the respective stimuli for an additional culture period of 7 days. The TCR BV gene repertoire was evaluated before and after restimulation with the antigen. As shown in figure 3.5, the TCR BV2 segment was overexpressed in the TSST-1 stimulated cells, but not in the PHA stimulated T-cell repertoire. The contribution of the TCR BV2 segment in the overall V gene repertoire of these cells increased from 19% to 35% after an additional stimulation with the superantigen. Note that the TSST-1 stimulated cells also overexpressed the TCR BV4, and to a lesser extent the TCR BV19 gene segment.

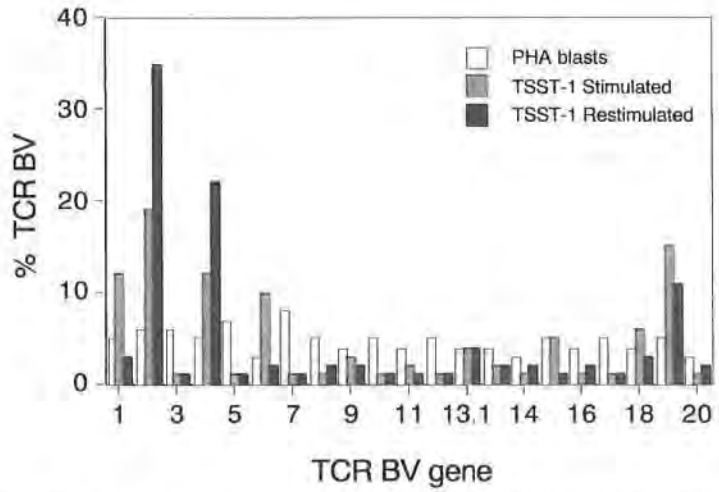


Figure 3.4: TCR V gene repertoire of PHA blasts and TSST-1 stimulated and restimulated PBMC. PBMC from an healthy subject were stimulated with PHA and TSST-1 in one or two stimulation cycles, and the resulting lymphocytes were subjected to PCR-ELISA TCR repertoire analysis. The expression of each individual TCR BV gene is expressed as a fraction of the total A_{450}.

As expected, the Vβ2 overexpressing T-cell population could also be identified by flowcytometry with a Vβ2 specific monoclonal antibody (E22E7.2 dfrom Immunotech, Marseille, France) (Table 3.1). However, with flowcytometry, the percentage of cells stained with the anti-Vβ2 antibody was 71% after one and 83% after two stimulation cycles, whereas PCR-ELISA analysis revealed only 19% and 35% of the T-cells expressing the TCR BV2 gene.

Table 3.1 : TCR BV 2 expression in the peripheral blood of a healthy subject stimulated with PHA or TSST-1 as studied by PCR-ELISA and flowcytometry.

Stimulus	**PCR-ELISA** *BV2 mRNA expression as percentage of total A_{450}*	**Flowcytometry** *BV2 surface expression as percentage of total T-cells*
PHA	7	7
TSST-1 (1 cycle)	19	71
TSST-1 (2 cycles)	35	83

PBMC of a healthy subject were stimulated with PHA or TSST-1. A fraction of TSST-1 stimulated cells were restimulated after 1 week for an additional period of 7 days. Cell cultures were tested by PCR-ELISA for the expression of Vβ2 specific mRNA, and by flowcytometry using a Vβ2 specific monoclonal antibody (Immunotech, France).

Analysis of the TCR BV gene repertoire of Jurkat cells mixed with PHA stimulated T-cell blasts

Next, we determined the TCR BV gene repertoire of an artificial cell mixture consisting of a clonally expanded T-cell population (Jurkat cells) on a polyclonal background (PHA blasts). To this end, PHA stimulated T-cell blasts were mixed with a T-cell clone expressing a known TCR Vβ protein. Jurkat cells are immortalized T-cells which express Vβ8 (26). The PHA stimulated T-cell blasts were mixed with Jurkat cells at 3:7 and 1:1 and the TCR V gene profile of these cell mixtures ($2x10^6$) was compared with both the Jurkat cells and the PHA blasts alone. Figure 3.5 illustrates that the TCR BV8 segment was easily identified as an overrepresented V gene among the Jurkat cells and the mixtures of the Jurkat cells with the PHA stimulated T-cell blasts. PCR-ELISA revealed that the TCR BV8 mRNA represented 53% of the total TCR mRNA pool in the Jurkat cells, 30% in the 3:7 mixture of Jurkat and PHA blasts, and 28% in the 1:1 mixture of Jurkat cells and PHA blasts.

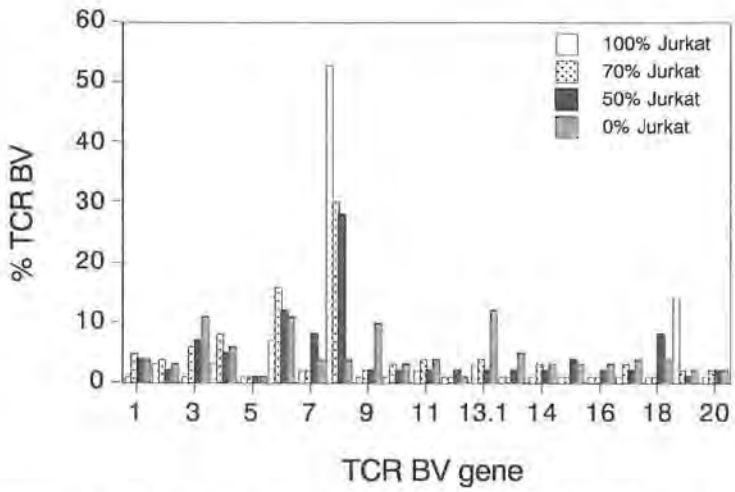


Figure 3.5: TCR BV gene repertoire of Jurkat cells mixed at different proportions with PHA blasts. Jurkat cells and PHA stimulated T-cells from a healthy subjects were mixed as: 100% Jurkat cells, 70% Jurkat cells, 50% Jurkat cells, and 0% Jurkat cells. Two million cells from each sample were subjected to PCR-ELISA TCR repertoire analysis. The data are expressed as the fraction of total A_{450}.

Analysis of the TCR BV gene repertoire of samples containing less than $1x10^6$ cells

In some samples such as tissue biopsies only a few T-cells are present. This could be problematic since at least $1x10^6$ cells are necessary to perform a typical TCR BV gene repertoire analysis using the PCR-ELISA. To overcome this problem we tested whether the sensitivity of

the PCR-ELISA could be increased by using DIG labeled dNTP's in the amplification reaction. We mixed Jurkat cells with PHA stimulated T-cell blasts at 1:1 and used $5x10^5$, 10^5 and $5x10^4$ cells for the PCR-ELISA. We used either 25%, 50%, 75% or 100% DIG labeled dNTP's in the amplification reaction, and tested whether it was still possible to identify the expanded BV8 subset of the Jurkat cells. With $1x10^6$ cells in a typical PCR-ELISA (without DIG dNTP's) we observed a TCR BV8 expression level of 28% (data not shown). Next, we tested lower cell amounts of the same mixture ($5x10^5$ cells, $1x10^5$ cells and $5x10^4$ cells) using various amounts of dNTP's as illustrated in figure 3.6. The data show that with $5x10^5$ cells, the TCR BV8 segment represented 27% of the total V genes when using 50% DIG labeled dNTP. With the lowest number of cells tested ($5x10^4$ cells) the TCR BV8 gene was still identified (24-28 %) when using 75% and 100% of the DIG labeling mix. With a lower percentage of DIG labeling mix, the signal to noise ratio becomes too low, leading to unreliable data (Figure 3.6).

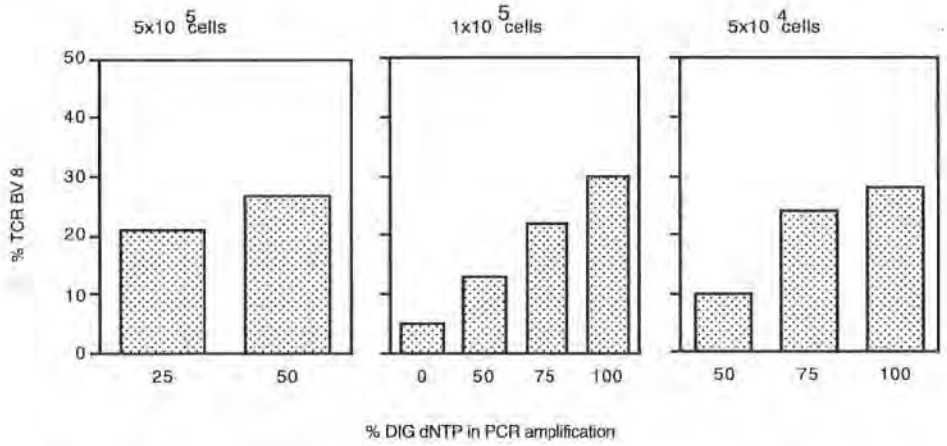


Figure 3.6: TCR BV gene usage of 1:1 mixtures of Jurkat and PHA blasts with low T-cell numbers. Jurkat cells and PHA stimulated T-cell blasts were mixed at 1:1 mixtures and PCR-ELISA TCR repertoire analysis was performed on $5x10^5$, 10^5 and $5x10^4$ cells. To increase the sensitivity of the PCR-ELISA the indicated amounts of DIG labeled dNTP were added during the amplification reaction. The percentage of expression of TCR BV8 corresponding to the Jurkat cells is shown for the various experiments.

TCR BV gene repertoire analysis of synovial fluid and synovial tissue cells of a patient with Rheumatoid Arthritis

To validate the PCR-ELISA system as a method for studying TCR BV gene repertoires we evaluated the TCR repertoire of lymphocytes isolated from blood, synovial fluid and synovial tissue of an RA patient. While synovial fluid samples may contain $2x10^5$ to $1x10^6$ mononuclear

cells (5-10 ml), synovial membrane biopsies contain a small number of cells only (typically 50 - 100,000 cells). As shown above, semiquantitative PCR-ELISA TCR repertoire analysis of samples containing low cell numbers can be performed using DIG labeled dNTP's. Figure 3.7 shows the TCR repertoire of PBMC, synovial fluid cells and synovial tissue. A cut-off value of 15% was arbitrarily chosen to define overrepresented TCR V genes. This value was chosen based upon a series of TCR repertoire analysis experiments on the PBMC of healthy subjects. In these control subjects no TCR expression above 15% of the total repertoire was observed (27). Interestingly, TCR BV4 appeared to be overrepresented in both the synovial fluid and synovial tissue of this patient, while TCR BV2 and BV18 was overrepresented in the synovial fluid only. In the blood TCR BV2, BV4, BV6 and BV18 represented more than 10% of the total repertoire, but none of these TCR's represented more than 15% of the total repertoire.

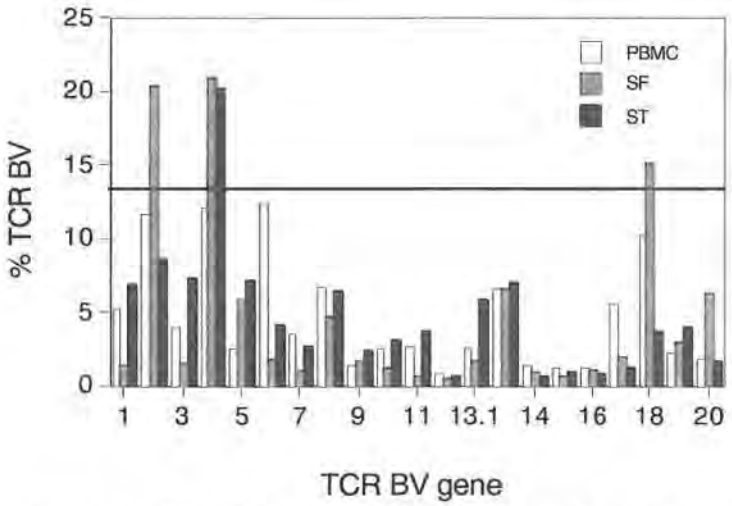


Figure 3.7: TCR BV gene usage in peripheral blood, synovial fluid and synovial tissue of an RA patient. Lymphocytes were isolated from the peripheral blood, the synovial fluid and synovial tissue of the same joint of an RA patient and used for TCR repertoire analysis by PCR-ELISA. An arbitrarily chosen cut-off of 15% was used to define overrepresented TCR V genes.

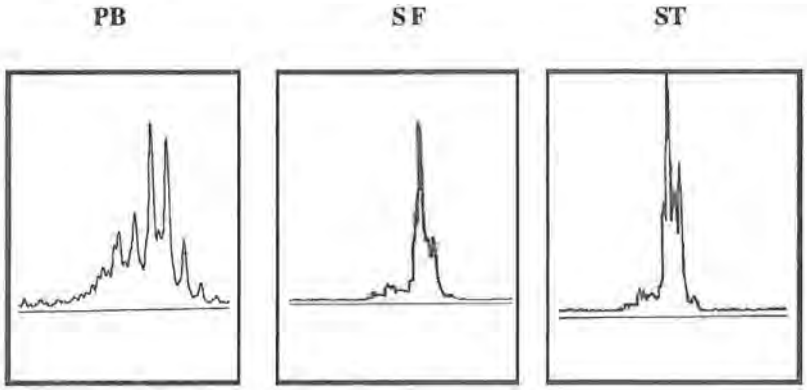
Immunoscope analysis of overrepresented TCR V gene segments provides information on the clonality of the corresponding T-cell population

The observation of overrepresented (expanded) TCR V gene elements in a pathological specimen does not necessarily correlate with a clonal expansion of this specific T-cell clone. The V gene specific T-cell expansion may indeed be caused by a poly-, oligo- or monoclonal T-cell activation and expansion process. The clonal origin of the T-cell family expressing the overrepresented BV gene can be studied by cloning the corresponding BV PCR products in a

plasmid vector, and by performing DNA sequence analysis of a representative number of recombinant plasmids after transformation into bacteria. However this is a labor intensive and time consuming strategy. An alternative method is provided by studying the fragment length profile of the hypervariable CDR3 region of these TCR V gene families. TCR BV gene families of polyclonal origin will exhibit a CDR3 fragment length profile with a Gaussian distribution, whereas monoclonal BV gene families have a restricted CDR3 fragment length distribution. The immunoscope technology as described by Pannetier et al (22) is an elegant and fast method to determine the CDR3 length profile of TCR transcripts after PCR amplification.

As an illustrative example, we have reamplified the PCR amplicons of the TCR BV4 gene family overrepresented in the synovial fluid and synovial tissue of the RA patient studied (Figure 3.7) with the BV4 gene specific primer and a fluorescently labeled TCR BC region specific primer. Fragment length distribution patterns of these amplicons are represented in Fig. 3.8. As illustrated in this figure, the TCR BV4 amplicons from both synovial fluid and synovial tissue consist of a single sized CDR3 fragment (Figure 3.8A) while the CDR3 length profile of the BV4 family in the blood (Figure 3.8A) represented a polyclonal pattern. However, the pattern differed slightly from a typical Gaussian profile, suggesting that one or more clones were also expanded in the blood. The CDR3 peaks in the synovial fluid and synovial tissue were of comparable length, suggesting that the same clone(s) is (are) present at both sites in the joint. Thus by CDR3 size analysis we could demonstrate that the TCR BV4 family, which was overrepresented in both the synovial fluid and the synovial tissue had a monoclonal origin at both sites in the affected joints of this patient. We also examined the CDR3 fragment size pattern of the TCR BV7 family, a TCR BV gene which is not overrepresented in the blood of this patient (see Fig. 3.7). As shown in Fig. 3.8 this TCR BV7 family displayed a polyclonal distribution.

A. TCR BV 4



B. TCR BV 7

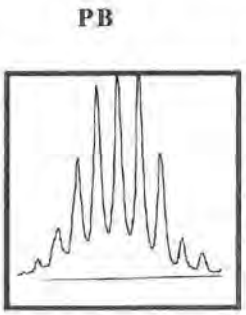


Figure 3.8: CDR3 fragment size distribution patterns of the TCR BV4 gene family in peripheral blood, synovial fluid and synovial tissue and the TCR BV7 gene family in the peripheral blood of an RA patient (Fig. 3.8). Amplicons of the overrepresented TCR BV4 gene family of an RA patient (Fig. 3.8) were PCR amplified for 25 cycles with a fluorescently labeled TCR C region specific primer, and fragment sizes of PCR amplicons from PB, SF and ST were determined using the 672 Genescan software (Panel A). Panel B represents the CDR3 fragment size distribution pattern of the TCR BV7 gene family, which was not overrepresented in the peripheral blood of that patient.

Discussion

Autoimmune T-cells are thought to play a crucial role in the pathogenesis of MS and RA. Although several candidate autoantigens have been intensively studied, the self antigens which are recognized by the pathogenic T-cells in these diseases are still not unequivocally identified. Since pathogenic T-cells are considered to accumulate in the affected organs in the early phase of the disease process, it would be of interest to identify the pathogenic T-cells by analyzing the TCR repertoires at the disease site. For instance, by comparing the TCR repertoires in the joints and the blood compartment in RA patients, it would be feasible to identify TCR genes which are overrepresented in the joints. Several groups have used this approach to search for T-cells which may be involved in the pathogenesis of RA (13,28). T-cells which accumulate in the joints may become the target of immunotherapeutic interventions.

Several technologies have been used to identify expanded T-cell subsets in the blood or tissues which are affected by an autoimmune disease. Most of these techniques utilize quantitative or semiquantitative PCR technology with family specific V gene primers. With these methods the amount of TCR transcript expressing a given V gene is determined instead of the numbers of T-cells bearing a particular TCR V gene element. When interpreting the PCR based data, it is important to consider that 'out-of-frame' transcripts are also amplified by PCR; and in addition, the ultimate TCR profile may also depend on post-transcriptional events. Another disadvantage of these techniques is their inability to accurately quantify the TCR mRNA molecules. Indeed, several parameters such as primer, nucleotide and template concentrations, reaction volume, and temperature differences between tubes in the cycler all influence the efficiency of the amplification reaction. Slight variations in these conditions may therefore lead to different results. Some protocols using competitive PCR with an internal standard were shown to be able to accurately quantify the levels of starting molecules, but these methods are very labor intensive and not feasible when many amplifications have to be performed in a single experiment, such as for TCR repertoire analysis (29). Therefore, TCR repertoires are generally determined by a semiquantitative approach. In these experiments PCR amplifications are performed using a panel of V region specific primers, and PCR products are subsequently quantified by, for example, Southern blot analysis using a radioactive labeled C region specific probe. Recently, several authors have used non-radioactive methods to quantify the PCR products, but some of these methods require expensive equipment such as TaqMan or a fluorescent automated DNA analyzer (14-16).

Hére we describe a simple, fast and sensitive method to quantify the PCR products with an ELISA based method. PCR amplification and subsequent hybridization with the DIG labeled TCR C region specific probe is performed in a microtiter plate. As a consequence, four different samples can be analyzed simultaneously in a single PCR plate. Fluorescently labeled PCR amplicons are then analyzed by simple ELISA using a typical ELISA reader. The total TCR AV and BV gene repertoire of 8 samples can easily be performed in one day. We have shown that

the sensitivity of the PCR-ELISA is increased significantly by using DIG labeled dNTP in the amplification reaction. Using the DIG labeled dNTP, we have been able to perform TCR BV gene repertoire analysis with samples of $5x10^4$ mononuclear cells only. With the present technique it is feasible to study tissue biopsies, synovial fluid of RA patients and cerebrospinal fluid samples of MS patients.

PCR-ELISA can easily identify expanded T-cell populations in two test systems; TCR BV2 cells were identified among TSST-1 stimulated blood lymphocytes, and Jurkat cells expressing TCR BV8 were traced back in cell mixtures of PHA stimulated T-cells and Jurkat cells. However, the results obtained by PCR-ELISA do not permita calculation of the exact number of T-cells bearing a particular BV gene. Indeed, FACS analysis revealed that 83% of TSST-1 stimulated cells were $V\beta2^+$ T-cells, whereas PCR-ELISA indicated that 35% of the T-cells were $BV2^+$. The difference may be due to post-transcriptional events, but may also be caused by variations in the PCR reaction conditions. For instance, although the efficiencies of the individual PCR reaction with the different primers are thought to be comparable as can be seen from the similar slopes of the amplification reactions in Fig. 3.2, small differences in reaction volume or temperature differences between the wells may still lead to small variations in the efficiency of the individual PCR amplifications. In addition, as for other RT-PCR based methods, out-of-frame transcripts will also lead to a PCR product which may obscure the final data. Finally, the relationship between template concentration and A_{450} values is not linear especially for high A_{450} values, thus preventing precise quantification of the expression levels of individual TCR V genes. Therefore, PCR-ELISA cannot be used to calculate the proportion of T-cells which express a given TCR BV or AV gene but, on the other hand, this technique is very well suited to identify TCR V genes which are significantly overrepresented in a tested lymphocyte population. In addition, this approach can be used to compare the TCR repertoire profiles between different samples of a single donor, for example when studying whether an expanded T-cell subset is present in the synovial fluid of the left knee versus the right knee, or in synovial fluid versus the blood of a patient with inflamed joints. As shown here for TSST-1, the technique can also be used to identify TCR genes which are preferentially stimulated by a superantigen. In this case, a comparison is made between the TCR repertoire of blood lymphocytes after stimulation with the superantigen versus a nonspecific stimulus such as PHA.

In this report it was shown that PCR-ELISA can be used to compare the TCR repertoires in inflamed joints of an RA patient, the synovial fluid and the synovial tissue. In the synovial fluid of the patient studied, 3 TCR BV genes were overrepresented: BV2, BV4 and BV18. Interestingly, the BV4 gene was also overrepresented in the synovial tissue of the same joint, but not in the blood. The CDR3 length profile of the TCR BV4 gene amplicons indicated that this BV4 gene population probably consisted of a single clone in the synovial fluid and the synovial tissue. However, to prove the clonality of these transcripts DNA sequence analysis of

the CDR3 segments should be performed. In addition, since CDR3 segments of identical length were observed in both the synovial fluid and membrane, the data suggest that the same clonally expanded T-cell population is present at both sites in the joints. However, T-cells with identical CDR3 region length but different AA composition can not be distinguished using the spectratyping technology. Therefore, CDR3 region sequence analysis is necessary to distinguish mono-, oligo- or poly-clonally expanded T-cell populations. Thus, PCR-ELISA can be used as a fast screening method to identify overrepresented TCR V gene segments, while subsequent analysis of CDR3 length profiles provides some important information about the clonal composition of these overrepresented V gene segments.

In conclusion, PCR-ELISA is a fast, simple and sensitive method to determine the TCR repertoire in blood or tissue biopsies. The method does not require radioactive labels, nor special expensive equipment. Only a PCR cycler and a common ELISA plate reader are necessary. The technique can be used to identify overrepresented TCR V gene elements, but does not permit the quantification of precise expression levels for a given TCR gene. However, this semiquantitative method is well suited to the comparison of TCR repertoires between different samples of the same patient. If combined with CDR3 fragment length analysis, the clonal composition of the overrepresented TCR V gene elements can be determined. Together, these two methods may provide valuable information in the study of autoreactive T-cells in autoimmunity.

Acknowledgments

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References

1. Monaco, J., Pathways for processing and presentation of antigens to T cells. *J. Leuk. Biol.* 57: 543, 1995.

2. Breit, T., Van Dongen, J., Unravelling human T-cell receptor junctional region sequences. Thymus 22:177, 1994

3. Davis, M.M., T cell receptor gene diversity and selection. *Ann. Rev. Biochem.* 59: 475, 1990.

4. Struyk, L., Hawes, G., Chatila M., Breedveld F., Kurnick J., van den Elsen P., T cell receptors in Rheumatoid Arthritis. *Arthritis Rheum.* 38(5): 577, 1995.

5. Ying, C., Janssens, J., Bleus, J., Vandevyver, C., Zhang, J., Raus, J., T cell receptor Vβ usage of tumor infiltrating lymphocyte lines cloned from human breast tumor and melanoma. *Int. J. Oncol.*: 7, 147, 1995.

6. O'Shea, UD., Hollowood, KM., Boylston, AW., Demonstration of the oligoclonality of an enteropathy associated T-cell lymphoma by monoclonal antibodies and PCR analysis of the T-cell receptor V-beta repertoire on fixed tissue. *Hum. Pathol.* 27(5): 509, 1996.

7. Oksenberg, JR., Panzara, MA., Begovich, AB., Mitchell, D., Erlich, HA., Murray, RS., Shimonkevitz, R., Sherritt, M., Rothbard, J., Bernard, C., Steinman, L., Selection for T-cell receptor Vβ–Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature* 362: 68, 1993.

8. Alam, A., Lambert, N., Lulé, J., Coppin, H., Masîères, B., de Préval, C., Cantagrel, A., Persistence of dominant T cell clones in synovial tissues during Rheumatoid Arthritis. *J. Immunol.* 156: 3480, 1996.

9. Bröker, B., Korthäuer, U., Heppt, P., Weseloh, G., De La Camp, R., Kroczek, R., Emmrich, F., Biased T cell receptor V gene usage in Rheumatoid Arthritis. *Arthritis Rheum.* 36(9): 1234, 1993.

10. Huchenq, A., Champagne, E., Sevin, J., Riond, J., Tkaczuck, J., Mazieres, B., Cambon-Thomsen, A., Cantagrel, A., Abnormal T cell receptor BV gene expression in the peripheral blood and synovial fluid of rheumatoid arthritis patients. *Clin. Exp. Rheumatol.* 13: 29, 1995.

11. Vandevyver, C., Mertens, N., van den Elsen, P., Medaer, R., Raus, J., Zhang, J., Clonal expansion of myelin basic protein-reactive T cells in patients with multiple sclerosis: restricted T cell receptor V gene rearrangements and CDR3 sequence. *Eur. J. Immunol.* 25: 958, 1995.

12. Padula, S., Sampieri, A., T-cell receptor use in early Rheumatoid Arthritis. *Ann. N.Y. Acad. Sc.* 756: 147, 1995.

13. Alam, A., Lulé, J., Coppin, H., Lambert, N., Mazières, B., De Préval, C., Cantagrel, A., T-cell receptor variable region of the β-chain use in peripheral blood and multiple synovial membranes during Rheumatoid Arthritis. *Hum. Immunol.* 42: 331, 1995.

14. Cottrez, F., Auriault, C., Capron, A., Groux, H., Analysis of the BV specificity of superantigen activation with a rapid and sensitive method using RT-PCR and an automatic DNA analyser. *J. Immunol. Methods* 172: 85, 1994.

15. Lang, R., Pfeffer, K., Wagner, H., Heeg, K., A rapid method for semiquantitative analysis of the human BV repertoire using TaqMan® PCR. *J. Immunol. Methods* 203: 181, 1997.

16. Manfras, B., Rudert, W., Trucco, M., Boehm, B., Analysis of the α/β T-cell receptor repertoire by competitive and quantitative family-specific PCR with exogenous standards and high resolution fluorescence based CDR3 size imaging. *J. Immunol. Methods* 210: 235, 1997.

17. Bettinardi, A., Imberti, L., Sottini, A., Primi, D., Analysis of amplified T cell receptor V beta transcripts by non-isotopic immunoassay. *J. Immunol. Methods* 146: 71, 1992.

18. Kohsaka, H., Taniguchi, A., Chan, PP., Ollier, WE., Carson, DA., The expressed T cell receptor gene repertoire of rheumatoid arthritis monozygotic twins: rapid analysis by anchored polymerase chain reaction and enzyme-linked immunosorbent assay. *Eur. J. Immunol.* 23(8): 1993, 1993.

19. van Boxel, J.A., Paget, S.A., Predominately T-cell infiltrate in Rheumatoid synovial membranes. *N. Engl. J. Med.* 293: 517, 1975.

20. Zwillich, S., Weiner, D., Wiliams, W., T cell receptor analysis in Rheumatoid Arthritis: What have we learnt. *Immunol. Res.* 13: 29, 1994.

21. Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zöller, M., Kourilsky, P., The sizes of the CDR3 hypervariable regions of the murine T-cell receptor β chains vary as a function of the recombined germ-line segments. *Proc. Natl. Acad. Sci. USA* 90: 4319, 1993.

22. Pannetier, C., Even, J., Kourilsky, P., T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today* 4: 176, 1995.

23. Martini, J-F., Villares, SM., Nagano, M., Delehaye-Zervas, MC., Eymard, B., Kelly, PA., Postel-Vinay, MC., Quantitative analysis by polymerase chain reaction of growth hormone receptor gene expression in human liver and muscle. *Endocrinol.* 136: 1355, 1995.

24. Morrison, C., Gannon, F., The impact of the PCR plateau phase on quantitative PCR. *Biochem. Biophys. Acta* 1219: 493, 1994.

25. Choi, Y., Lafferty, J., Clemants, J., Todd, J., Gelfand, E., Kappler, J., Marrack, P., Kotzin, B., Selective expansion of T cells expressing Vβ 2 in toxic shock syndrome. *J. Exp. Med.* 9: 982, 1990.

26. Rigaut, KD., Scharff, JE., Neville, DM., Selective killing of T cells by immunotoxins directed at distinct Vβ epitopes of the T cell receptor. *Eur. J. Immunol.*, 25: 2077, 1995.

27. VanderBorght A., Geusens, P., Vandevyver, C., Raus, J., Stinissen, P. Skewed T-cell receptor variable gene in the synovium of Rheumatoid arthritis patients. *Submitted.*

28. Jenkins, RN., Nikacin, A., Zimmermann, A., Meek, K., Lipsky, PE., T cell receptor Vβ gene bias in Rheumatoid Arthritis. *J. Clin. Invest.* 926: 2688, 1993.

29. Borgato, L., Beri, R., Biasi, D., Testoni, R., Cugola, L., Ceru, S., De Sandre, G., Lunardi, C., Analysis of the T cell receptor repertoire in Rheumatoid Arthritis. *Clin. Exp. Rheumatol.* 15(5):475, 1997.

Chapter 4

Skewed T-cell Receptor Variable Gene Usage in the Synovium of Early and Chronic Rheumatoid Arthritis Patients and Persistence of Clonally Expanded T-cells in a Chronic Patient

Based on:

Skewed T-cell Receptor Variable Gene Usage in the Synovium of Early and Chronic Rheumatoid Arthritis Patients and Persistence of Clonally Expanded T-cells in a Chronic Patient

Ann VanderBorght, Piet Geusens, Caroline Vandevyver, Jef Raus and Piet Stinissen.

Submitted for publication.

Abstract

Autoreactive T-cells may contribute to the pathogenesis of rheumatoid arthritis (RA). We studied the T-cell receptor (TCR) V gene repertoire in the blood and the synovium of early and chronic RA patients using PCR-ELISA to evaluate possible differences between these patient groups. Overrepresented TCR V genes (>15% of total repertoire) were observed in the synovium, but not in the blood of all RA patients (n=38). The number of overrepresented V genes was significantly higher in the synovium of chronic (n=31) versus early RA patients (n=7). The V gene profile was different among patients, and similar in both knees for patients with bilateral synovitis (5). The clonal composition of overrepresented TCR BV genes in an early and a chronic RA patient was further studied by CDR3 region sequence analysis. A high level of clonal diversity was found in the joints and the blood of the early RA patients, suggesting a polyclonal T-cell expansion. In the chronic RA patient, predominant clonal expansions were observed in the blood and the synovium, and some expanded clones were still present 2 years later. The observation of similar T-cell populations in both joints in patients with bilateral synovitis, and the persistence of clonally expanded T-cells for more than 2 years in the joints of a chronic RA patient may indicate a pathogenic role for these cells in the disease process.

Introduction

Increasing evidence suggests that autoimmune mechanisms involving autoreactive T-cells contribute to the pathogenesis of rheumatoid arthritis (RA) (1-3). Activated T-cells are found in the inflamed synovium and the infiltrating T-cells preferentially produce Th1 proinflammatory cytokines (3). Susceptibility to RA is associated with the HLA-DR1 and DR4 alleles and treatment with immunosuppressive drugs shows clinical effects in RA (4,5). However, the eliciting autoantigens remain unknown, but collagen type II, heat shock proteins, glycoprotein-39 and superantigens are possibly involved in the activation of the pathogenic T-cells (6-9).

Based on the assumption that disease relevant T lymphocytes may undergo expansion in the synovium, several groups have studied T-cell receptor (TCR) gene expression in the joints and blood of RA patients (8,10). If a biased TCR expression would be associated with the pathogenic T-cell populations, the corresponding TCR elements could be targeted by TCR specific immunotherapies, such as TCR peptide vaccination or T-cell vaccination (11,12). Unfortunately, many contradictory findings have been reported so far. Paliard and coworkers (13) observed elevated expression of BV14 genes in synovial fluid (SF), while Howell *et al.* (6) found an increased expression of BV3, BV14 and BV17 in IL2-receptor positive T-cells in the joints. Sottini *et al.* (14) reported preferential usage of BV 7 genes in the SF, and Struyk *et al.* (15) observed a heterogeneous usage of TCR V genes in the SF of 12 RA patients, with an increased expression of AV10 in most patients. Some authors described an oligoclonal expansion of $CD4^+$ T-cells in the joints, while others found an oligoclonal expansion of the $CD8^+$ subset (16-21). Some of the contradictory findings are potentially related to differences in patient populations, such as HLA background or disease duration, or the use of unstimulated versus *in vitro* expanded cells from the SF. In addition, various technical approaches such as Southern blot analysis (6), polymerase chain reaction (PCR) amplification (14,15,22), or staining with TCR V gene specific antibodies were applied (23). Some of these methods provide limited quantitative data, while flowcytometry depends on the availability of V gene specific monoclonal antibodies.

We used a sensitive and powerful PCR-ELISA (24) to identify overrepresented TCR V gene elements in the synovium of two groups of RA patients, a group with short disease duration (n=7) (less than 1 year) and a group with chronic disease (n=31) (mean duration 14 y). To avoid *in vitro* bias, fresh unstimulated and unseparated SF cells and paired peripheral blood mononuclear cells (PBMC) were used. Skewed TCR V gene expression was found in the joints of patients with RA but not in their blood, which is in line with previous observations. None of the BV genes was however consistently overexpressed in the joints of all, or a subgroup of patients. Interestingly, the TCR V gene skewing was more pronounced in patients with early

RA compared to chronic RA. Overrepresented TCR BV gene families of an early and a chronic RA patient were further characterized by CDR3 sequence analysis. Oligo- and polyclonally expanded T-cells were observed in the synovium of the early RA patient, while major clonal expansions were observed in the joints of the chronic RA patient. Identical CDR3 sequences were identified in both knees of the early and chronic RA patient. In the chronic patient, clonally expanded TCR sequences persisted for two years, suggesting that the corresponding T-cell population plays a role in the progression of the synovial inflammation in this patient.

Patients and methods

Characteristics of the study population

Thirty-eight patients with RA as defined by the criteria of the American College of Rheumatology were included. Seven RA patients with a disease duration of less than 1 year were grouped as early RA, while the other 31 patients with a longer disease duration (mean disease duration of 14 years) were grouped as chronic RA patients. All RA patients were treated at the time of sampling with either disease modifying antirheumatic drugs (DMARD), non-steroidal anti-inflammatory drugs (NSAID), or corticosteroids as summarized in Table 4.1. Nine patients with other rheumatic diseases (5 with osteoarthritis, 4 with psoriatic arthritis) and four healthy individuals were included as control subjects. The patients and controls were sampled for peripheral blood (PB) alone, or for PB and paired SF of one or both knees at the same time. Synovial tissue (ST) was obtained from five patients undergoing synovectomy. The characteristics of the study populations are listed in Table 4.1. Written informed consent was obtained from all patients.

Isolation of T-cells and PCR-ELISA of TCR AV and BV genes

T-cells were isolated from peripheral blood, synovial fluid and synovial tissue samples as described in 2.1. Semiquantitative TCR AV and BV gene repertoire analysis was performed using PCR-ELISA as described in 2.4. These TCR V genes which constituted more than 15% of the total TCR V gene repertoire were defined as overrepresented V genes, based upon the V gene expression levels in a panel of PB samples obtained from healthy subjects (see Results).

Table 4.1. Characteristics of patients and control subjects *

group	subjects	synovitis		sampling			HLA-DR1/DR4 +	RF	patients treated with				
							number of HLA	positive					
		bilateral	unilateral	PB	SF	ST	typed subjects -		DMARD	NSAID	steroids	MTX	no
Early RA	7	1	6	7	7	0	3/6	3	5	1	1	0	0
Chronic RA	31	4	27	31	31	4	15/24	15	22	4	0	4	1
Psoriatic Arthritis	4	0	4	4	4	1	1/2	0	0	2	0	1	1
Osteoarthritis	5	0	5	5	5	0	2/3	0	0	0	3	0	2
Normal Subjects	10	NA	NA	10	NA	NA	3/10	NA	NA	NA	NA	NA	NA

* NA: not applicable; PB: peripheral blood; SF: synovial fluid; ST: synovial tissue; RF: rheumatoid factor;
NSAID: non-steroidal anti-inflammatory drugs; MTX: Methotrexate, DMARD: Disease modifying antirheumatic drugs

Sequence analysis of TCR rearrangements

PCR amplicons of overrepresented TCR V gene families were cloned in bacteria and prepared for CDR3 region sequence analysis as described in 2.5.1.

Results

Overrepresented TCR V genes are found in the joints but not in the blood of RA patients

TCR V gene expression was studied by PCR-ELISA (24). Lymphocyte mRNA is transcribed to cDNA and amplified in individual PCR reactions using primers specific for the TCR AV and BV gene segments, in the presence of digoxigenin (DIG)-labelled dUTP's. The DIG-labelled PCR products are quantified by ELISA (24). This method was first applied to unstimulated PBMC from ten healthy subjects. Figure 4.1 shows TCR V gene profiles in the peripheral blood of 4 healthy subjects. All subjects showed a heterogeneous usage of all AV and BV genes. Since none of these segments constituted more than 15% of the total AV or BV gene repertoire in a given donor, we have arbitrarily chosen a cut-off value of 15% as the threshold value to define an overrepresented TCR V gene element.

The amount of starting T-cell specific mRNA copies is quite variable in SF, synovial tissue and PB samples, which may interfere with the semiquantitative analysis. We therefore first studied the TCR mRNA content of all samples by PCR-ELISA (see 2.4 in chapter 2) and subsequently used equal amounts of TCR specific cDNA from the joints and the PB of each subject to identify overrepresented TCR V genes by PCR-ELISA.

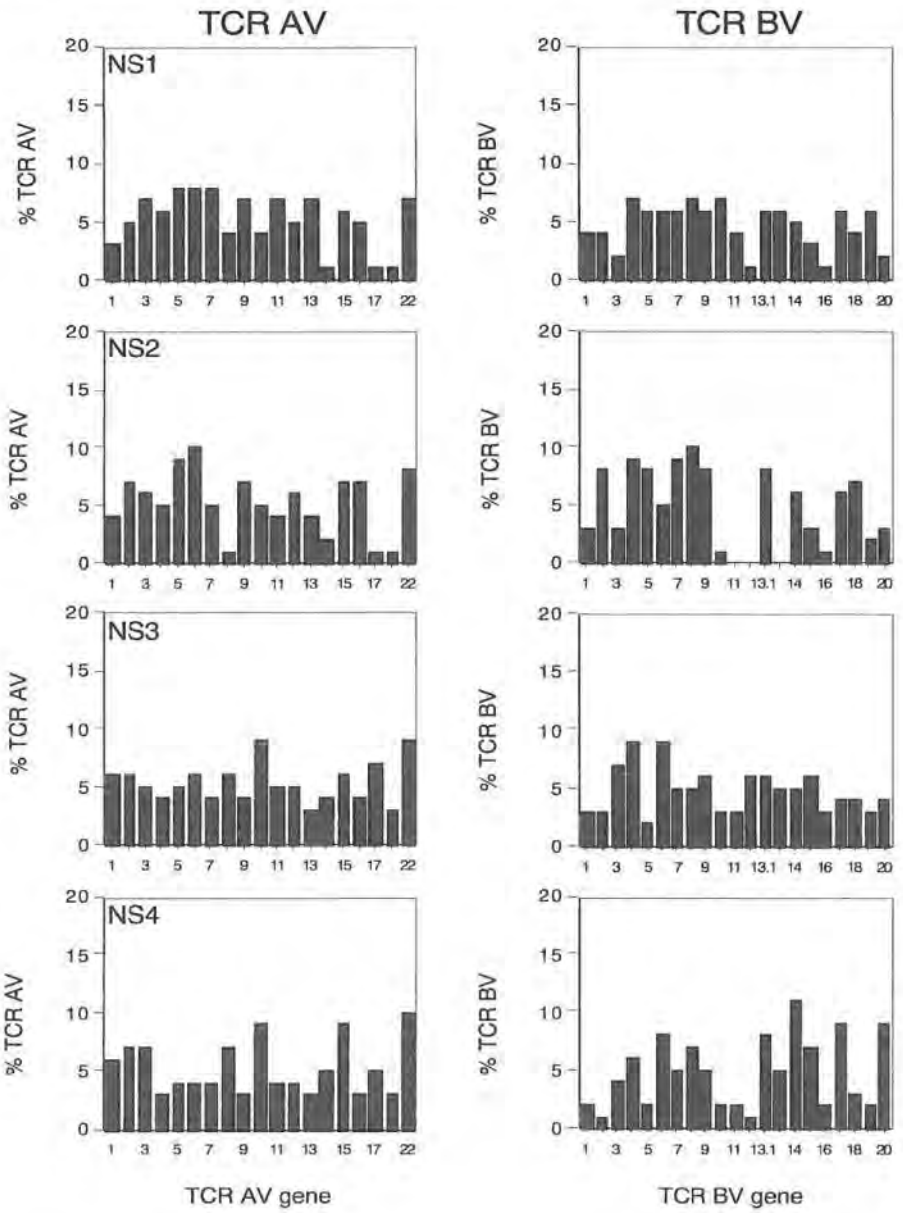

Figure 4.1. TCR V gene repertoire analysis of the blood lymphocytes of 4 healthy subjects. Fresh PBMC were subjected to mRNA isolation and cDNA synthesis, and further amplified in individual PCR amplifications using primers specific for each TCR AV and BV gene segment. The PCR products were quantified by ELISA. The expression of each TCR V gene element is represented as a fraction of the total V gene expression.

PCR-ELISA was performed with PB and paired SF samples of 7 early and 31 chronic RA patients, 5 osteoarthritis and 4 psoriatic arthritis patients were used as controls (Table 4.1). Figure 4.2 shows the typical TCR V gene expression profiles of a patient with early RA (RA-5) and a patient with chronic RA (RA-11). The TCR AV 5 and 22 genes, and BV 2 and 6 genes were overrepresented (>15%) in the SF of patient RA-5, while AV3 and AV15 and BV10, BV13.1 and BV18 were overrepresented in the synovial fluid of the chronic RA patient RA-11. Thus, while the TCR V gene usage in the blood of these patients was rather heterogeneous, a restricted TCR V gene usage was observed in their SF.

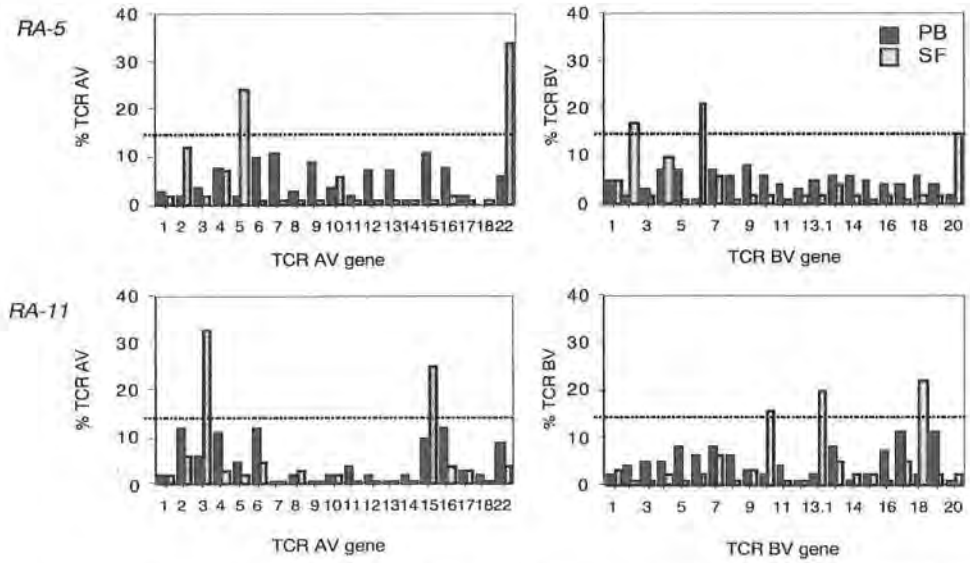

Figure 4.2. TCR V gene repertoire in SF and PB of an early (RA-5) and a chronic (RA-11) patient. Fresh mononuclear cells of the SF and PB were submitted to PCR-ELISA analysis to determine the TCR V gene expression repertoire. The expression of each individual TCR V gene element is represented as the fraction of the total TCR V gene expression.

A similar heterogeneous usage of all TCR V gene elements (all below 15%) was observed in the PBMC of the 38 RA patients and 9 control patients (5OA, 4 PsA) (data not shown). In contrast, overrepresented TCR V gene segments were identified in the synovial samples of all RA and control patients tested (Table 4.2).

Table 4.2: Synovial TCRV gene usage in the joints of RA patients and controls

Patient	Duration (Y)	HLA DR4/DR1	Sample	TCR AV genes overexpressed	TCR BV genes overexpressed
A. RA patients					
Early RA patients					
RA-1	<1	-/-	SF	22	15, 17
RA-2	<1	ND	SF	5	7
RA-3	<1	-/-	SF	6, 7, 11, 12	2, 9, 10
RA-4	<1	+/-	SF	15	5, 10
RA-5	<1	+/-	SF	5, 22	2, 6
RA-6	<1	+/-	SF	22	7, 9
RA-7	<1	-/-	SFR	5	4, 8, 16
			SFL	5, 16	2, 8, 16
Chronic RA patients					
RA-8	38	-/-	SF	2, 5, 22	4, 6, 7, 9, 10
RA-9	7	-/-	SF	9, 15	1, 8, 12, 13.1, 13.2
RA-10	19	ND	SF	3, 5, 9, 13, 16	9, 12, 20
RA-11	>35	ND	SF	3, 15	10, 13.1, 18
RA-12	3	ND	SF	2, 3, 16	2, 4, 7, 17, 19
RA-13	13	+/-	SF	1, 6, 7, 15	-
RA-14	15	+/-	SF	5, 11, 18	1, 7, 13.1
RA-15	12	-/+	SF	3, 7, 8, 16	1, 13.2, 8
RA-16	32	-/+	SF	12, 17, 22	2
RA-17	6	+/-	SF	2, 4, 12	9, 12
RA-18	4	-/+	SF	2, 17	13.1, 19
RA-19	7	+/-	SF	11, 12, 13, 18	1, 11, 16
RA-20	14	ND	SF	7, 13	6, 8, 11, 13.2
RA-21	17	ND	SF	4, 5, 7	3, 5, 13.1, 13.2
RA-22	4	ND	SF	2, 11, 22	5, 8, 12, 14, 19
RA-23	8	-/-	SF	7, 13, 17, 22	2, 8
RA-24	11	-/-	SF	9, 10, 12, 17	2, 9, 13.1
RA-25	37	-/-	SF	5, 12, 14, 18	7, 13.1, 13.2
RA-26	6	-/-	SF	10, 18	2, 6, 18
RA-27	4	-/-	SF	4, 8, 15	4, 6, 8
RA-28	11	-/-	SF	2, 5, 8	8, 18
RA-29	17	-/-	SF	4, 5, 9, 15	6, 16
RA-30	26	+/-	SF	2, 22	2, 10, 15, 20
RA-31	32	+/-	SF	6, 7, 11, 12	1, 7, 9, 15, 18
RA-32	4	+/-	SFL	2, 5, 16	8, 13.1, 17, 18
			SFR	2, 3, 15, 16	14, 17, 18, 19
RA-33	2	+/-	SMR	5, 8, 10, 22	3, 15, 17, 18
			SFR	5, 8, 15, 16	12
RA-34	8	+/-	SFL	6, 7, 10, 15	2, 5
			SFR	8, 15	6, 8, 13.1
RA-35	12	+/-	SF	12, 15	2, 13.1, 14
			Lavage	4, 15	13.1
			SM	15	7, 14
RA-36	9	+/-	SFR	1, 6, 10, 15	2, 8, 9
			SMR	2, 7, 10, 11	4, 6, 13.2
			Lavage	6, 7, 11	7, 13.2
RA-37	19	+/-	SFL	3, 5	9, 14, 15, 16
			SFR	3	1, 7, 13.1, 15, 16
RA-38	12	ND	SFR	9, 12, 13, 15	1, 4, 10, 15, 18
			SFL	9, 12, 15, 16	1, 4, 7, 18
			SMR	7, 9	1, 4, 7, 10

Table 4.2: Continued

Patient	HLA DR4/DR1	Sample	TCR AV genes overexpressed	TCR BV genes overexpressed
B. Other rheumatic diseases				
OA-1	-/-	SF	5, 6, 7, 13	2, 18
OA-2	ND	SF	7, 15	2, 13.1, 13.2, 16
OA-3	+/-	SF	3, 10, 15, 17	4
OA-4	ND	SF	2, 7	1, 9
OA-5	+/-	SF	7, 11, 16	2, 4, 7,9 ,11
PsA-1	-/-	SF	8, 14	16
PsA-2	+/-	SF	4, 5, 7	4, 13.2, 18
PsA-3	ND	SF	5, 22	2, 13.2, 20
PsA-4	ND	SF	9, 10, 12, 17, 18	18
		SM	9, 12, 17, 18, 22	13.1
		Lavage	10, 15	-

The number of overrepresented TCR AV and BV genes in the joints ranged from 1 to 5, with a mean number of 3.2 AV and 3.4 BV genes in the RA population, and 3.1 AV and 4.4 BV genes in the joints of the control patients (osteoarthritis and psoriatic arthritis) (Table 4.3).

Table 4.3: Mean number of TCRV genes expressed in synovial cavity samples of RA patients and control

		TCR V genes expressed in the joint (Mean ± SD)			
Patient group		AV		BV	
RA	all (n=38)	3.20 ± 0.2		3.40 ± 0.2	
	Early (n=7)	1.60 ± 0.2	*	2.12 ± 0.1	*
	Chronic (n=31)	3.50 ± 0.2		3.70 ± 0.2	
Non RA controls	all (n=9)	3.08 ± 0.2		4.36 ± 1.0	

* $p<0.05$

The TCR V gene skewing pattern varies significantly among the RA patients

The TCR AV and BV genes which are overrepresented in the SF vary significantly among RA patients (Table 4.2). No particular TCR V gene was overrepresented in more than 50% of the RA derived SF samples (n=49), nor in the HLA-DR1/DR4$^+$ subgroup (n=26) (Figure 4.3). However, some V genes including AV15 and AV5 were overrepresented in more than 30% of the RA derived synovial samples. AV15 was overrepresented in SF of 42% of the DR1/DR4$^+$ RA patients. Other V genes, including AV9, AV14, BV3, BV4, BV11 and BV20, were less frequently overrepresented in the DR1/DR4$^+$ RA patients as compared to the total RA group. No V gene element was commonly overrepresented in the SF of the 3 DR1/DR4$^+$ early RA patients. Thus, our data indicate a lack of TCR BV gene expression bias in RA patients, and do not confirm the previous reported overrepresentation of the BV 3/14/17 genes (6,16). In the non-RA control group (n=9), nearly all V gene segments were overrepresented in at least one patient studied. The small number of samples analysed (9) does not allow to draw conclusions on preferentially expressed V genes in this group.

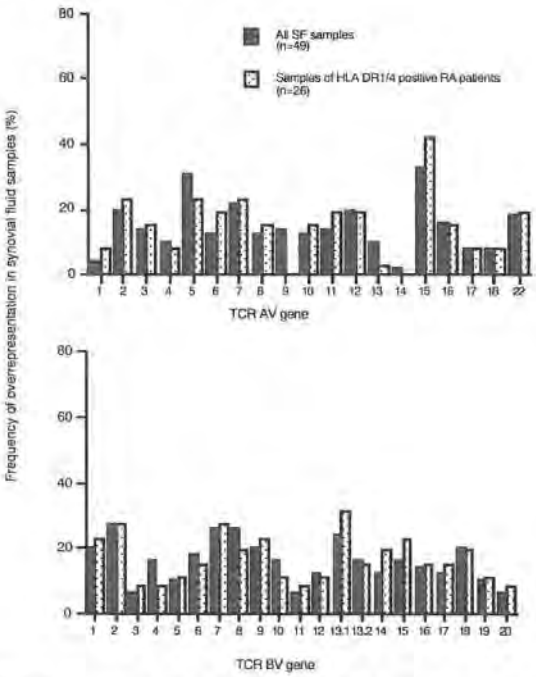

Figure 4.3. Frequency of overrepresentation of individual TCR AV and BV genes in synovial fluid samples of RA patients. The frequency of overrepresentation (%) of each TCR AV and BV gene element was calculated for all synovial samples studied for the group of RA patients (38 patients, 49 synovial samples) and the subgroup of RA patients expressing HLA-DR1/DR4 alleles (18 patients, 26 synovial samples).

The TCR V gene expression profile is similar in synovial fluid and tissues of both affected knees of RA patients with bilateral synovitis

We studied SF and synovial tissue (ST) from the same diseased joint in 4 RA patients (RA-33, -35, -36, -38) and paired SF samples of both knees in 5 patients with bilateral synovitis (RA-7, -32, -34, -37, -38). In these patients, the TCR V gene profile at both disease sites was highly comparable (Table 4.2). For example, in patient RA-7 AV5, BV8 and BV16 were overrepresented in the SF of both knees. In patient RA-38, two separate samples of the same cavity (fluid and tissue of the right knee) as well as fluid of the opposite knee (SFL) were analyzed. In the right knee, AV9 and BV1, BV4 and BV10 were overrepresented in both the synovial tissue and fluid. The AV9, AV12 and AV15 genes and BV1, BV4 and BV18 expressed in the synovial fluid of the right knee (SFR) were also overrepresented in the synovial fluid of the left knee (SFL) of this patient. These data suggest that similar T-cell populations are represent at different sites of the same cavity, and in both knees of patients with bilateral synovitis.

The TCR V gene expression is more restricted in the joints of early versus chronic RA patients

Next, we studied the TCR V gene restriction in relation to the disease duration of the patients. We compared the mean number of overrepresented V gene elements in early RA patients (< 1y. disease duration) versus chronic RA patients (> 1y.) with a mean disease duration of 14 y. The mean number of BV genes and AV genes was significantly higher (Student T test: $p < 0.05$) in chronic RA patients (AV: 3.5, BV: 3.7) as compared to early RA patients (AV: 1.6, BV: 2.1) (Table 4.3). The mean number of TCR AV and BV genes overrepresented in the joints of early RA patients was also lower than in the non-RA control population (osteoarthritis and psoriatic arthritis patients) (AV: 3.1, BV: 4.4). The difference was statistically significant for the AV genes but not for the BV genes.

Similar TCR V genes are overrepresented in the joints of a chronic RA patient for at least two years

In one RA patient (RA-36) we evaluated the TCR repertoire in the joints with an interval of two years. A SF sample from both inflamed knees was obtained at a disease duration of 7y., and a SF and tissue sample was obtained from the right knee at a second sampling two years later. The patient was treated with disease modifying antirheumatic drugs (DMARD) before and during the observation period. The disease activity was comparable at both time points.

Interestingly, out of 5 TCR AV and 5 BV genes overrepresented in the right knee at the first sampling, 2 AV genes (AV2 and AV10), and 2 BV genes (BV2 and BV6) were still overrepresented two years later (Table 4.4). The total number of overrepresented TCR V genes was decreased after two years in this patient. It is unclear whether the administration of DMARD influenced the TCR V gene expression profile in the joints of this patient.

Table 4.4: TCR V gene usage in the joints of a chronic RA patient (RA-36) determined at two timepoints

Patient RA-36

	Sample	TCR AV genes overrepresented	TCR BV genes overrepresented
timepoint 1 (1994)	PBMC	none	none
	SFL	**2**, 3, 5, **10**, 14	**2**, **6**, 8, 13.1, 18
	SFR	**2**, 3, 5, **10**, 12	**2**, **6**, 12, 13.1, 17
timepoint 2 (1996)	PBMC	none	None
	SFR	1, 6, **10**, 15	**2**, 8, 9
	SMR	**2**, 7, **10**, 11	4, **6**, 13.2

SFL(R): Synovial fluid of the left (right) knee, SMR: synovial membrane of the right knee

CDR3 sequence analysis of overrepresented V gene elements in the synovium of two RA patients

To provide information on the clonal composition of an overrepresented V gene family, we cloned the PCR products in a plasmid vector and sequenced 10 to 15 randomly selected recombinant clones. For this experiment we selected a representative patient of the early RA (RA-7) and chronic RA (RA-36) group. Table 4.5 lists the clonally expanded CDR3 sequences and identical CDR3 sequences shared between different samples.

In the early RA patient RA-7 the BV2, BV8 and BV16 and BV4, BV8 and BV16 gene families were overrepresented in respectively the SFL and SFR. Overall a high level of diversity was observed among these sequences, although some sequences were represented more than once among these families in the SF (Table 4.5). Some uniquely represented CDR3 sequences were shared between both knees, or between the blood and synovium. No repetitive sequences were

observed in the blood BV2 (10 clones), BV4 (10 clones) and BV8 (4 clones) families, but one BV16 sequence was found in 3 out of 5 CDR3 sequences although this family was not overrepresented in the blood. To test whether repetitive sequences could also be found among other BV genes in the blood, we analysed the PB derived BV19 (11 clones), BV13.1 (15 clones) and BV11 (14 clones) families but no repetitive sequences could be identified (data not shown). Taken together, in this early patient the expanded BV genes of the left and right knee contain many diverse CDR3 sequences, suggesting that they represent T-cell populations of oligo- or polyclonal origin.

Interestingly, some sequence motifs were frequently found among the CDR3 sequences in this patient. The XGGX motif was frequently observed in the CDR3 sequences of the BV2 and BV8 gene families that were overrepresented in the SF of this patient (Table 4.6). This motif was found in 15 / 35 BV2 CDR3 sequences of the SF and blood. The motif was also seen in 8 / 37 BV8 sequences of the SF, and 3 / 5 BV16 sequences of the blood. We also determined whether this motif was present in CDR3 sequences of BV genes that were not overrepresented in the SF, and found the motif in 5 / 15 and 3 / 14 CDR3 sequences of the BV13.1 and BV11 genes respectively. Out of 38 XGGX sequences observed, serine was found in 26% and arginine in 13% at the first X position, while serine, alanine and asparagine were found at the last X position in 21%, 30% and 11% of all XGGX sequences. In 7 out of 38 XGGX sequences this motif was SGGA. The GG sequence in the motif was in most cases encoded by the BD1 gene segment (not shown), so it seems that this D-region element was preferentially rearranged among TCRβ chains found in the SF and blood of this patient.

Table 4.5. V-D-J sequence analysis of overrepresented TCR V genes in the joints of two RA patients

A. Patient RA-7 (early RA)

TCR V gene			Clonally expanded V-D-J sequences[1]				Identical V-D-J sequences shared between samples[2]			
	Sample	No	V	N-D-N	J	Freq	V	N-D-N	J	Freq
BV2	PBMC	10	none				none			
	SFR	10	none				CSA	PRGG	NTGEL	1/10
	SFL	15	none				CSA	PRGG	NTGEL	1/15
BV4	PBMC	10	none				YLCS	PNDRS	EAFFG	1/10
	SFR	13	YLCSV	DAR	NYGYT	2/13	YLCS	PNDRS	EAFFG	1/13
							YLCSV	LD	NTGEL	1/13
							YLCSV	AGP	NEKLF	1/13
	SFL	12	none				YLCSV	LD	NTGEL	1/12
							YLCSV	AGP	NEKLF	1/12
BV8	PBMC	4	none				CASS	TNAGDRE	TGELF	1/4
	SFR	12	CASS	TPPGTVY	TGELF	2/12	CASS	TPPGTVY	TGELF	2/12
							CASS	YIPSGN	TDTQY	1/12
							CAS	RADRVS	SYEQY	1/12
							CASS	LTTGGR	STDTQ	1/12
							CASS	SGTGE	YEQYF	1/12
	SFL	25	CASS	LIASGGA	NEQFF	2/25	CASS	TPPGTVY	TGELF	1/25
			CASS	EPGRYT	YEQYF	2/25	CASS	YIPSGN	TDTQY	1/25
			CASS	LRHGPEE	NYGYT	2/25	CAS	RADRVS	SYEQY	1/25
			CASS	FSYF	GANVL	2/25	CASS	LTTGGR	STDTQ	1/25
			CASS	TNAGDRE	TGELF	2/25	CASS	TNAGDRE	TGELF	2/25
							CASS	SGTGE	YEQYF	1/25
BV16	PBMC	5	CAS	HWTSGGA	ETGYF	3/5	CAS	QDTA	EAFFG	1/5
	SFR	ND								
	SFL	8	CAS	QNRGQGR	SPLHF	3/8	CAS	QDTA	EAFFG	1/8
							CAS	RIRQHDIS	NEQFF	2/8

Table 4.5. Continued

B. Patient RA-36 (chronic RA)

TCRV gene	Sample	No	Clonally expanded V-D-J sequences[1] V	N-D-N	J	Freq	Identical V-D-J sequences shared between samples[2] V	N-D-N	J	Freq
First sampling										
BV2	PBMC	21	CSA	PQGGT	NTGELF	9/21	CSA	PQGGT	NTGELF	9/21
			CSA	RVDSL	YNEQFF	8/21	CSA	RVDSL	YNEQFF	8/21
			CSA	RDTESGAR	YTGYFG	2/21	CSA	VRVDSL	YNEQFF	1/21
	SFL	15	CSA	RVDSL	YNEQFF	8/15	CSA	RVDSL	YNEQFF	8/15
			CSA	PQGGT	NTGELF	2/15	CSA	PQGGT	NTGELF	2/15
			CSA	VRVDSL	YNEQFF	1/15				
BV4	SFL	11	CSV	DPGPAV	NEQFF	9/11	CSV	**DPGPAV**	**NEQFF**	**9/11**[3]
							CSV	**NRDGG**	**GYTFG**	**1/11**[4]
	SFR	10	CSV	DPGPAV	NEQFF	9/10	CSV	**DPGPAV**	**NEQFF**	**9/10**
							CSV	**NRDGG**	**GYTFG**	**1/10**
Second sampling (two years later)										
BV2	PBMC	17	CSA	NNRGPGAKNIQY	FGAG	3/17	NA			
			CSA	DPAVP	NEQFF	14/17	NA			
BV4	SM	20	CSVE	QWEKD	TEAFFG	2/20	CSV	**DPGPAV**	**NEQFF**	**2/20**
			CSV	DPGPAV	NEQFF	2/20	CSA	RS	SYNEQ	1/20
			CSVE	VDRA	YGYTF	2/20	CSV	RRGGD	TGELFF	1/20
			CSV	DSGRGTA	YGYTF	2/20				
	PBMC	15	CSV	LD	NTGELF	3/15	CSV	**DPGPAV**	**NEQFF**	**3/15**
			CSV	DPGPAV	NEQFF	3/15	CSA	RS	SYNEQ	1/15
							CSV	RRGGD	TGELFF	1/15

[1] Only CDR3 sequences (V-nDn-J) found twice or more were listed in this column. [2]CDR3 sequences identified at more than one site (SFL, SFR, PB) were listed in this column together with their frequency. [3]Underlined sequences were observed at both samplings. [4]The NRDGG sequence was observed at a frequency of 1/15 in the PBMC at the second sampling. No = number of plasmids sequenced

In the chronic patient RA-36 we studied the CDR3 sequences of the BV2 and BV4 families which were predominantly expressed in the synovium at either the first or second sampling. Major clonal expansions were observed in the left and right knee SF, but also in the blood (Table 4.5B). In the BV2 family some of these expansions accounted for 1/3 to 1/2 of all sequences, while in the BV4 gene family a single CDR3 sequence was observed in more than 90% of sequences from the left and the right knee. The two CDR3 sequences observed among 11 clones from the left knee were all shared with the right knee. Furthermore, three BV2 CDR3 sequences were shared between the blood and left knee. The RVDS motif was observed in four independent BV2 CDR3 sequences, and was represented in 10/21 sequences in the blood, and 10/15 sequences in the left knee SF. In this patient we evaluated the persistence of these clones two years later. Interestingly, the two BV4 CDR3 sequences which accounted for all BV4 sequences seen in the left and right knee at the first sampling were still identified in the synovial membrane and blood after two years. These sequences were however found at a lower frequency at the second time point suggesting that other BV4 T-cells also infiltrated the joints of this patient.

Table 4.6: Conserved nDn region sequence motifs in early RA patient (RA-7)

BV family	PB		SFL		SFR	
	Sequence	Freq	Sequence	Freq	Sequence	Freq
BV2	LTPSGGS	1/10	GGGSVGT	2/15	RTSGGA	1/10
	RGGTSG	1/10	RAASGGLQG	1/15	RGGQG	1/10
	REQGGSH	1/10	PRGGN	1/15	PRGGN	1/10
	KIGGS	1/10	REVSGGI	1/15	RDPGGS	1/10
	KGGN	1/10			AGGRGDS	1/10
	XGGX	**5/10**	**XGGX**	**5/15**	**XGGX**	**5/10**
BV4	NRDGGG	1/10	VAGGN	1/12	RRGGD	1/13
			DRTGGL	1/12		
	XGGX	**1/10**	**XGGX**	**2/12**	**XGGX**	**1/13**
BV8	none		LIASGGA	2/25	SLTNGGA	1/12
	none		LQGGA	1/25	LRSGGAR	1/12
	none		LTTGGR	1/25	LTTGGR	1/12
	none		LVVGGGD	1/25		
			XGGX	**5/25**	**XGGX**	**3/12**
BV16	HWTSGGA	3/5				
	XGGX	**3/5**				
BV13.1	VGGAD	1/15				
	YGGST	1/15				
	TKGGS	1/15				
	RGGGLV	1/15				
	YVGGER	1/15				
	XGGX	**5/15**				
BV11	VGGAD	1/14				
	RQTGTGG	1/14				
	ELGGL	1/14				
	XGGX	**3/14**				

Discussion

The role of T lymphocytes in the pathogenesis of RA is controversial. Some observations indicate that monocytes and synoviocytes are the key elements in the pathogenesis of RA, while T-cell responses are an unimportant by-product of synovial inflammation and destruction (25). Other findings suggest that autoreactive T-cells are the key pathogenic players in RA (10). Perhaps both scenario's should be combined in a unifying hypothesis where T-cells provoke the initial inflammatory response, while monocytes and synoviocytes play an important role in the secondary destructive phase of the disease. If this holds true, studies of pathogenic T-cell populations in RA may need to be focused particularly on patients with a recent disease onset. We therefore included both early and chronic RA patients in our study. We also decided to use unstimulated and unseparated lymphocytes for our TCR studies, because we tried to avoid manipulations which may activate T-cells or induce *in vitro* bias.

We observed different TCR V gene expression patterns in the joints and peripheral blood of RA patients. No TCR V gene expansions representing more than 15% of the total repertoire were seen in the blood, while one to five overrepresented TCR V gene elements were observed in the joints of all RA patients. This means that only a limited subset of the peripheral T-cells has infiltrated into the synovium of these patients. Interestingly, similar TCR V genes were overrepresented in both joints of patients with bilateral synovitis. Since no expanded TCR V genes were found in the blood, and overrepresented TCR V genes are already seen in patients with a very short disease duration, it is possible that some of these T-cells are involved in the disease process. The number of overrepresented TCR V gene elements was significantly higher in the joints of chronic versus early RA patients. Thus, the TCR repertoire seems to be more skewed in the early phase of the disease, suggesting that the random profile as found in the peripheral blood of the patients is distorted. This distortion may be caused by an increased expression of a specific subset of T-cells as a consequence of an antigenic stimulation or, alternatively, can be due to a depletion of the other T-cells found in the repertoire. This observation is consistent with the hypothesis that determinant spreading mechanisms may lead to the activation and infiltration of a larger variety of autoreactive T-cells (26).

One striking observation from the TCR repertoire analysis is that similar TCR V genes were overrepresented in both knees of five patients with bilateral synovitis. The expansion of T-cells with similar V gene expression suggests that identical T-cell populations are present in both joints. This can however only be confirmed by CDR3 region sequence analysis of the overrepresented TCR genes in the joints of these patients. Therefore we determined the CDR3 sequences of overrepresented BV genes of the SF and blood of two representative patients, one with early RA and one with chronic RA. Clonal analysis of the overrepresented V gene families in these two patients revealed different pictures. In the early RA patient the overrepresented

TCR BV gene families in the joints were of oligo- or polyclonal origin, while some TCR sequences were identified in both affected joints. Interestingly, a short aminoacid motif (XGGX) in the CDR3 region was found at an increased frequency among the overrepresented TCR families in the joints and the peripheral blood. Further studies need to resolve whether the increased frequency of this motif in the CDR3 region of SF T-cells is caused by T-cell sensitisation induced by one or a limited number of structurally related epitopes. Since the GG peptide is encoded by the TCR D region element BD1, it is however also possible that the frequent usage of this motif by T-cells in the SF and blood of this patients is simply due to the preferential rearrangement of this TCR D region element by the T lymphocytes in the patient. Shared CDR3 sequence motifs in the joints of RA patients were also observed in previous studies (27-29). Alam and coworkers (30) observed a related GXXG motif in the CDR3 of 30% of the dominant clones isolated from the synovial tissue of two RA patients. In line with our observations it was shown that the clones analysed by Alam et al. (30) which expressed the conserved motif were generally not clonally expanded in the synovium. Therefore, despite the frequent use of a common motif in the CDR3 sequences, it is possible that the polyclonal TCR V gene expansion in the joints of the early RA patient reflect T-cell activation induced by microbial superantigens, which lead to deletion or expansion of T-cells in a BV-specific manner (31).

In the chronic RA patient major clonal expansions were observed in the joints among the $V\beta2^+$ and $V\beta4^+$ T-cells. These expansions accounted for the vast majority of CDR3 region sequences identified among these subsets in the joints. This TCR pattern is compatible with *in vivo* T-cell activation induced by a few antigenic determinants only. The T-cell activation most likely occurred in the blood since the BV2 clonally expanded sequences were also detected in the circulation. One of the clonally expanded CDR3 sequences was still present in the joints and blood after two years, while other "new" additional CDR3 sequences were found in the joints at the second time point. The persistence of the clonally expanded T-cell population for a period of two years suggests a possible role of these T-cells in the disease process of this patient. Note that the number of overrepresented V genes was slightly decreased at the second sampling, however the patient already had a disease duration of 7 years at the first sampling and was treated with DMARD during the period between the samplings. The clonally expanded cells in this patient may be targeted by specific T-cell targeted immunotherapies, such as TCR or T-cell vaccination, in order to test whether the depletion of these T-cells would have an effect on the disease progression in this patient (32,33). Our results are in agreement with previous reports (28,30). Kato and coworkers (28) also described a persisting though gradually decreasing clonal expansion in a patient with chronic RA.

Based on our data we hypothesise that different T-cell activation pathways may be operating in RA. In the early RA patient the TCR expression pattern is compatible with a T-cell activation

process induced by microbial superantigens. Perhaps the synovial T-cell repertoire in this patient reflects the peripheral pool of superantigen stimulated T lymphocytes. If some of these cells would cross-react with a synovial autoantigen, they may become reactivated and will overgrow other synovial T-cells which are not reactivated by a cross-reactive epitope. Such a pattern is seen in the chronic RA patient where only a few antigenic epitopes may have been responsible for the persistent expansion of the T-cells in the joints. In subsequent stages, determinant spreading may lead to activation and expansion of other T-cell clonotypes in the synovium. Intra- and intermolecular antigen spreading (determinant spreading) was previously described in experimental autoimmune encephalomyelitis, an animal model of MS (34). Further studies need to address whether this hypothetical scenario may also operate in other RA patients. This may however explain the previously reported contradictory observations, since the TCR repertoire diversity will change during disease progression in individual patients.

TCR V gene skewing was also observed in the joints of patients with osteoarthritis, which is mainly considered to be a noninflammatory process. However, TCR V gene restriction in osteoarthritis patients was also reported by Zwillich et al. (35). Further studies need to address whether the TCR restriction in osteoarthritis patients is caused by T-cell sensitisation to cartilage antigens released as a consequence of mechanical destruction. Restricted TCR V gene expression was also observed in the joints of 4 patients with PsA, suggesting that similar T-cell mediated immune mechanisms may operate in RA and PsA. Similar observations were made by Tassiulas et al (36), who described clonal expansion in both the joints and the skin lesions of Psoriatic arthritis patients. In this sense PsA and OA patients may not be optimal controls for T-cell studies in RA.

In conclusion, the observed TCR V gene skewing in the synovium of all RA patients, the similar TCR V gene profiles in both affected joints of patients with bilateral synovitis, and the clonal expansion and persistence of T-cell clonotypes in the joints of a chronic RA patient are in agreement with an important role for T-cells in the pathogenesis of RA. However, the expanded V genes differ among patients with RA and can therefore not be targeted by a uniform TCR V gene-directed therapy. Our detailed clonal analysis of overrepresented V genes in two RA patients showed possible different T-cell activation pathways in an early and chronic RA patient. Although T-cell directed therapies might therefore be effective in RA, such therapies may need to be customised for individual patients. While in some patients a CDR3 specific approach may be effective in depleting the majority of expanded synovial T-cells, broader BV family-specific approaches may be required for other RA patients.

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References

1. Struyk, L., Hawes, G., Chatila, M., Breedveld, F., Kurnick, J. and van den Elsen, P. T-cell receptors in Rheumatoid Arthritis. *Arthritis Rheum.* 38:577-589, 1995.

2. van Boxel, J.A. and Paget, S.A. Predominant T-cell infiltrate in Rheumatoid synovial membranes. *N.Engl.J.Med.* 293:517-520, 1975.

3. Panayi, G.S. The immunopathogenesis of Rheumatoid Arthritis. *Rheumatol.Rev.* 1:63-74, 1992.

4. Nepom, G.T., Gersuk, V. and Nepom, B.S. Prognostic implications of HLA genotyping in early assessment of patients with Rheumatoid Arthritis. *J.Rheumatol.* 23 suppl 44:5-9, 1996.

5. Perdriger, A., Guggenbuhl, P., Chales, G., et al. The role of HLA DR-DR and HLA DR-DP interactions in genetic susceptibility to Rheumatoid Arthritis. *Human Immunol.* 46:42-48, 1996.

6. Howell, M.D., Diveley, J.P., Lundeen, K.A., et al. Limited T-cell receptor β chain heterogeneity among interleukin-2 receptor-positive synovial T-cells suggests a role for superantigen in Rheumatoid Arthritis. *Proc.Natl.Acad.Sci.USA* 88:10921-10925, 1991.

7. Celis, L., Vandevyver, C., Geusens, P., Dequeker, J., Raus, J. and Zhang, J. Clonal expansion of mycobacterial heat-shock protein reactive T-lymphocytes in the synovial fluid and blood of Rheumatoid Arthritis patients. *Arthritis Rheum.* 40:510-519, 1997.

8. Fox, D.A. The role of T-cells in the immunopathogenesis of Rheumatoid Arthritis: new perspectives. *Arthritis Rheum.* 40:598-609, 1997.

9. Verheijden, G., Rijnders, A., Bos, E., et al. Human cartilage glycoprotein-39 as a candidate autoantigen in Rheumatoid Arthritis. *Arthritis Rheum.* 40:1115-1125, 1997.

10. Kinne, R.W., Palombo-Kinne, E. and Emmrich, F. T-cells in the pathogenesis of Rheumatoid Arthritis. Villains or accomplices? *BBA* 1360:109-141, 1997.

11. Moreland, L.W., Heck, L.W., Koopman, W.J., et al. Vβ T-cell receptor peptide vaccine. Results of a phase I dose-finding study in patients with Rheumatoid Arthritis. *Ann.N.Y.Acad.Sci.* 756:211-214, 1995.

12. Breedveld, F.C., Struyk, L., van Laar, J.M., Miltenburg, A.M., de Vries, R.R. and van den Elsen, P. Therapeutic regulation of T-cells in Rheumatoid Arthritis. *Immunol.Rev.* 144:5-16, 1995.

13. Paliard, X., West, S.G., Lafferty, J.A., et al. Evidence for the effect of a superantigen in Rheumatoid Arthritis. *Science* 253:325-329, 1991.

14. Sottini, A., Imberti, L., Gorla, R., Cattaneo, R. and Primi, D. Restricted expression of T-cell receptor Vβ but not Vα genes in Rheumatoid Arthritis. *Eur.J.Immunol.* 21:461-466, 1991.

15. Struyk, L., Kunick, J.T., Hawes, G.E., et al. T-cell receptor V-gene usage in synovial fluid lymphocytes of patients with chronic Rheumatoid Arthritis. *Hum.Immunol.* 37:237-251, 1993.

16. Yikin, L., Guang-Rong, S., Tumang, J.R., Crow, M. and Friedman, S.M. CDR3 sequence motifs shared by oligoclonal Rheumatoid Arthritis synovial T-cells. *J.Clin.Invest.* 94:2525-2531, 1994.

17. Khazaei, H.A., Lunardi, C. and So, A.K. CD4 T-cells in the rheumatoid joint are oligoclonally activated and change during the course of the disease. *Ann.Rheum.Dis.* 54:314-317, 1995.

18. Davey, M.P., Burgoine, G.A and Woody, C.N. TCRB clonotypes are present in CD4+ T-cell populations prepared directly from rheumatoid synovium. *Hum.Immunol.* 55:11-21, 1997.

19. Jendro, M.C., Ganten, T., Matteson, E.L., Weyand, C.M. and Goronzy, J.J. Emergence of oligoclonal T-cell populations following therapeutic T-cell depletion in Rheumatoid Arthritis. *Arthritis Rheum.* 38:1242-1251, 1995.

20. Hakoda, M., Ishimoto, T., Yamamoto, K., et al. Clonal analysis of T-cell infiltrates in synovial tissue of patients with Rheumatoid Arthritis. *Clin.Immunopathol.* 57:387-398, 1990.

21. Hingorani, R., Monteiro, J., Furie, R., et al. Oligoclonality of Vβ 3 TCR chains in the CD8+ T-cell population of Rheumatoid Arthritis patients. *J.Immunol.* 156:852-858, 1996.

22. Maruyama, T., Saiti, I., Miyake, S., et al. A possible role of two hydrophobic amino acids in antigen recognition by synovial T-cells in Rheumatoid Arthritis. *Eur.J.Immunol.* 23:2059-2065, 1993.

23. Bröker, B., Korthäuer, U., Heppt, P., et al. Biased T-cell receptor V-gene usage in Rheumatoid Arthritis. *Arthritis Rheum.* 36:1234-1243, 1993.

24. VanderBorght, A., Van de AA, A., Geusens, P., Vandevyver, C., Raus, J. and Stinissen, P. Identification of overrepresented TCR genes in blood and tissue biopsies by PCR-ELISA. *J.Immunol.Methods* 223:47-61, 1999.

25. Firestein, G.S. and Zvaifler, N. How important are T-cells in chronic rheumatoid synovitis? *Arthritis Rheum.* 33:768-773, 1990.

26. Fisher, D.K., Opalka, B., Hoffman, A., Mayr, W. and Haubeck, H.D. Limited heterogeneity of rearranged T-cell receptor Vα and Vβ transcripts in synovial fluid T-cells in early stages of Rheumatoid Arthritis. *Arthritis Rheum.* 39:454-462, 1996.

27. Struyk, L., Hawes, G.E., Mekkers, H.M.M., Tak, P.P., Breedveld, F.C. and van den Elsen, P. Molecular analysis of the T-cell β chain repertoire in early Rheumatoid Arthritis: heterogeneous TCR BV gene usage with shared amino acid profiles in CDR3 regions of T-lymphocytes in multiple synovial tissue needle biopsies from the same joint. *Eur.J.Clin.Invest.* 26:1092-1102, 1996.

28. Kato, T., Kurokawa, M., Masuko-Hongo, K., et al. T-cell clonality in synovial fluid of a patient with Rheumatoid Arthritis: persistent but fluctuant oligoclonal T-cell expansions. *J.Immunol.* 159:5143-5149, 1997.

29. Struyk, L., Hawes, G.E., Dolhain, R.J., et al. Evidence for selective *in vivo* expansion of synovial tissue-infiltrating CD4+CD45RO+ T-lymphocytes on the basis of CDR3 diversity. *Int.Immunol.* 6:897-907, 1994.

30. Alam, A., Lambert, N., Lulé, J., et al. Persistence of dominant T-cell clones in synovial tissues during Rheumatoid Arthritis. *J.Immunol.* 156:3480-3485, 1996.

31. Drake, C. and Kotzin, B.L. Superantigens: biology, immunology and potential role in disease. *J.Clin.Immunol.* 12:149-162, 1992.

32. Vandenbark, A.A., Chou, Y.K., Whitham, R., et al. Treatment of multiple sclerosis with T-cell receptor peptides: results of a double-blind pilot trail. *Nature Medicine* 2:1109-1115, 1996.

33. Zhang, J.W., Medaer, R., Stinissen, P., Hafler, D. and Raus, J. MHC restricted clonotypic depletion of human myelin basic protein-reactive T-cells by T-cell vaccination. *Science* 261:1451-1454, 1993.

34. Lehman, P.V., Forsthuber, T., Miller, A. and Sercarz, E.E. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155-158, 1992.

35. Zwillich, S.H., Fang, Q., Kieber-Emmons, T., et al. V alpha gene usage in rheumatoid compared with osteoarthritis synovial tissue T-cells. *DNA and Cell.Biol.* 13:923-931, 1994.

36. Tassiulas, I., Duncan, SR., Cantola, M., Theophilopoudos, AN;, Boumpas, DT., Clonal characteristics of T-cell infiltrates in skin and synovium of patients with psoriatic arthritis. *Hum. Immunol.* 60(6), 479-491, 1999.

Chapter 5

Dynamic T-cell Receptor Clonotype Changes in Synovial Tissue of Early Rheumatoid Arthritis Patients Are Influenced by Treatment With Cyclosporin A (Neoral®)

Based on:

Dynamic T-cell receptor clonotype changes in synovial tissue of early rheumatoid arthritis patients are influenced by treatment with Cyclosporin A (Neoral)

Ann VanderBorght, Filip De Keyser, Piet Geusens, Marc De Backer, M. Malaise, Dominique Baeten, E. Vanden Bosch, Eric M. Veys, Jef Raus and Piet Stinissen

Submitted for publication

Abstract

Objective: To study the TCR changes in synovial membrane over a 16 week period in early RA patients and to evaluate the influence of cyclosporin A (CSA) on the TCR repertoire in early RA patients.

Methods: Synovial tissue biopsies and paired blood samples were obtained from 12 early RA patients at two time points, and 4 early RA patients at one time point. Seven patients were treated with CSA (Neoral-Sandimmun®, 3 mg/kg/day) and 5 patients with placebo for 16 weeks. TCR V gene repertoires were analyzed by semiquantitative PCR-ELISA, and CDR3 spectratyping was used to compare TCR clonotype distributions of expanded T-cell populations in blood and biopsies at both time points.

Results: TCR specific mRNA was detected in all synovial tissue biopsies at the first sampling, but only in biopsies of 8/12 RA patients 16 weeks later (4/7 CSA group, 4/5 placebo group). Overrepresented TCR BV genes (>15 %) were found in the biopsies of 9/12 patients at the first time point, and in 6/12 patients after 16 weeks (3/7 CSA, 3/5 placebo). BV genes overrepresented in synovial tissue were not overrepresented in blood. The overrepresented BV genes in synovial tissue were different at both time points in all placebo patients, whereas some BV families remained overrepresented at the second sampling in biopsies of three CSA-treated patients. CDR3 spectratyping demonstrated TCR clonotype persistence in synovial biopsies of three CSA-treated patients but in none of the control patients.

Conclusions: Skewed TCR profiles were found in the synovial tissue of most early RA patients. In the control RA patients, the TCR repertoire changed during the 16 week follow-up, with no evidence for T-cell persistence. These data suggest a dynamic process of T-cell recruitment in the joints of these patients, possibly due to activation of new T-cell clones. This process apparently is influenced by CSA-treatment, since synovial tissue T-cells were either no longer detected, or contained persisting TCR clonotypes 16 weeks after treatment with CSA. Further studies need to resolve whether the dynamic repertoire changes seen in the joints of the control patients contribute to the chronicity of the disease process. These data may have important consequences on the design of T-cell targeted therapies for RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the synovial membranes of multiple joints. Although RA is considered to be a typical autoimmune disease, its etiology is still unknown (1). Several lines of evidence suggest that T lymphocytes are involved in the initiation and the perpetuation of RA, although the disease-mediating lymphocytes have not been identified yet (2-4). Since little information is available on candidate autoantigens in RA, many groups have compared the T-cell receptor (TCR) repertoire of synovial T-cells and blood derived T-cells, to identify disease-relevant TCR V genes or T-cell subtypes (reviewed in (3,5)). However, these studies have yielded conflicting information, which can be due to the use of different techniques (various PCR protocols versus flowcytometry), to the use of different study material (synovial fluids or membranes, with or without *in vitro* selection), or to the genetic and clinical heterogeneity of the patient populations studied. Another important parameter that may complicate the interpretation of these data is the variable disease duration of the RA patients included. We and others have shown that the TCR repertoire in the synovium becomes more diverse along the progression of the disease (6-10). This process may be caused by the infiltration of irrelevant 'bystander' T-cells, or to the influx of newly activated T-cells by locally sequestered antigens, a process termed determinant spreading (11). Determinant spreading has been shown to operate in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (11). In addition, it has been hypothesized that T-cells play a prominent role in the early stages of the disease process only, while T-cell independent mechanisms are involved in later pathogenic steps of RA (4,12). Furthermore, pathogenic T-cells may only be present at increased levels in early autoimmune lesions, as demonstrated by analysis of new and old brain lesions in rodents with EAE (13). Studies aimed at defining the role of T-cells as possible initiators of the disease process should therefore be focused on patients at an early phase of the disease.

We have evaluated TCR V gene repertoire changes and TCR clonotype persistence in the synovial tissue and paired blood of patients with early RA. In addition, we examined whether treatment with cyclosporin A microemulsion (CSA, Neoral-Sandimmun®) influences these TCR changes. As a result of the enormous diversity created in the hypervariable region of the TCR chains, each T-cell expresses a unique TCR clonotype, and these clonotypes can be used to trace individual T-cell subsets along the disease process. Twelve early RA patients treated with either Neoral (n=7) or placebo (n=5) were studied to evaluate the effects of CSA treatment on the TCR profile in the joints. All patients were sampled for blood and synovial tissue at entry and after 16 weeks of treatment. TCR V gene expression was studied by PCR-ELISA (14), while persistence of TCR clonotypes was confirmed by CDR3 spectratyping (15). CSA is effective in improving clinical parameters in RA patients (16, 17). The microemulsion-based formulation of CSA (Neoral®) has an increased bioavailability, and has been shown to have a

similar safety, tolerability and efficacy profile as the original preparation (Sandimmun®) (18,19). CSA is an immunosuppressive drug which specifically blocks T-lymphocyte activation through inhibition of the antigen induced secretion of IL-2 and other inflammatory cytokines, such as IFN-γ and IL-4, at the transcriptional level (18-20). Thus, our study was aimed to provide information on the TCR repertoire changes in the synovial tissue of patients during a 16 weeks follow-up period, while a subgroup of patients was treated with an agent that prevents or suppresses *in vivo* activation of new T-cells.

Patients and Methods

Patient population and study design

The objectives of this study were to study TCR repertoire in blood and synovial membrane in early RA patients, and to analyze the TCR repertoire changes in synovial membrane over a period of 16 weeks in early RA patients. In addition, the effects of CSA-treatment on the TCR expression of peripheral blood and joint T lymphocytes were studied in a subgroup of patients, and correlated with clinical efficacy. Eighteen early RA patients with a disease duration of less than one year from three centers were included in a double-blinded, placebo-controlled study (Table 5.1). The patients either met the 1987 revised ACR criteria (17 patients) (21), or presented with synovitis and/or inflammatory polyartralgia, and were positive both for one of the RA-related subtypes of HLA-DR4 or HLA-DR1 and for rheumatoid factor (one patient, ELB). All patients had oral piroxicam as the only treatment before entering the study, and this treatment was continued during the study. After giving signed informed consent, patients were randomized and 12 patients were treated with a cyclosporin A microemulsion (Neoral-Sandimmun), and 6 patients were treated with placebo for 16 weeks. Peripheral blood and synovial membrane tissue was sampled at entry and at week 16. Synovial tissue was obtained by fine needle arthroscopy (22). Approximately 15 small biopsies were obtained at each arthroscopy and samples were preferentially taken in macrosopically inflamed areas of the synovial membrane. Patients were clinically examined by blinded rheumatologists at week –3, -1 (baseline), and at week 2, 4, 6, 8, 10, 12 and week 16. Three patients on CSA therapy were withdrawn from the study before week 16 because of an increase of serum creatinine (n=1), gastric complaints (n=1) and flare up of disease (n=1). Two other patients who were followed up until week 16 discontinued the study medication prematurely because of an increase of serum creatinine (1 CSA) and thrombocytopenia (1 placebo). One placebo treated patient violated the study protocol because of the administration of an intra-articular diprophos (6.03 g/ml β methazondipropionaat and 2.63 g/ml β methazondinatriumfosfaat) injection.

Blood and synovial tissue was available for TCR analysis from 7 CSA-treated and 5 placebo-treated RA patients at both time points (Table 5.1). Two of these patients (ELB and AH) discontinued the study medication at week 9 and week 12 respectively, but were still included in the TCR analysis. Patient ELB was treated with CSA and patient AH with placebo.
The study protocol was approved by the ethical committees of all participating centers.

TCR repertoire screening and CDR3 spectratype analysis

RNA extraction and cDNA synthesis was performed as described in 2.3. TCR V gene usage was performed by semiquantitative PCR-ELISA as described in 2.4.(23). CDR3 region fragment size analysis was performed as described in 2.5.2.

Table 5.1: Patient characteristics

Patient Age/Sex	Group	Sampling start	Sampling end	Swollen joints		Tender joints		Included in TCR analysis		Comments
		PB/ST	PB/ST	start	end	start	end	start	end	
ADS 54/M	CSA	+/+	+/+	15	9	10	0	yes	yes	
ANB 67/F	CSA	+/+	+/+	6	4	10	7	yes	yes	
LOV 46/F	CSA	+/+	+/+	19	6	26	5	yes	yes	
PAG 46/M	CSA	+/+	+/+	7	7	6	9	yes	yes	
BEV 40/F	CSA	+/+	+/+	7	3	11	5	yes	yes	
MAV 37/F	CSA	+/+	+/+	10	8	13	4	yes	yes	
ELB 60/F	CSA	+/+	+/+	0	0	2	2	yes	yes	Stopped medication after wk 9 [1]
MJS 62/M	CSA	+/+	-/-	1	0	6	4	yes	no	
GBV 46/F	CSA	+/+	-/-	11	4	15	13	yes	no	Stopped medication after wk8 [1]
DEA 18/F	CSA	+/+	-/-	14	NT	16	NT	yes	no	Stopped medication after wk 11 [1]
GAJ 65/F	CSA	+/+	+/-	7	7	28	24	no	no	Stopped medication after wk 1 [1]
FAG 37/F	CSA	-/-	+/+	2	0	2	0	no	no	
NIG 59/M	Placebo	+/+	+/+	11	3	17	7	yes	yes	
DAL 35/M	Placebo	+/+	+/+	13	13	23	23	yes	yes	
LED 68/F	Placebo	+/+	+/+	7	8	6	1	yes	yes	
GEM 64/F	Placebo	+/+	+/+	4	4	3	2	yes	yes	
AH 70/M	Placebo	+/+	+/+	3	2	3	2	yes	yes	Stopped medication after wk 12 [1]
JAL 43/F	Placebo	+/+	+/-	12	7	13	6	yes	no	Extra medication [2]

[1]: The reasons for ending the medication protocol are summarized in the Results.
[2]: Intra-articular diprophos injection. NT: not tested, due to drop out. All patients were HLA DR1+ or DR4+.

Results

Clinical results

A reduced number of swollen and tender joints was observed in 11 out of 18 patients (8/12 CSA group and 3/6 placebo group) (Table 5.1). This reduction was more pronounced in the CSA-treated versus placebo-treated group. The clinical efficacy data of the intention-to-treat population (N=18) are summarized in table 5.2. Although these data show a statistically significant improvement of some clinical parameters in the CSA-treated patients as compared to the placebo treated patients, the small size of this explorative study does not allow to draw conclusions on the clinical efficacy. Hence, these results should be interpreted with caution because this small study was statistically not sufficiently powered to demonstrate clinical efficacy. Detailed clinical data from this limited trial will be published in a separate report (in preparation).

Table 5.2 : Clinical results (intention-to-treat population, n=18)

		Intention-To-Treat analysis[1]			
		CSA (n=12)		Placebo (n=6)	
		Number	P-value	Number	P-value
Swollen joints (n)	Entry[2]	8.3 ± 1.67	0.007	8.3 ± 1.75	NS
	End[2]	4.4 ± 0.99		6.2 ± 1.67	
Tender joints (n)	Entry	12.1 ± 2.39	0.01	10.8 ± 3.34	NS
	End	6.6 ± 2.08		6.8 ± 3.38	
Pain (VAS)	Entry	4.8 ± 0.86	NS	5.2 ± 1.10	NS
	End	3.1 ± 0.72		5.0 ± 1.42	
Patient global assessment (VAS)	Entry	5.2 ± 0.66	NS	5.6 ± 1.22	NS
	End	4.0 ± 0.87		4.3 ± 1.18	

[1]: Mean values ± standard error of the mean; [2]: entry = week −1, end = week 16
VAS = Visual Analogue Scale; NS = Not significant

TCR BV expression in blood and synovial tissue biopsies at the first time point

RNA was extracted from PB and synovial tissue biopsies of 16 early RA patients and reverse transcribed into cDNA. The integrity of the cDNA, and the T-cell specific mRNA content of the samples was analyzed by control PCR amplifications with primers specific for the house-keeping gene (β2 microglobulin) and primers specific for the TCR BC region. Successful control amplifications were obtained for all PBMC and biopsy samples (data not shown).
PCR-ELISA was then used to analyze the TCR BV gene repertoire at the first time point. The TCR BV gene repertoire was highly diverse in PB and synovial tissue of all patients. All patients expressed at least 12 different BV genes in the blood (mean: 17.6). Fewer BV genes were expressed in the synovial biopsies (mean: 14.8). This reduction was more pronounced in the biopsies of patients LOV, LED and GEM, in which expression of only 5, 7 and 9 BV genes was detected, respectively.

To study possible T-cell expansions, we subsequently identified the BV genes that were overrepresented in PB or synovial tissue. Overrepresented TCR BV genes were arbitrarily defined as representing more than 15% of the total repertoire, based on previous data showing individual BV gene expression levels of less than 15% in the blood of healthy individuals (23). Overrepresented BV genes were identified in biopsies of 13/16 patients, and in PB of 5/16 patients (Table 5.3).

Table 5.3: Overview of preferentially used TCR BV genes before and after treatment

A. Early RA patients examined at two time points

CSA-treated group				Placebo group			
Patient	sample	BV genes overrepresented[1]		Patient	sample	BV genes overrepresented	
		At entry	After 4 months			At entry	After 4 months
ADS	PBMC	none	none	NIG	PBMC	none	9
	Biopsy	2,3	2		Biopsy	17, 18	2, 9
ANB	PBMC	1,17	none	DAL	PBMC	2, 3	none
	Biopsy	2,4	none		Biopsy	none	2
LOV	PBMC	none	5,13.2	LED	PBMC	none	none
	Biopsy	1,13.2	6,13.2		biopsy	2, 9	4
PAG	PBMC	none	none	GEM	PBMC	none	none
	Biopsy	6,7,13.2,17	6		biopsy	13.2	none
BEV	PBMC	none	1,4	AH	PBMC	none	none
	Biopsy	none	no T-cell mRNA[2]		Biopsy	none	no T-cell mRNA
MAV	PBMC	none	none				
	Biopsy	2,13.2	no T-cell mRNA				
ELB	none	ND	ND				
	Biopsy 1	6,9	no T-cell mRNA				
	Biopsy 2	ND	no T-cell mRNA				

B. Early RA control patients examined at a single time point

Patient	sample	BV genes overrepresented
MJS	PBMC	8
	Biopsy	2,3,4,18
JAL	PBMC	12
	Biopsy	2,4,13.1,18
GBV	PBMC	9
	Biopsy	2,4,12,18
DEA	PBMC	18
	Biopsy 1	4,18
	Biopsy 2	4

[1]: Lists the TCR BV genes that were overrepresented (more than 15% of the total BV gene expression) in the sample. [2]: Control PCR amplifications were performed with primers specific for β_2 microglobulin to check the integrity of the isolated RNA, and with primers specific for the constant region of the TCR β-chain (TCR BC) to evaluate the TCR mRNA content. All β_2 microglobulin PCR amplifications were successful. TCR BC PCR's were not successful, for some samples, indicating that no TCR mRNA is present. ND: not done

The mean number of overrepresented BV genes was higher in biopsies (2.06±0.35) than in PB (0.44±0.18, $p<0.05$). Some BV genes were found to be overrepresented in biopsies of a high proportion of the early RA patients. For example, BV2 was overrepresented in biopsies of 44% of the patients, while BV4 and BV18 were overrepresented in synovial tissue of 31% of the patients.

The overrepresented BV genes (>15%) in PB differed from those in the biopsies of all patients except patient DEA, who had an overexpression of BV18 in blood and synovial biopsy (Table 5.3, Fig. 5.1 and 5.2). However, in five patients one or more BV genes were expressed at a level of 10-15 % in both blood and synovial tissue (Fig.5.1 and 5.2). For example, the BV2 and BV13.2 genes were expressed at 10-15% in blood and synovial tissue of patients ADS and NIG respectively (Fig. 5.1). In conclusion, the TCR repertoire of the early RA patients was diverse in both PB and synovial tissue, although overrepresentation of some TCR BV genes was more often detected in the synovial tissue, indicating a preferential accumulation of some T-cell subtypes at the disease site.

Comparison of the TCR BV expression in synovial biopsies and lavage in four patients

To asses whether TCR BV gene repertoires are similar in synovial tissue and paired lavage fluid, TCR profiles of paired samples from 4 control patients were analyzed. In one patient, synovial tissue was analyzed from two different biopsies of the same joint. As illustrated in Fig. 5.2, the TCR BV expression profile was similar in the lavage fluid and synovial tissue in patients DEA, JOL and MJS, but was slightly different in patient GBV. Eight BV genes were expressed at more than 10% in both the synovial tissue and the lavage fluid (Fig. 5.2). The TCR BV gene repertoires in the two biopsy samples of patient DEA were highly comparable. Increased expression of the BV4 and BV18 genes was seen in both biopsy samples, although some TCR BV genes expressed at low level (<5%) were only seen in one sample. In conclusion, although the TCR expression profile of the biopsies may reflect the T-cell composition at one site in the joint only, it seems to correlate with the profile at another site in the joint and with the profile in the lavage fluid, suggesting that analysis of synovial tissue biopsies provides a reasonable estimate of the TCR expression profile in the joint.

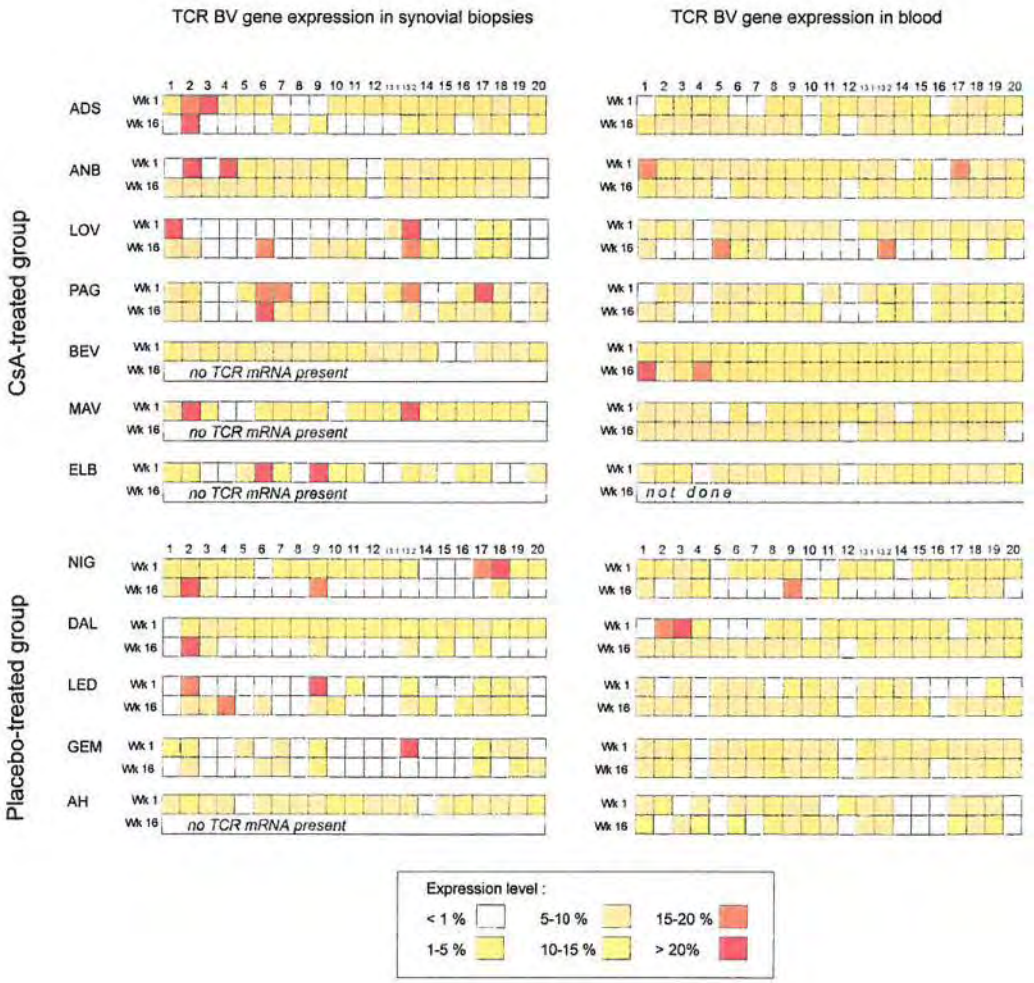

Figure 5.1: T-cell receptor V gene expression profile at two timepoints in synovial tissue and peripheral blood of early RA patients treated with CSA (Neoral) or placebo.

TCR BV gene expression in blood and synovial biopsies after 4 months

To study the fluctuations in the TCR profile over time, PBMC and synovial tissue biopsies of 12 patients were analyzed after 4 months. The control β2-microglobulin PCR amplification was successful for all PBMC and biopsy samples. The TCR BV specific control PCR was successful for all PBMC samples, but only for 8/12 biopsy samples, indicating that 4 biopsy samples did not contain sufficient TCR specific mRNA for further analysis by PCR-ELISA. In one patient (ELB) two independent synovial biopsies from the same joint were negative for the control TCR specific PCR. TCR mRNA negative biopsies were more frequently observed in the CSA-treated patients (3/7) than in the placebo group (1/5) (Not significant).

As for the first sampling, also at the second time point, most TCR repertoire profiles were heterogeneous, in blood as well as synovial tissue samples. In PB the total number of expressed BV genes (>1%) remained similar at both time points, irrespective of the treatment (Fig. 5.1). In the synovial tissue, one patient expressed more BV genes at the second time point, while the remaining patients had either less (3 patients), or a similar (3 patients) number of BV genes. The reduction of expressed BV genes in the synovial tissue was higher in the placebo group (mean: 14.4 before, 7.5 after) than in the CSA-treated group (mean: 14.4 before, 12.8 after).

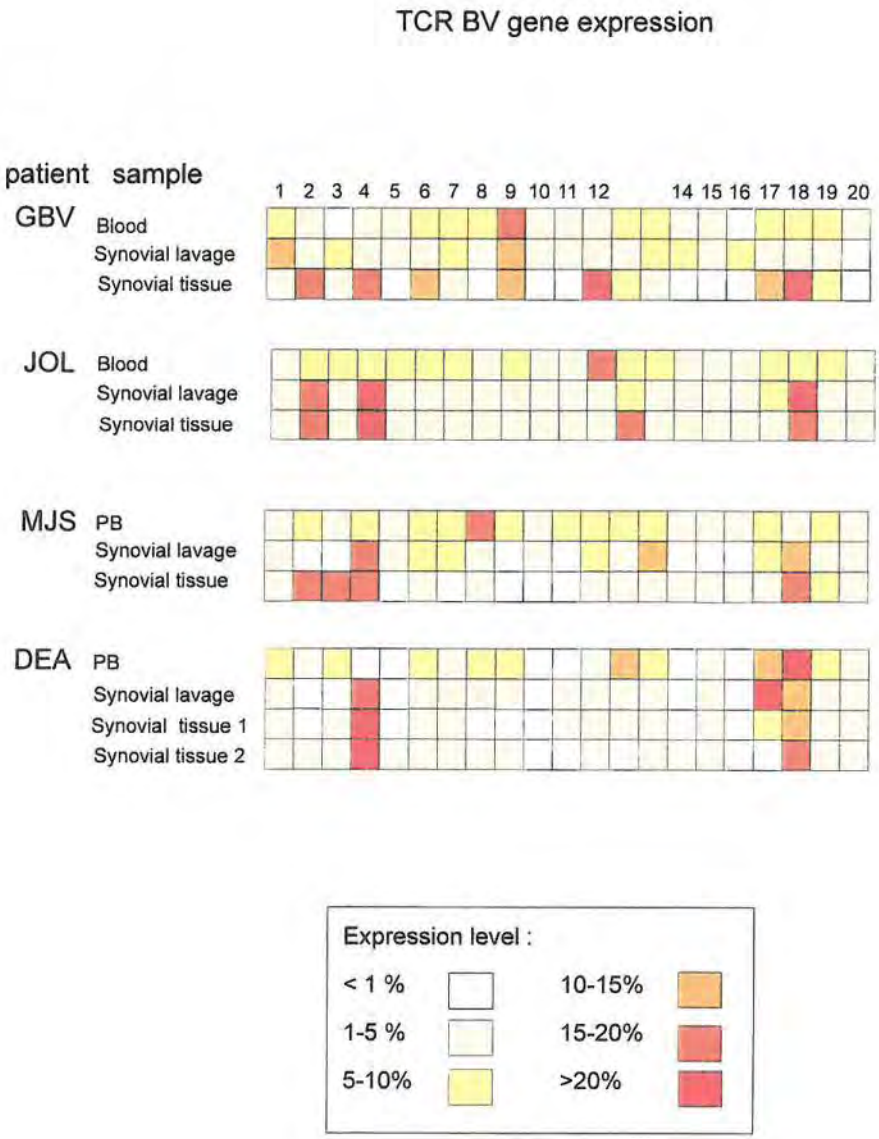

Figure 5.2: T-cell receptor V gene expression profiles in synovial tissue and lavage fluid of four early RA patients.

We then examined possible changes in the number of BV genes that were overrepresented (>15%) in the patient groups. Overrepresented BV genes were found in 3/12 PBMC samples (2/7 CSA-group and 1/5 placebo group), which is comparable with the data at entry. Overrepresented BV genes were identified in most biopsy samples that contained TCR mRNA: in 3/4 samples of the CSA-group and 3/4 samples of the placebo-group. A significant reduction in the number of overrepresented TCR BV genes was observed in synovial biopsies of the CSA-treated patients ($p<0.05$), but to a lesser extent in the control group (Table 5.4).

Table 5.4: Mean number of overrepresented TCR V genes at entry and at week 16 after treatment with CSA or placebo

		Mean number of overrepresented TCR BV genes (± standard error)		
Sample	Group	At entry	At week 16	
Peripheral blood				
	Total group (n=12)	0.25 ± 0.20	0.43 ± 0.23	
	CSA-treated (n=7)	0.29 ± 0.29	0.67 ± 0.39	
	Placebo-treated (n=5)	0.40 ± 0.40	0.20 ± 0.20	
Synovial biopsy				
	Total group (n=12)	1.13 ± 0.29	0.50 ± 0.22	*(p < 0.05)*
	CSA-treated (n=7)	2.00 ± 0.44	0.57 ± 0.30	*(p < 0.05)*
	Placebo-treated (n=5)	1.00 ± 0.44	0.80 ± 0.37	

Patients with no TCR specific mRNA as tested by a control PCR amplification with BC specific primers were scored as having no overrepresented BV genes.

In conclusion, a significant fraction of patients treated with CSA no longer had TCR specific mRNA present in the synovial biopsies, suggesting either an inhibition of TCR mRNA expression in the synovial tissue, or the absence (or reduction below the detection limit) of T-cells in these tissues.

Persistence of overrepresented TCR BV genes in synovium and blood

We then studied whether some BV gene families persisted in the joints of the early RA patients during a 4 month-follow-up period, and whether overrepresented BV gene families consisted of

mono, oligo- or polyclonally expanded T-cells. Persistence of BV families at the disease site is a possible indication that the corresponding T-cells may be involved in the disease process. The pattern of overrepresentation was different in the PB of most patients at both samplings. Patient PAG of the CSA-treated group, was the only patient who expressed similar BV genes (BV13.2 and BV19) in PB at increased levels (10-15%) at both time points (Fig. 5.1).

In synovial tissue, differences were seen for the CSA-treated patients as compared to the placebo controls. The CSA-treated patients ADS, LOV and PAG, who had sufficient TCR mRNA in the biopsies at the second sampling, had an overrepresentation (>15%) of one or two TCR BV genes in the synovial tissue. Some overrepresented BV genes identified at the first sampling were no longer overrepresented at the second sampling. However, in these three CSA-treated patients at least one BV gene was overrepresented at both time points (Table 5.3). In contrast, none of the BV genes was overrepresented at both samplings in the biopsies of the placebo group. These data suggest that some dominant T-cell populations persisted in the synovial tissue of CSA-treated patients, but not in tissue of placebo controls.

Confirmation of T-cell persistence in synovium by CDR3 spectratyping

CDR3 spectratype analysis was used to further confirm the persistence of overrepresented TCR BV gene families in the CSA-treated group. This technique provides information on the clonal distribution of individual T-cell populations based on the analysis of the fragmentlength of PCR amplified CDR3 regions, which are depicted as peaks in the profile. In addition, the heights of the peaks correspond to the intensity of the bands. When bands of the same CDR3 length are shared between T-cell subsets with identical BV (or BV-BJ) expression, this is a strong indication that identical T-cell clonotypes are present in these samples (15). CDR3 spectratype analysis was performed for 23 BV families overrepresented at either time point in the synovial tissue of 5 CSA-treated and 4 placebo-treated patients (Table 5.5 and Fig. 5.3). These BV gene specific CDR3 spectratypes were studied in the blood and tissue samples at both time points.

The first type of information from this analysis is whether the T-cells of a particular BV family are of a poly-, oligo- or mono-clonal origin. Spectratypes with a Gaussian distribution profile with at least 4 bands represent a polyclonal T-cell population. A less heterogeneous profile with 2-4 bands suggests an oligoclonally expanded T-cell population, whereas a CDR3 region profile representing a single band including the majority of the peak area strongly suggests a monoclonal T-cell population. Previous studies have shown that dominant peaks represent clonal expansions within a particular BV family (15). All CDR3 profiles in the blood were poly- or slightly oligoclonally restricted at both time points, in both patient groups (Table 5.5). Some polyclonal spectratypes did not show a typical Gaussian distribution, but contained bands at increased frequency, suggesting that they correspond to T-cell populations which are expressed

at increased levels in the blood. For example, the BV9 gene spectratype of patient LED at entry contained a band of increased peak height (Fig 5.3). At the second time point the CDR3 profiles in blood were either less, more or equal heterogeneous, but there were no obvious differences between the two patient groups (Table 5.5). As all CDR3 profiles in PB were highly complex, it is difficult to draw conclusions on the persistence of T-cell clonotypes, since different T-cell clonotypes with the same CDR3 length can be present in these heterogeneous samples.

In the synovial tissue biopsies, however, the majority of analyzed BV genes were of oligoclonal (52%) or monoclonal (33%) origin. TCR BV gene families that were overrepresented in biopsies (>15%) were predominantly mono- or oligoclonal, indicating that only a limited number of T-cell clonotypes were represented in these expanded T-cell populations. The diversity of about halve of the synovial CDR3 spectratypes remained unchanged at the second time point, while the remaining profiles became either broader or more skewed, irrespective of the treatment. Interestingly, in all synovial spectratypes of BV families analyzed at both time points of the three CSA-treated patients who had sufficient TCR mRNA in the biopsies for PCR analysis, persistence of at least one CDR3 band was observed (Table 5.5). In marked contrast, no persistent CDR3 bands were observed in the synovial tissue of placebo control patients.

In conclusion, the CDR3 spectratype analysis confirmed the persistence of T-cell clonotypes along the 4 month follow-up period in synovial tissue of the CSA-treated patients, while dynamic CDR3 profile changes with no persisting clonotypes were found in the synovial tissue of placebo controls.

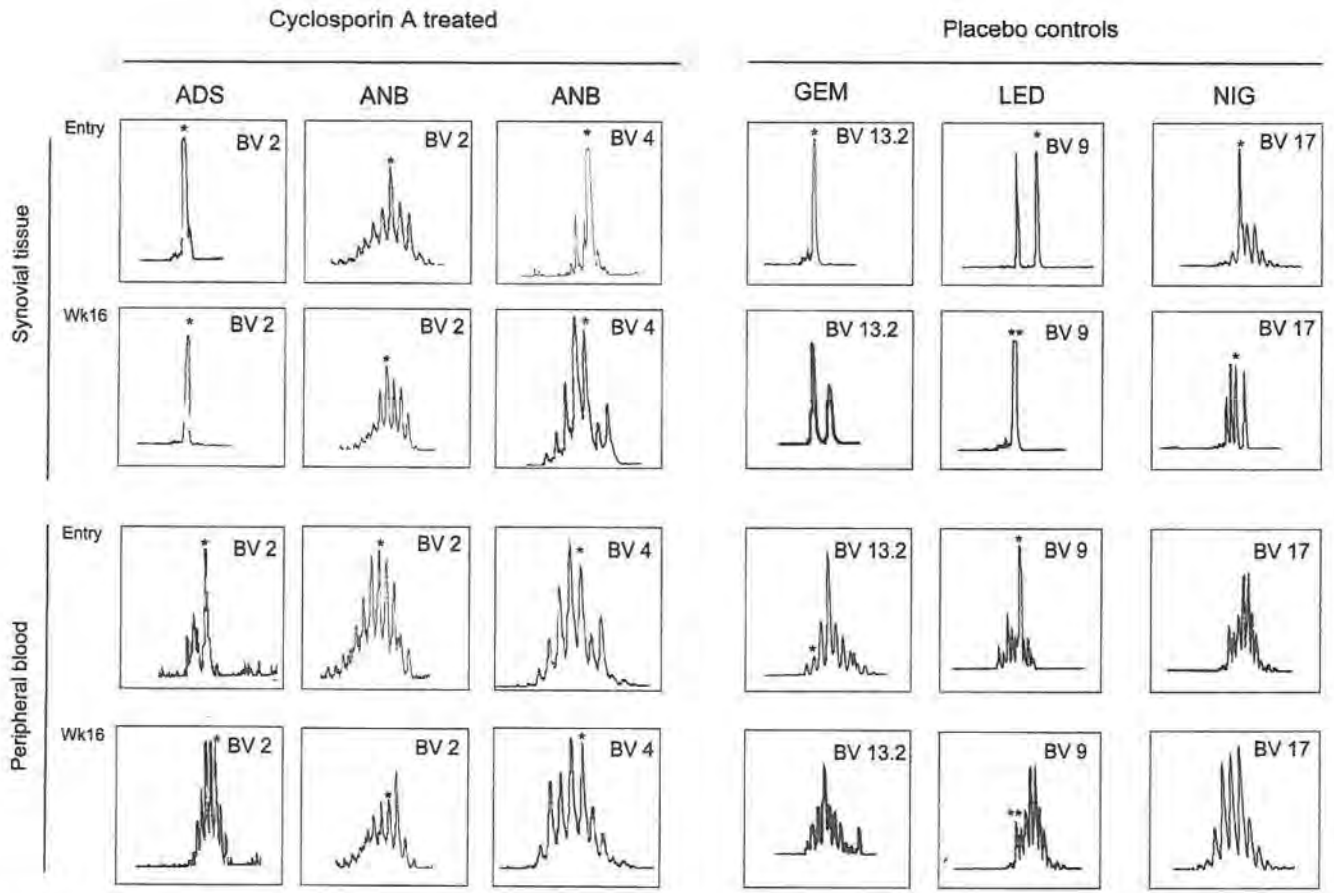

Figure 5.3: CDR3 spectratype analysis of selected TCR BV gene families that were overrepresented in blood or synovial tissue at the first or second sampling
In the CSA-treated patients, peaks with identical CDR3 length in blood and synovial tissue are marked with *. In the placebo control group, CDR3 peaks in the synovial tissue identified at the first timepoint were no longer found at the second timepoint, except for patient NIG, the peak marked with * was found in the synovial tissue at both timepoints. Some CDR3 peaks were identified in the blood and the synovial tissue at the same timepoint (marked with * or **).

Table 5.5. CDR3 spectratype analysis of relevant BV genes in synovial tissue and blood

CSA-treated group

Patient	BV gene	sample	BV expression (%)		CDR3 spectratype analysis			
			Entry	wk 16	CDR3 profile[1] entry	wk16	Profile changes	persistence in[2] in synovial tissue?
ADS	BV2	PBMC	10-15	5-10	oligo	poly	broadening	
		Biopsy	>15	>15	mono	mono	no change	Yes
ANB	BV2	PBMC	1-5	5-10	poly	poly	no change	
		Biopsy	>15	5-10	poly	poly	no change	Yes
	BV4	PBMC	5-10	5-10	poly	poly	no change	
		Biopsy	>15	5-10	oligo	poly	broadening	Yes
LOV	BV1	PBMC	5-10	5-10	poly	NT		
		Biopsy	>15	5-10	mono	mono	no change	No
	BV6	PBMC	5-10	10-15	poly	poly	no change	
		Biopsy	1-5	>15	mono	oligo	broadening	Yes
	BV13.2	PBMC	5-10	>15	poly	poly	no change	
		Biopsy	>15	>15	oligo	oligo	no change	Yes
PAG	BV6	PBMC	5-10	5-10	poly	oligo	skewing	
		Biopsy	>15	>15	oligo	oligo	no change	Yes
	BV7	PBMC	5-10	10-15	poly	poly	broadening	
		Biopsy	>15	5-10	poly	oligo	skewing	Yes
	BV13.2	PBMC	10-15	10-15	oligo	oligo	skewing	
		Biopsy	>15	5-10	oligo	oligo	no change	Yes
	BV17	PBMC	5-10	5-10	poly	oligo	skewing	
		Biopsy	>15	5-10	oligo	mono	skewing	Yes
ELB	BV6	PBMC	5-10	5-10	poly	NT	NA	
		Biopsy	>15	no T-cell mRNA	mono	NT	NA	
	BV9	PBMC	5-10	5-10	poly	NT	NA	
		Biopsy	>15	no T-cell mRNA	oligo	NT	NA	

Table 5.5 : continued

Placebo group

Patient	BV gene	sample	BV expression (%)		CDR3 spectratype analysis			
					CDR3 profile		Profile	persistence in
			Entry	wk 16	entry	wk16	changes	in synovial tissue?
NIG	BV2	PBMC	1-5	>1	poly	NT	NA	
		Biopsy	1-5	>15	mono	oligo	broadening	No
	BV9	PBMC	1-5	>15	poly	poly	no change	
		Biopsy	1-5	>15	mono	oligo	broadening	No
	BV17	PBMC	1-5	10-15	poly	poly	no change	
		Biopsy	>15	<1	oligo	NT	NA	
	BV18	PBMC	<1	5-10	NT	poly	NA	
		Biopsy	>15	1-5	oligo	oligo	no change	No
DAL	BV2	PBMC	>15	5-10	poly	poly	no change	
		Biopsy	1-5	>15	oligo	oligo	no change	No
LED	BV2	PBMC	<1	1-5	NT	poly	NA	
		Biopsy	>15	5-10	oligo/mono	oligo	broadening	No
	BV4	PBMC	<1	<1	NT	NT	NA	
		Biopsy	<1	>15	NT	mono	NA	
	BV9	PBMC	10-15	5-10	oligo	poly	broadening	
		Biopsy	>15	5-10	oligo	mono	skewing	No
GEM	BV9	PBMC	5-10	5-10	poly	poly	no change	
		Biopsy	10-15	1-5	oligo	oligo	no change	No
	BV13.2	PBMC	5-10	5-10	poly	poly	no change	
		Biopsy	>15	<1	mono	NT	NA	
	BV17	PBMC	5-10	5-10	poly	poly	no change	
		Biopsy	10-15	10-15	poly	oligo	skewing	No

CDR3 spectratypes were determined by amplification of CDR3 regions of selected BV genes using fluorescently labeled PCR primers, and subsequent separation of amplified products on high resolution polyacrylamide gels and analysis by an automated DNA sequence analyzer. The fragment lengths were calculated by using appropriate size standards and Genescan software.

[1]. The CDR3 profile was considered polyclonal (poly), oligoclonal (oligo), or monoclonal (mono) when respectively more than 4, between 2 and 4, or only a single band was observed. [2]. If bands of the same length (the same mobility) were present in the synovial tissue samples at entry and week 16, then it was assumed that T-cells with corresponding CDR3 regions persisted in the synovium. NT: not tested, NA: not applicable.

Discussion

The purpose of this study was to analyze the TCR repertoire changes over a 16 week period in the synovial membrane of early RA patients and to evaluate the influence of cyclosporin A (CSA) on the TCR repertoire in early RA patients. This may provide additional information on the possible T-cell mediated pathogenesis of RA. TCR repertoire analysis was performed on synovial tissue because this is the major site of inflammation in RA patients. T-cells that are potentially involved in the disease process are therefore most likely found in synovial tissue of early RA patients (23). We have used needle arthroscopy for obtaining synovial tissue samples in patients with early arthritis. This is a non-invasive, safe and well tolerated approach (22). The samples obtained, however, contain small amounts of cellular material only. To analyze TCR repertoires in these biopsies highly sensitive PCR-based techniques are required. We have previously shown that PCR-ELISA is a useful method to study TCR V gene usage of biopsy samples (9, 14). A strong bias could be that the cellular composition of these needle biopsies may be different when samples are taken from different sites in the joint. However, previous studies have shown that multiple blinded samples taken by needle arthroscopy provide a good estimate of the synovial membrane inflammation (24). In addition, shared amino acid profiles in CDR3 regions have been reported in multiple synovial tissue needle biopsies from the same joint (25). Furthermore, this non-invasive technique allows for longitudinal studies of synovial tissue samples from the same joint of an RA patient. Our data showed a similar TCR BV gene profile for synovial tissue and synovial lavage fluid obtained at the same time in three out of four RA patients studied. In addition, similar BV gene profiles were observed at two sites in the joint of a patient. Together these results indicate that these blinded needle biopsies are useful to study TCR BV gene expression at different time points.

Our results demonstrate biased TCR BV gene repertoires in synovial tissue of the majority of early RA patients. Some BV genes were more frequently found to be overrepresented in synovial tissue, including BV2, BV4 and BV18, while no BV gene was uniformly overrepresented in all early RA patients. Our data do not confirm the previously reported overexpression of the BV3, BV14 and BV17 families in RA patients (26). The BV genes that were overrepresented in the synovial tissue were generally not overrepresented in blood, indicating a preferential accumulation of some T-cell populations in synovial tissue. The observed skewed but variable TCR repertoire in the joints of early RA patients is consistent with previous reports (7, 10, 23, 27, 28).

The comparison of TCR repertoires at both time points revealed some interesting findings. Although overrepresented BV genes were found in the blood of some patients, the pattern of overrepresented BV genes was different in most patients at both time points. In the synovial tissue, a biased TCR repertoire was observed at both time points. The TCR pattern became

slightly more diverse in the control group, whereas a similar or more skewed repertoire was evident in most of the CSA treated patients at the second time point. In the five placebo patients, different BV genes were overrepresented at both time points. This was confirmed by CDR3 spectratype analysis, which showed different TCR clonotypes for the overrepresented BV families at both time points in the synovial tissue of these patients. Most of the TCR clonotypes present at the first sampling were no longer observed at the second sampling, suggesting that the corresponding T-cell populations were depleted from the synovial tissue. This is remarkable since some of these TCR families represented more than 15% of the total TCR repertoire at the first time point. Together, these data are consistent with the accumulation of different T-cell subsets in the synovial tissue at both time points, suggesting a dynamic process of T-cell activation or T-cell recruitment in the joints. Our data are in agreement with the report of Khazaei et al. (29) who studied BV12 and BV14 TCR clonotypes in two RA patients in CD4 T-cells isolated from synovial fluid at two time points with an interval of 3 or 9 months. In these patients, with disease duration's of one and four years, different dominant clones were found at both time points. Persistence of dominant clones was however described in some chronic RA patients. Alam and coworkers (30) studied TCR CDR3 sequences in two RA patients with long-standing disease. They also found different dominant clones at two time points in the synovial tissue of these patients, although some clones persisted over time. Similar observations were made in our previous study, which showed persistence of dominant clones in synovial fluid of two chronic RA patients (9). Taken together, the current information may indicate that some T-cell clones persist in the joints of patients with severe long-standing RA, while dynamic T-cell clonotype changes are taking place in the joints of early RA patients.

What causes these dynamic TCR repertoire changes in the early RA control patients? There is evidence that activated autoimmune T-cells may undergo rapid apoptosis at the disease site. For example, it has been shown that freshly isolated synovial T-cells are highly susceptible to apoptosis (12). Likewise, it was demonstrated that disease inducing autoimmune T-cells undergo rapid apoptosis in the central nervous system of rodents with EAE (31). The antigen specific apoptosis process is potentially induced by cytokines (e.g. TGF-β) or may be induced by the liberation of free, unprocessed antigen (31). We therefore hypothesize that the predominant clones are depleted in the synovial tissue through rapid apoptosis. However, as a result of the local or peripheral activation of new T-cells, other T-cell clones accumulate in the joints of these patients. These observations are in agreement with the determinant spreading concept that was previously described in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (11). In this concept new antigens or epitopes released as a consequence of the ongoing tissue destruction and inflammation may induce activation and expansion of other T-cell clonotypes in the synovium. Such a process of dynamic T-cell activation may play a role in the perpetuation of the disease. On the other hand it is also possible that these newly T-cells are bystander cells with no major role in the disease process.

In contrast to the control RA patients, persistence of dominant clones was observed in four patients treated with CSA, although some TCR clonotypes were present at only one time point. These data indicate that the dynamic clonotype changes in synovial tissue, as seen in the control RA patients, were suppressed or prevented by treatment with CSA. CSA is known to inhibit early events of T-cell activation, most likely by inhibition or down regulation of the expression of IL-2, IL-4, IFN-γ and the IL-2 receptor, but may also suppress apoptosis (19, 32, 33, 34, 35). Based on its mode of action, it can be speculated that CSA treatment may have suppressed activation of new T-cell clones in the blood or synovium of these patients, while clearance through apoptosis may have been reduced, resulting in a more stable TCR expression pattern at both time points. In line with this hypothesis is the observation that no TCR mRNA was detected in synovial tissue of 3 out of 7 RA patients after CSA treatment, although this was also noted in 1 control patient. Although the expression of TCR mRNA may have been selectively inhibited in these samples, the most likely explanation for this observation is that these tissue samples contain few T-cells only.

Our findings may explain the inconsistent findings of previous studies. If the TCR repertoire changes rapidly over time in the joints of early RA patients, analysis of TCR repertoires will reveal different information at various phases along the disease course. In addition, these observations have important consequences for the design of immunotherapies targeted at potentially pathogenic T-cell subsets in RA. To be effective such therapies may need to target T-cells in a V gene independent manner, especially in early RA patients. The optimal scenario would be to follow-up TCR repertoires in the joints of individual RA patients, and then to use either V gene specific or unspecific therapies. However, these patient-specific approaches are probably not realistic for routine clinical application. Alternatively, our data suggest that RA patients may be treated with an agent which prevents activation of new T-cell clones, in combination with a therapy that depletes dominant T-cell clones at the same time. T-cell vaccination or TCR peptide vaccination can be used as an approach to deplete the dominant T-cell populations (26, 36), while CSA is an attractive drug to prevent activation of new T-cell subsets. Although CSA has been shown to be effective in early RA patients, the major adverse effects are considered as an important drawback for current use in early RA patients (37).

In conclusion, our data have shown that the TCR repertoire in the synovial tissue of early RA patients is biased, and that this pattern undergoes dynamic changes during a period of 16 weeks. These dynamic repertoire changes were influenced by CSA treatment, suggesting that

CSA may affect T-cell activation or recruitment of T-cells into the joints of early RA patients. These data provide new information about the possible T-cell mediated pathogenesis of RA, and may have important consequences for the design of new T-cell targeted therapies for RA.

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References

1. Sewell, K.L. and Trentham, D.E. Pathogenesis of Rheumatoid Arthritis. *Lancet* 341:283-286, 1993.

2. Smolen, J.S., Tohidast-Akrad, M., Gal, A., et al. The role of T-lymphocytes and cytokines in Rheumatoid Arthritis. *Scand.J.Rheumatol.* 25:1-4, 1996.

3. Fox, D.A. The role of T-cells in the immunopathogenesis of Rheumatoid Arthritis: new perspectives. *Arthritis Rheum.* 40:598-609, 1997.

4. Panayi, G.S. T-cell dependent pathways in Rheumatoid Arthritis. *Curr. Opin Rheumat.* 9(3):236-240, 1997.

5. Struyk, L., Hawes, G., Chatila, M., Breedveld, F., Kurnick, J. and van den Elsen, P. T-cell receptors in Rheumatoid Arthritis. *Arthritis Rheum.* 38:577-589, 1995.

6. Bucht, A., Larsson, P., Weisbrot, L., et al. Expression of interferon-gamma (IFN-γ), IL-10, IL-12 and transforming growth factor-beta (TGF-β) mRNA in synovial fluid cells from patients in early end late phases of Rheumatoid Arthritis. *Clin. Exp. Immunol.* 103:357-367, 1996.

7. Fischer, D.C., Opalka, B., Hoffmann, A., Mayr, W. and Haubeck, H.D. Limited heterogeneity of rearranged T-cell receptor Vα and Vβ transcripts in synovial fluid T-cells in early stages of Rheumatoid Arthritis. *Arthritis Rheum.* 39(3):454-462, 1996.

8. Lim, A., Toubert, A., Pannetier, C., et al. Spread of clonal T-cell expansions in Rheumatoid Arthritis patients. *Hum. Immunol.* 48(1-2):77-83, 1996.

9. VanderBorght, A., Geusens, P., Vandevyver, C., Raus, J. and Stinissen, P. Skewed T-cell receptor variable gene in the synovium of Rheumatoid Arthritis patients: Clonal expansion and persistence of synovial T-cells, Submitted 1999.

10. Elewaut, D., De Keyser, F., Van Den Bosch, F., et al. Enrichment of T-cells carrying β7 integrins in inflamed synovium tissue from patients with early spondylarthropathy, compared to Rheumatoid Arthritis. *J. Rheumatol.* 25:1932-1937, 1998.

11. Lehman, P.V., Sercarz, E.E. and Forsthuber, T. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today* 14:203-208, 1993.

12. Hasunuma, T., Kato, T., Kobata, T. and Nishioka, K. Molecular mechanism of immune response, synovial proliferation and apoptosis in Rheumatoid Arthritis. *Springer Semin. Immunopathol.* 20:41-52, 1998.

13. Cross, A.H., O'Mara, T. and Raine, C.S. Chronologic localization of myelin-reactive cells in the lesions of relapsing EAE: Implications for the study of multiple sclerosis. *Neurol.* 43:1028-1033, 1993.

14. VanderBorght, A., Van der Aa, A., Geusens, P., Vandevyver, C., Raus, J., Stinissen, P. Identification of overrepresented T-cell receptor genes in blood and tissue biopsies by PCR-ELISA. *J Immunol. Methods*, 223, 47-61, 1999.

15. Pannetier, C., Even, J. and Kourilsky, P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today* 16(4):176-181, 1995.

16. Dougados, M., Awada, H. and Amor, B. Cyclosporin in RA: a double blind, placebo controlled study in 52 patients. *Ann. Rheum. Dis.* 47:127-133, 1998.

17. Wels, G. and Tugwell, P. Cyclosporin A in Rheumatoid Arthritis: overview of efficacy. *Br. J. Rheumatol.* 32(suppl 1):51-56, 1993.

18. Granelli-Piperno, A. Lymphokine gene expression in vivo is inhibited by cyclosporin A. *J. Exp. Med.* 171:533-544, 1990.

19. Russel, G., Graveley, R., Seid, J., Al-Humidan, A.K. and Skjodt, H. Mechanisms of action of cyclosporin and effects on connective tissues. *Semin. Arthritis. Rheum.* 21(suppl 3):16-22, 1992.

20. Yocum, D. Immunological actions of cyclosporin A in Rheumatoid Arthritis. *Br. J. Rheumatol.* 32:38-41, 1993.

21. Arnett, F.C., Edworthy, C., Bloch, D.A., et al. The American Rheumatism Association 1987 revised criteria for the classification of Rheumatoid Arthritis. *Arthritis Rheum.* 31:315-324, 1988.

22. Baeten, D. Van den Bosch, F., Elewaut, D., Stuer, A., Veys, EM., De Keyser, F., Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. *Clin Rheumatol* in press.

23. Ramanujam, T., Luchi, M., Schumacher, H.R., et al. Detection of T-cell receptors in early Rheumatoid Arthritis synovial tissue. *Pathobiol.* 63:100-108, 1995.

24. Bresnihan, B. Pathogenesis of joint damage in Rheumatoid Arthritis. *J. Rheumatol.* 26(3):717-719, 1999.

25. Struyk, L., Hawes, G.E., Mekkers, H.M.M., Tak, P.P., Breedveld, F.C. and van den Elsen, P. Molecular analysis of the T-cell β chain repertoire in early Rheumatoid Arthritis: heterogeneous TCR BV gene usage with shared amino acid profiles in CDR3 regions of T-lymphocytes in multiple synovial tissue needle biopsies from the same joint. *Eur. J. Clin. Invest.* 26:1092-1102, 1996.

26. Moreland, L.W., Morgan, E.E., Adamson, T.C., et al. T-cell receptor peptide vaccination in Rheumatoid Arthritis. A placebo controlled trial using a combination of BV3, BV14 and BV17 peptides. *Arthritis Rheum.* 41(11):1919-1929, 1998.

27. Goronzy, J.J., Bartz-Bazzanella, P., Hu, W., Jendro, M.C., Walser-Kuntz, D.R. and Weyand, C.M. Dominant clonotypes in the repertoire of peripheral CD4+ T-cells in Rheumatoid Arthritis. *J. Clin. Invest.* 94(5):2068-2076, 1994.

28. Zwillich, S. and Weiner, W. T-cell receptor analysis in Rheumatoid Arthritis: What have we learnt. *Immunol. Res.* 13(1):29-41, 1994.

29. Khazaei, H.A., Lunardi, C. and So, A.K. CD4 T-cells in the rheumatoid joint are oligoclonally activated and change during the course of the disease. *Ann. Rheum. Dis.* 54:314-317, 1995.

30. Alam, A., Lambert, N., Lulé, J., et al. Persistence of dominant T-cell clones in synovial tissues during Rheumatoid Arthritis. *J. Immunol.* 156:3480-3485, 1996.

31. Bauer, J., Wekerle, H. and Lassmann, H. Apoptosis in brain-specific autoimmune disease. *Curr. Opin. Immunol.* 7:839-843, 1995.

32. Brunner, T., Yoo, NJ., LaFace, D., Ware, CF., Green, DR. Activation-induced cell death in murine T-cell hybridomas. Differential regulation of Fas(CD95) versus Fas ligand expression by cyclosporin A and FK506. *Int Immunol* 8(7): 1017-1026, 1996.

33. Shi, YF., Sahai, BM., Green, DR. Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. *Nature* 339(6226): 625-626, 1989

34. Sugano, N., Ito, K., Murai, S. Cycolsporin A inhibits H_2O_2-induced apoptosis of human fibroblasts. *FEBS Lett* 447(2-3): 274-276, 1999.

35. Yazdanbakhsh, K., Choi, JW., Li, Y., Lau, LF., Choi, Y. Cyclosporin A blocks apoptosis by inhibiting the DNA binding activity of the transcription factor Nur77. *Proc Natl Acad Sci USA*, 92(2): 437-441, 1995

36. Zhang, J.W., Medaer, R., Stinissen, P., Hafler, D. and Raus, J. MHC restricted clonotypic depletion of human myelin basic protein-reactive T-cells by T-cell vaccination. *Science* 261:1451-1454, 1993.

37. Panayi, G.S. and Tugwell, P. The use of cyclosporin A microemulsion in Rheumatoid Arthritis: conclusions of an international review. *Br. J. Rheumatol.* 36(7):808-811, 1997.

Chapter 6

Cytokine mRNA Quantification in Synovial Tissue of Early Rheumatoid arthritis Patients: Effect of Cyclosporin A (Neoral®) Treatment

Based on:

Cytokine mRNA Quantification in Synovial Tissue of Early Rheumatoid Arthritis Patients: Effects of Cyclosporin A (Neoral®) Treatment.

Ann VanderBorght, Filip De Keyser, Piet Geusens, Marc De Backer, Eric Veys, Jef Raus and Piet Stinissen.

In preparation.

Abstract

Objective: To study the influence of cyclosporin A (CSA) on the cytokine mRNA levels in early RA patients.

Methods: Synovial tissue biopsies and paired blood samples were studied from 10 early RA patients at two timepoints, and 6 early RA patients at one timepoint. In this double blinded study six of these patients were treated with CSA (Neoral-Sandimmun®) and 4 patients with placebo for 16 weeks. cDNA prepared from PBMC and synovial tissue was used for cytokine mRNA (IL-4, IL-10, IFN-γ, TNF-α) quantification. β2-microglobulin mRNA levels were used to correct for the variability of the cell numbers in the samples.

Results: Cytokine mRNA levels for IL-4, IL-10, IFN-γ and TNF-α were detectable in synovial tissues of most of the early RA patients. The cytokine profiles in synovial tissues were dominated by type 2 patterns with high IL-10 and IL-4 mRNA levels and low IFN-γ mRNA levels. After CSA treatment a reduction of the IFN-γ mRNA levels was observed in synovial tissues of all patients (6 / 6) and a reduction of IL-4, IL-10, and TNF-α mRNA levels in four patients. The remaining two CSA treated patients had increased IL-4, IL-10, and TNF-α mRNA levels in blood and synovial tissue. This type 2 pattern was even more pronounced after CSA treatment. The cytokine mRNA of the placebo control group also did change but the patterns in tissues and blood samples were more diverse.

Conclusions: Cytokine mRNAs are present in T-cells of synovial tissues of early RA patients. The pattern is dominated by type 2 cytokines. After CSA therapy, two distinct patterns of cytokine changes in synovial tissue were observed. Four patients had reduced mRNA levels of all cytokines in synovial tissue, while two patients had reduced IFN-γ mRNA levels, but increased IL-4 and TNF-α mRNA levels. Most of the changes in the synovial tissue correlated well with the cytokine mRNA levels in the blood. These results provide further information about the in vivo effects of CSA treatment on synovium of early RA patients.

Introduction

Cytokines play an important role in the autoimmune pathogenesis of rheumatoid arthritis (RA) (1). The monocyte-derived proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) were found to be expressed at high levels in the joints of RA patients. Therefore they have been selected as candidate therapeutic targets in RA (2). Despite the presence of activated T lymphocytes in synovial tissues of RA patients, a low expression of T-cell-derived cytokines such as interferon-γ (IFN-γ) and IL-2 has been found (3). In contrast, T-cell clones isolated from RA synovia produced large amounts of IFN-γ but no IL-4 (4). These data suggest that synovial T-cells belong to the proinflammatory type 1 subset.

We were interested to study the expression of T-cell cytokines in synovial tissues of patients with a short disease duration (< 1 year, early RA), since there is evidence indicating that T-cell mediated autoimmune processes are most important in early phases of the disease (5). In addition, the cytokine expression in a subgroup of patients has been studied after treatment with cyclosporin A (CSA, neoral). The effects of CSA treatment on the cytokine expression in the joints has been evaluated in early RA patients treated with either CSA or placebo. These patients were sampled for blood and synovial tissues at entry and after 16 weeks of treatment. Synovial tissue was sampled by needle arthroscopy. We have previously shown that CSA influences the T-cell receptor (TCR) expression profile in the joints, and leads to a significant reduction of the TCR mRNA in the biopsies of most patients after treatment (6). CSA is an immunosuppressive drug which specifically blocks T lymphocyte activation through inhibition of the antigen induced secretion of IL-2 and other inflammatory cytokines, such as IFN-γ and IL-4, at the transcriptional level (7). Thus, our study was aimed to provide information on the cytokine expression in synovial tissues of early RA patients, and to evaluate cytokine mRNA changes in the synovial tissues of patients during a follow-up period of 16 weeks, while a subgroup of patients was treated with an agent that prevents or suppresses *in vivo* activation of new T-cells.

Our study was focused on cytokines that are produced by T-cells, but also by other immune cells: IL-4, IFN-γ, IL-10 and TNF-α. The analysis of these cytokines also allows to discriminate between T1 and T2 phenotypes in the synovial tissue, since IFN-γ is a T1-specific cytokine, while IL-4 and IL-10 are mainly produced by T2 T lymphocytes (4). A sensitive PCR-based technique was used to determine the levels of these cytokines in unstimulated blood mononuclear cells and synovial tissue cells. One of the problems associated with PCR-based quantification of mRNA is that quantification can only be performed at the exponential phase and not the plateau phase of the reaction. However, most of these protocols use end-point analysis which do not allow to examine whether the reaction reached the plateau phase at the final analysis step. In this study we used a real-time quantitative PCR system to amplify cDNA made from isolated mRNA and quantify the PCR products with the double strand DNA binding

dye SYBR Green I (LightCycler, Roche) (8). The system allows to identify the 4 or 5 cycles in the linear phase of the amplification curve, allowing for accurate measurement of PCR products. Amplification artefacts do not interfere with the analysis since the measurements are performed above the melting temperature of these products. We have applied this new technology to study the mRNA levels of the T-cell derived cytokines IL-4, IL-10, IFN-γ and TNF-α in synovial tissues of early RA patients and to compare these levels with the concentrations of the cytokines in blood. In addition, our data provide new information about the effects of CSA treatment on the cytokine mRNA levels in the joints of early RA patients.

Materials and Methods

Patient population and study design

The objectives of this study were to determine cytokine mRNA levels in blood and synovial membranes of early RA patients, and to analyse the changes in cytokine expression in the synovial membranes over a period of 16 weeks. In addition, the effects of CSA-treatment on the cytokine expression in peripheral blood and T lymphocytes of the joints were studied in a subgroup of patients. Eighteen early RA patients from three RA centres, with a disease duration of less than one year were included in a double-blinded, placebo-controlled study (6). The patients (n=17) either met the 1987 revised ACR criteria (9) or showed symptoms of synovitis and/or inflammatory polyartralgia (n=1), all patients were positive both for one of the RA-related subtypes of HLA-DR4 or HLA-DR1 and for rheumatoid factor. All of them had oral piroxicam as the only treatment before entering the study, and this treatment was continued along the study. After signing their informed consent, patients were randomised. Twelve patients were treated for 16 weeks with a cyclosporin A microemulsion (Neoral-Sandimmun), and 6 patients were treated with placebo. Peripheral blood and synovial membrane tissues were sampled at entry and at week 16. Synovial tissues were obtained by fine needle arthroscopy (10). Approximately 15 small biopsies were obtained at each arthroscopy and samples were preferentially taken in macroscopically inflamed areas of the synovial membrane. The study protocol was approved by the ethical committees of all participating centres.

RNA extraction and cDNA synthesis

Analysis of samples and interpretation of primary data was done in a blinded manner. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll-Hypaque density gradient centrifugation as described in 2.1. Synovial tissue biopsies were immediately frozen at -70°C. Out of 15 biopsies taken during the needle arthroscopy RNA was extracted from 5 randomly selected biopsies as described in 2.1. Total RNA was extracted from $2x10^6$ PBMC with the Rneasy method and reverse transcribed into first-strand cDNA using oligo-dT as described in 2.3 (11).

Quantification of cytokine mRNA

PCR amplification and quantification of cytokine messengers were performed as described in 2.6.2. Primer sequences are described in table 2.1. Serial dilution's of the pQA (for β_2-microglobulin, TNF-α, IL-4, IFN-γ) and pQB (for IL-10) vector cDNA (SANOFI Elf Bio Recherches, Labege, France) were used as an external quantification standard (12).

Quantification of PCR amplicons was performed as described in 2.6.2

Statistical analysis

To assess differences in the cytokine levels between patients and controls the Mann-Whitney test was used. The differences in cytokine mRNA levels between paired patient samples, as well as within each treatment group at different timepoints were tested using the Wilcoxon rank test.

Table 6.1: Patient characteristics

Patient	Age/Sex	Group	Sampling start	Sampling end	Included in cytokine analysis start	Included in cytokine analysis end	comments
			PB/ST	PB/ST			
ADS	54/M	CSA	+/+	+/+	yes	no	
ANB	67/F	CSA	+/+	+/+	yes	yes	
LOV	46/F	CSA	+/+	+/+	yes	yes	
PAG	46/M	CSA	+/+	+/+	yes	yes	
BEV	40/F	CSA	+/+	+/+	yes	yes	
MAV	37/F	CSA	+/+	+/+	yes	yes	
ELB	60/F	CSA	+/+	+/+	yes	yes	Stopped medication after wk 9 [1]
MJS	62/M	CSA	+/+	-/-	yes	no	
GBV	46/F	CSA	+/+	-/-	no	no	Stopped medication after wk8 [1]
DEA	18/F	CSA	+/+	-/-	yes	no	Stopped medication after wk 11 [1]
GAJ	65/F	CSA	+/+	+/-	no	no	Stopped medication after wk 1 [1]
FAG	37/F	CSA	-/-	+/+	no	no	
NIG	59/M	Placebo	+/+	+/+	yes	yes	
DAL	35/M	Placebo	+/+	+/+	yes	no	
LED	68/F	Placebo	+/+	+/+	yes	yes	
GEM	64/F	Placebo	+/+	+/+	yes	yes	
AH	70/M	Placebo	+/+	+/+	yes	yes	Stopped medication after wk 12 [1]
JAL	43/F	Placebo	+/+	+/-	no	no	Additional medication [2]

[1]: The reasons for ending the medication protocol are summarized in the Results.
[2]: Intra-articular diprophos injection (6.03 g/ml β methazondipropionaat and 2.63 g/ml β methazondinatriumfosfaat)

Results

Clinical data

Clinical data are briefly summarized in chapter 5.

Quantification of cytokine mRNA

RNA isolated from blood mononuclear cells or synovial tissues was reverse transcribed into cDNA and then used as template for quantitative cytokine PCR with a new real-time analysis system: LightCycler™ (Roche). This system has some advantages as compared to previous methods. Quantification of PCR amplicons is performed by fluorescence of a double strand DNA binding dye: SYBR Green I. SYBR Green I binds only to double strand DNA with no sequence specificity, while unbound SYBR Green I has a low background fluorescence. PCR amplification and detection is performed in glass capillaries. Correct PCR products are discriminated from PCR artefacts by measuring fluorescence at a temperature slightly above the melting temperature of the correct PCR product. PCR artefacts such as primer-dimers are shorter than the wanted PCR products and thus have a lower melting temperature. These artefacts will therefore be at a single stranded conformation at the analysis temperature and will not bind SYBR Green I.

Cytokine mRNA levels in blood and synovial tissues of early RA patients and in blood of healthy controls

Cytokine mRNA levels of IFN-γ, TNF-α, IL-4 and IL-10 were determined in peripheral blood mononuclear cells ($2x10^6$) of early untreated RA patients (n=16) and healthy controls (n=5). The clinical characteristics of these patients are listed in Table 6.1. The mean levels of all tested cytokine mRNAs (IFN-γ, TNF-α, IL-4 and IL-10) were higher in the blood of the early RA patients than in the blood of the healthy controls (Fig. 6.1). The differences were statistically significant for IL-4 ($p=0.008$) but not for the other cytokines tested ($p>0.05$). Interestingly, increased levels of both type 1 and type 2 cytokines were observed in the blood of early RA patients.

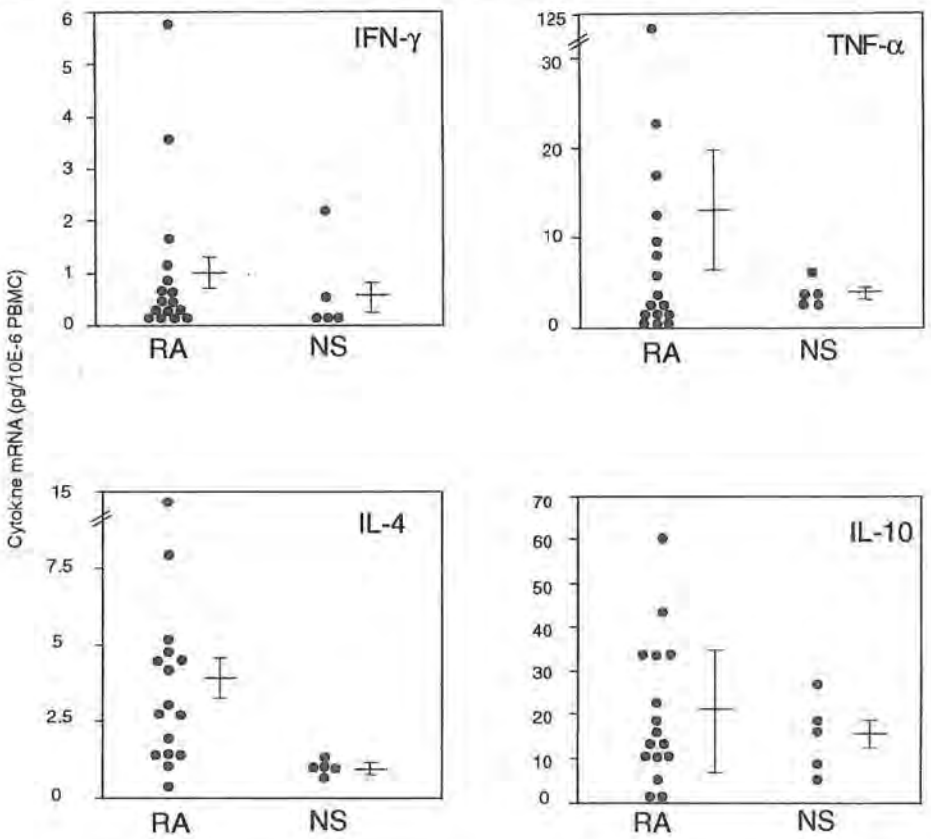

Figure 6.1. Cytokine mRNA levels in blood mononuclear cells isolated from early RA patients (n=16) and healthy control subjects (n=5).

Next, cytokine mRNA levels were compared between blood and synovial tissue of the early RA patients (n=14), for two patients there was insufficient material available for analysis). However, the number of cells in the synovial tissue samples was not known, and this number may vary significantly between samples. To compare synovial tissue biopsies with blood samples, a control PCR was performed for each cDNA sample with primers specific for the housekeeping gene β2-microglobulin. The cytokine mRNA content was expressed as the amount of cytokine mRNA (pg) in a sample that contains 1 pg β2 microglobulin mRNA (arbitrary units). As shown in Fig. 6.2 the mean IFN-γ, TNF-α, IL-4 and IL-10 mRNA levels were all increased in the synovial biopsy samples of the early RA patients as compared to their paired blood samples. The differences were statistically significant for IL-4 ($p=0.026$), but not for IFN-γ, TNF-α and IL-10 ($p>0.05$). It should be noted that TNF-α, IL-4 and IL-10 mRNA levels were increased in synovial tissue versus blood for the majority of patients tested, while the IFN-γ mRNA levels were generally lower in synovial tissues than in blood. Together, the data suggest a preferential expression of the type 2

cytokines IL-4 and IL-10 in synovial tissue, and low expression of the type 1 cytokine IFN-γ.

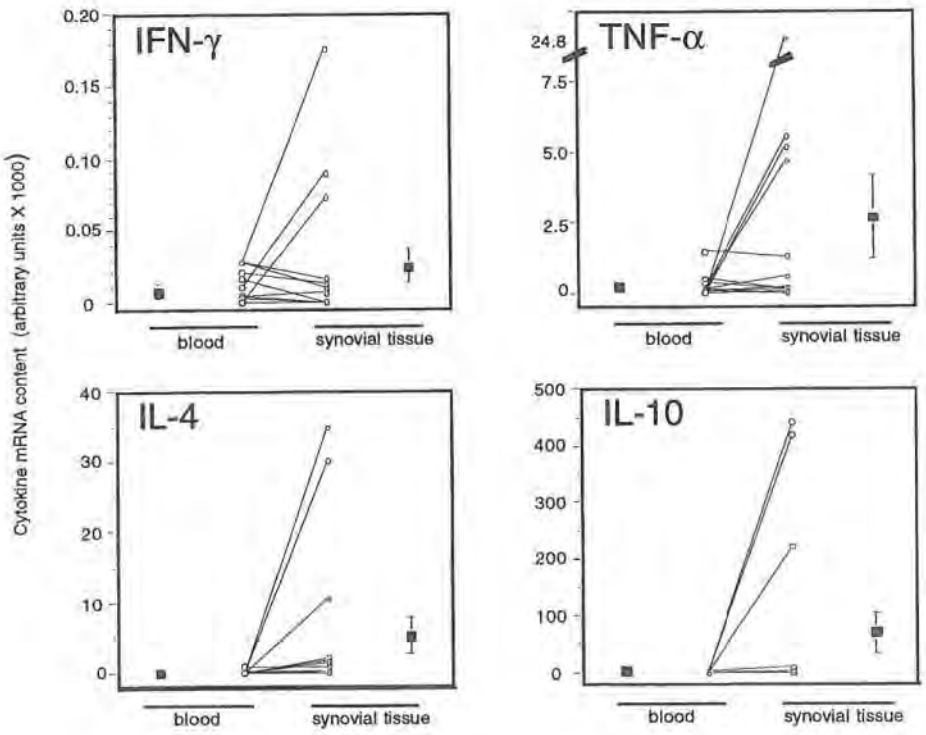

Figure 6.2: Cytokine mRNA levels in blood mononuclear cells and synovial tissue of 16 early RA patients.

Cytokine mRNA changes in synovial tissue and blood over a 16 week period

Twelve early RA patients were treated with CSA, while 6 patients received placebo (6). Three patients on CSA therapy were withdrawn from the study before week 16 because of an increase of serum creatinine, gastric complaints and flare up of disease. Two other patients discontinued the study medication prematurely because of an increase of serum creatinine (1 CSA) and thrombocytopenia (1 placebo). One placebo treated patient violated the study protocol because of an intra-articular diprophos injection. Blood and synovial tissues were available for cytokine mRNA analysis from 6 CSA-treated and 4 placebo-treated RA patients at both time points (Table 6.1). Detailed clinical data from this limited

trial will be published in a separate report (in preparation). The changes in cytokine mRNA levels are presented in Fig. 6.3 and 6.5.

IFN-γ mRNA

The IFN-γ mRNA levels were reduced in the blood of 4/6 CSA patients and in synovial tissue of all CSA treated patients ($p>0.05$ for blood and $p=0.046$ for synovial tissue) (Fig. 6.3, top panel). In some of the CSA treated patients (ANB, LOV, ELB, PAG) no IFN-γ mRNA was detectable in the synovial tissues at week 16. In contrast, the mean IFN-γ mRNA levels remained more or less stable in the blood and synovial tissues of the control group ($n=4$, $p>0.05$). However, major fluctuations were observed among individual patients.

TNF-α mRNA

There were no statistically significant differences in the TNF-α mRNA content in the blood and the synovial tissue. Although, in the CSA-treated group the mean TNF-α mRNA levels were reduced in blood ($p>0.05$) but increased in the synovial tissues ($p>0.05$) at the second timepoint. (Fig. 6.3, bottom panel). The increase in the synovial tissues was however due to an increase of the levels in two patients who also had an increased TNF-α mRNA value in the blood at week 16 (PAG and LOV). Four CSA patients with reduced TNF-α levels in synovial tissues also had reduced IFN-γ levels in their blood. The TNF-α mRNA levels were lower at week 16 in the blood of all placebo control patients, while TNF-α mRNA levels were increased at that timepoint in synovial tissue of 3 / 4 placebo patients resulting in an increased mean TNF-α mRNA level in synovial tissues of the control group ($p>0.05$).

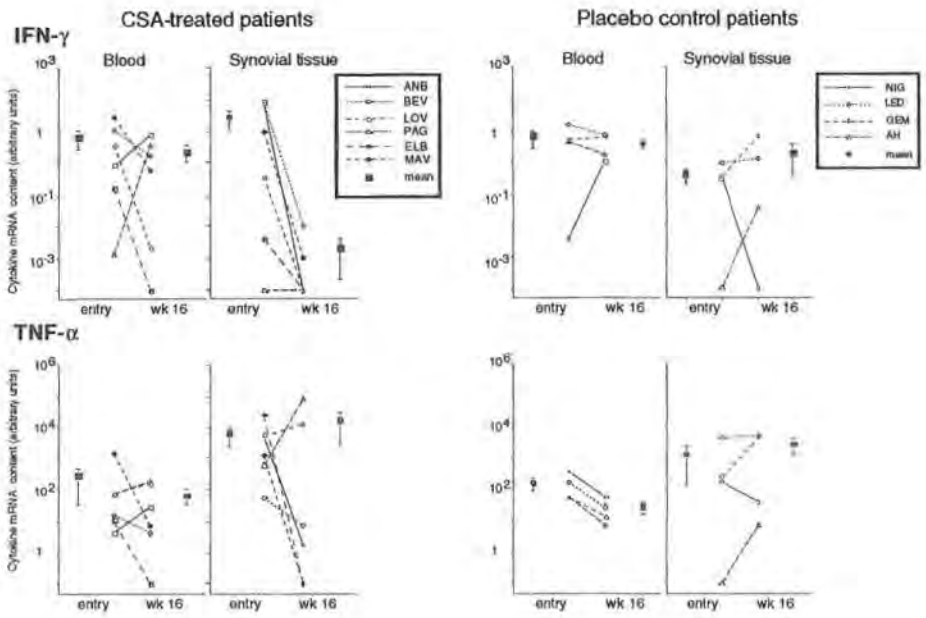

Figure 6.3. IFN-γ and TNF-α mRNA levels in synovial tissues of early RA patients before and after treatment with cyclosporin A or placebo.

IL-4 mRNA

At the second timepoint the mean IL-4 mRNA levels were increased in blood ($p>0.05$) and synovial tissues ($p>0.05$) in the CSA-treated patients (Fig. 6.4, top panel). The increased mean values were mainly due to 2 patients (LOV and PAG) since 4 / 6 CSA-treated patients had a reduced IL-4 mRNA level after treatment in their synovial tissue, and three of these patients also had a reduced IL-4 mRNA level in blood at week 16. Note that patients LOV and PAG also had increased TNF-α mRNA levels in blood and synovial tissue after CSA treatment. In the placebo control patients, a reduction of the mean IL-4 mRNA levels was observed in blood and synovial tissue, although 2 / 4 placebo patients had an increased IL-4 mRNA level in the synovial tissue at week 16 ($p>0.05$).

IL-10 mRNA

In synovial tissue IL-10 mRNA levels were reduced ($p>0.05$) in the majority (5 / 6) of CSA treated patients at the second timepoint (Fig. 6.4, bottom panel). A modest reduction of the mean IL-10 mRNA levels was also found in the blood ($p>0.05$) of these patients, although 3 patients (PAG, LOV, ANB) had an increased IL-10 mRNA level in the blood at week 16.

Interestingly, these 3 patients also had increased IL-4 mRNA level in the blood while two of these patients (PAG and LOV) also had increased TNF-α mRNA levels in their blood. The mean IL-10 mRNA levels were increased in blood and synovial tissues of the placebo patients. In three of these patients the IL-4 mRNA changes (increase or decrease) correlated with the changes of the IL-10 mRNA levels.

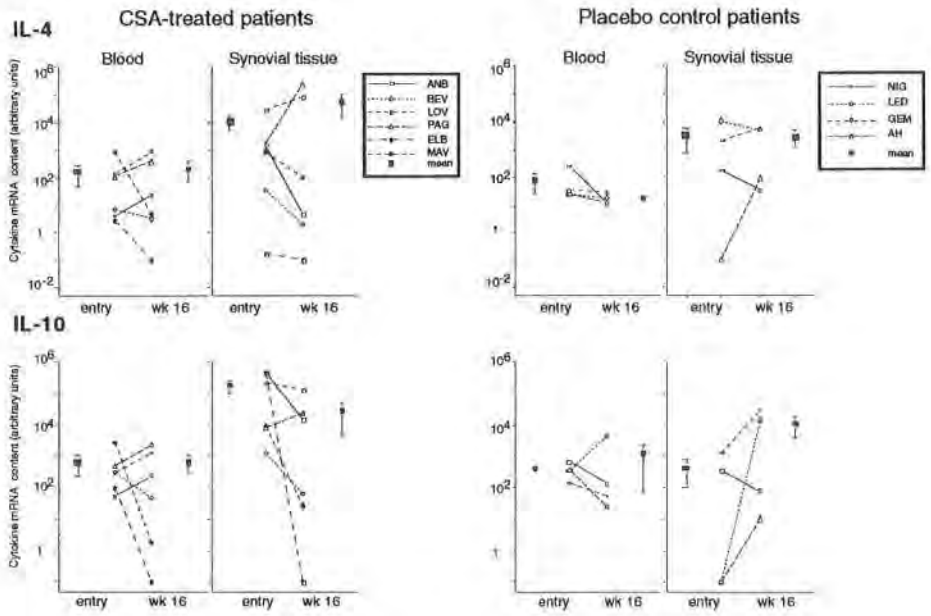

Figure 6.4. IL-4 and IL-10 mRNA levels in synovial tissues of early RA patients before and after treatment with cyclosporin A or placebo.

Together the data suggest that the cytokine mRNA levels may change in the synovial tissue over a 4 month period as observed in the placebo control patients. In the CSA treated group the changes in the blood cytokine mRNA levels tended to correlate with changes of the cytokine mRNA levels in the synovial tissues. All CSA treated patients had a significant reduction of the IFN-γ mRNA level in their synovial tissue after treatment, while the TNF-α, IL-4 and IL-10 mRNA levels were reduced in the synovial tissue in the majority of these CSA treated patients (4 and 5 out of 6 patients respectively). Interestingly, 4 CSA treated patients (ANB, BEV, ELB, MAV) had a reduction of all four tested cytokines in the synovial tissue at week 16. At week 16, three of these patients also had reduced mRNA levels of all tested cytokines in their blood. Two patient (LOV and PAG) had an increased IL-4 and TNF-α mRNA level in synovial tissues at week 16, and this correlated well with the levels in their blood compartment. A similar observation was made in the control group,

where patient NIG had a reduced level of all tested cytokines, and patients GEM and AH had increased levels of all cytokines tested in the synovial tissue at week 16.

T1 / T2 phenotype in the joints before and after treatment

Both type 1 and type 2 cytokine mRNAs were identified in the synovial tissues of the patients. However, higher levels of the type 2 cytokines IL-4 and IL-10 were found in the joints as compared to the levels of the T1 IFN-γ cytokine, indicating a local type 0 or type 2 biased environment. To test whether the treatment induced a shift of the cytokine phenotype in the synovial tissues, changes in IFN-γ (T1 marker cytokine) and IL-4 (T2 marker cytokine) mRNA levels were evaluated in individual patients. Fig. 6.5 summarizes the cytokine data in a slightly different manner. The IFN-γ mRNA levels which were already low in the synovial tissues at entry were even further reduced in all patients. In two patients (PAG and LOV) an increase of IL-4 mRNA was seen after CSA treatment. The IL-4 mRNA was reduced in the synovium of the remaining four patients, but was still much higher than the IFN-γ mRNA levels at the same timepoint. Together, these data suggest a further shifting to a type 2 phenotype in the synovial tissues after CSA treatment. Similar shifts were observed in the blood of the CSA treated patients, with the exception of patient ANB who had increased IL-4 and IFN-γ mRNA levels after treatment.

The picture is more diverse in the synovial tissues of the control group. The IL-4 mRNA levels were still higher than the IFN-γ levels in all patients at the second time point, suggesting a type 2 biased profile. In one patient this pattern however was slightly shifted to the type 1, with an increased IFN-γ level and a reduced IL-4 mRNA level.

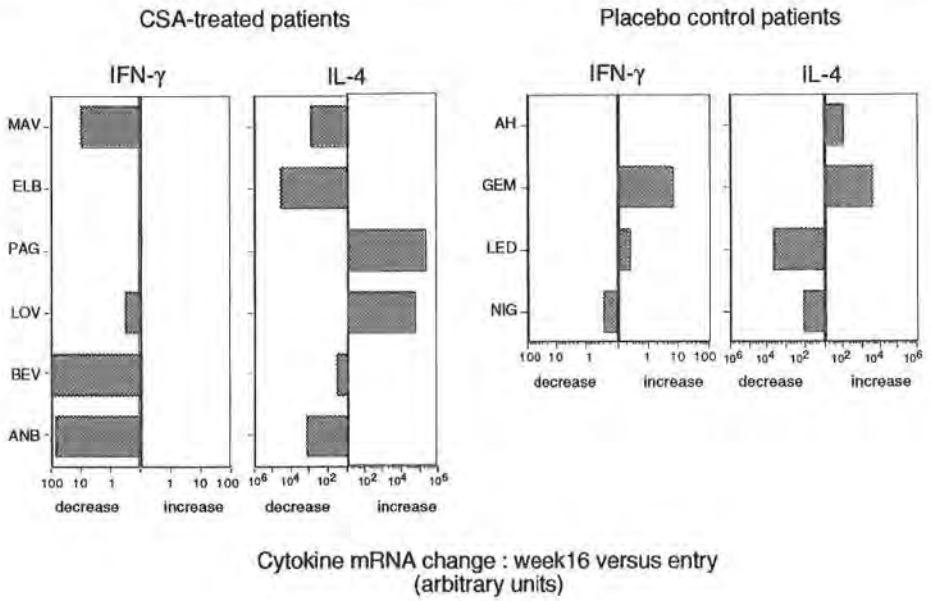

Figure 6.5. Cytokine profile changes in CSA treated and placebo controlled patients.

Discussion

It has been suggested that the difficulties to identify T-cell related cytokines in the synovium of RA patients may relate to technical issues or to the disease duration of the patients studied (13). T-cells may play a prominent role in the early stages of the disease process, while T-cell independent mechanisms could be involved in later pathogenic steps of RA (14). The chances to identify T-cell derived cytokines may therefore be higher in patients with a recent disease onset (early RA). We observed significant mRNA levels of all tested cytokines (IL-4, IL-10, IFN-γ and TNF-α) in the synovial tissue of early RA patients (disease duration less than one year). The cytokine profiles in the synovial tissue of these early RA patients was dominated by type 2 cytokines, since high levels of IL-4 and IL-10 mRNA were observed as compared to the IFN-γ mRNA levels. Our data are in agreement with previous reports which also demonstrated high levels of IL-10 or IL-10 mRNA in synovial tissue of RA patients (15, 16). These findings also correspond to recent reports of Steiner et al. (17) and Kirkham et al. (18) who also identified IL-4, IL-10, IFN-γ and TNF-α mRNA in synovial tissues of RA patients but are not supported by other reports which failed to identify T-cell related cytokines in the synovium (19, 20, 21) Taken together, our data indicate that the synovial tissues of early RA patients contains both type 1 and type 2 cytokines, but predominantly type 2 cytokines. It seems however that the inhibitory potential of the T2 cytokines may not be sufficient to control the disease activity (4).

In the second part of the study we evaluated the effects of treatment with cyclosporin on the cytokine mRNA levels in the synovial tissue of the early RA patients. Previous studies have shown that CSA reduces the IL-2 and IFN-γ production by T-cells *in vitro* (22, 23, 24), and that circulating IL-10 levels decrease significantly after *in vivo* CSA therapy (25). However, to our knowledge there have been no studies so far on the *in vivo* effects of CSA therapy on the cytokine expression in the synovium of RA patients. The present study demonstrates a reduction of the IFN-γ mRNA levels in the synovium of all early RA patients after CSA treatment, and a reduction of IL-4, IL-10 and TNF-α mRNA levels in the majority (4/6) of CSA treated patients. The cytokine profile was further shifted to a type 2 pattern in the majority of CSA treated patients, while this effect was not observed in the placebo controlled patients. Interestingly, a correlation was observed between the fluctuations of individual cytokines in these patients. For instance, two CSA treated patients who had increased IL-4 mRNA levels in the synovial tissues at week 16 also had an increased TNF-α level at that timepoint, and one of these patients had increased IL-10 mRNA levels at week 16. These patients also had increased TNF-α, IL-4 and IL-10 mRNA levels in their blood mononuclear cells at week 16. It would be interesting to study whether these two patients may show a different clinical response to the CSA therapy (non-responders), although the short term follow up data do not support such a difference. The reduced cytokine mRNA

levels in synovial tissues of four patients are possibly due to CSA treatment. However, it should be noted that a reduction of all tested cytokines was also observed in one placebo controlled patient. In addition, although there was a correlation between cytokine mRNA changes of most cytokines in blood and synovial tissues from most of the CSA treated patients, there were some notable exceptions, indicating that CSA treatment may have different effects on circulating mononuclear cells and synovial tissue cells in individual patients.

Although this study does not allow to identify the cell types that are responsible for the production of these cytokines, the findings of this report can be correlated with the observations of our previous study where the TCR characteristics of the same study population were evaluated at both timepoints (6). This study showed a dynamic process of T-cell recruitment in the joints of the placebo controlled RA patients, possibly due to activation of new T-cell clones. This process was influenced by CSA-treatment, since synovial tissue T-cells were either no longer detected, or contained persisting TCR clonotypes 16 weeks after treatment with CSA (6). Interestingly, three patients (BEV, ELB, MAV) out of six CSA treated RA patients studied in the current report, had no TCR mRNA in the synovial tissue at week 16, suggesting that no or very few T-cells were present in the synovial biopsies. In line with the reduced number of T-cells is the current finding that these patients also had low mRNA levels of all four cytokines studied. However, despite the lack of TCR mRNA in these biopsies, IL-4, IL-10, TNF-α and IFN-γ mRNA levels were still observed in these tissues, suggesting that these cytokines may have been produced by non-T-cell populations. This is further illustrated by the data of one of the placebo controlled patients (AH). The synovial tissue biopsy of this patient also had no TCR mRNA, although high mRNA levels of some cytokines were found in that tissue sample. It has been reported that IL-10 in the synovium of RA patients may be mainly of monocytic origin, while IL-4 can also be produced by mast cells, basophils and eosinophils (4). IFN-γ may be produced by $\gamma\delta$ T-cells and NK cells, while activated macrophages are the predominant producers of TNF-α in the synovium (26). Thus the reduced cytokine mRNA levels in most of the CSA treated patients can be attributable at least to some extend to a reduced number of T-cells in the synovial tissue. However, our data suggest that CSA may also reduce the cytokine mRNA production of other cell types in the synovial tissue by a direct or indirect mechanism. Further immunohistochemical analysis is required to identify the main cell types responsible for the cytokine mRNA production in the synovial tissue of these patients.

In conclusion, T1 and T2 cytokines can be detected in synovial tissue biopsies of early RA patients by a powerful quantitative PCR approach. Our data provide further information about the mechanism of action of CSA. After CSA treatment a reduction of the IFN-γ mRNA levels was observed in all patients, and a reduction of IL-4, IL-10 and TNF-α mRNA in most patients. The cytokine profile was dominated by type 2 cells in the synovial

tissue of these patients, and this profile was even further shifted to the T2 direction after CSA treatment. The findings also suggest that the patients may not respond in the same manner to CSA treatment, as suggested in two patients by the increased TNF–α and IL-4 mRNA levels in blood and synovial tissue at study end point. Further studies are necessary to evaluate whether this may correlate with reduced clinical effectiveness in these patients, and whether a reduction of the potentially pathogenic cytokine TNF-α may be accomplished by combined treatment with an TNF-α targeting approach (26).

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References

1. 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* 54:654-661, 1995;

2. Arend, WP., Dayer, JM. Inhibition of the production and affects of interleukin-1 and tumor necrosis factor α in Rheumatoid Arthritis. *Arthritis Rheum* 38(2):151-160, 1995

3. Firestein, GS., Zvaifler, NJ., How important are T-cells in chronic rheumatoid synovitis? *Arthritis Rheum* 33: 768-772, 1990.

4. Miossec, P., van den Berg, W. Th1/Th2 cytokine balance in arthritis. *Arthritis Rheum* 40(12): 2105-2115, 1997.

5. Bresnihan, B. Pathogenesis of joint damage in rheumatoid arthritis. J Rheumatol 26(3): 717-719, 1999.

6. VanderBorght, A., De Keyser, F., Geusens, P., De Backer, M., Malaise, M., Baeten, D., Van den Bosh, F., Veys, E., Raus, J., Stinissen, P. Dynamic T-cell receptor clonotype changes in synovial tissue of early rheumatoid arthritis patients are influenced by treatment with cyclopsporin A (Neoral). Submitted.

7. Barrera, P., Boerbooms, AM., van de Putte, LBA., van der Meer, JWM. Effects of antirheumatic agents on cytokines. *Sem Arthritis Rheum* 25(4): 234-253, 1996.

8. Rasmussen, R., Morrison, T., Herrmann, M. Wittwer, C., Quantitative PCR by continuous fluorescence monitoring of a double strand DNA specific binding dye. *Biochemica* 2: 8-11, 1998.

9. Arnett, FC., Edworthy, C., Bloch, DA. The American Rheumatism Association 1987 revised criteria for the classification of Rheumatoid Arthritis. *Arthritis Rheum* 31: 315-324, 1988.

10. Baeten, D., Van den Bosch, F., Elewaut, D., Steur, A., Veys, EM., De Keyser, F. Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. *Clin Rheumatol.* in press.

11. VanderBorght, A., Van der Aa, A., Geusens, P., Vandevyver, C., Raus, J., Stinissen, P. Identification of overrepresented T-cell receptor genes in the blood and tissue biopsies by PCR-ELISA. *J Immunol Methods* 223: 47-61, 1999.

12. Vandevyver, C., Motmans, K., Stinissen, P., Zhang, J., Raus, J. Cytokine mRNA profile of myelin basic protein reactive T-cell clones in patients with multiple sclerosis. *Autoimmunity* 28: 77-89, 1998.

13. Klareskog, L., Ronnelid, J., Holm, G. Immunopathogenesis and immunotherapy in rheumatoid arthritis: an area in transition. *J Intern Medic* 1995: 238: 191-206

14. Panayi, GS: T-cell-dependent pathways in rheumatoid arthritis. *Curr Opin Rheumatol* 9(3): 236-240, 1997

15. Cush, JJ., Splawski, JB., Thomas, R., McFarlin, JE., Schulze-Koops, H., Davis, LS., Fujita, K., Lipsky, P. Elevated interleukin-10 levels in patients with Rheumatoid Arthritis. *Arthritis Rheum.* 38(1): 96-104, 1995.

16. Katsiskis, PD., Chu, CQ., Brennan, FM., Maini, RN., Feldmann, M. Immunoregulatory role of interleukin-10 in rheumatoid arthritis. *J Exp Med* 179: 1517-1527, 1994.

17. Steiner, G., Tohidast-Akrad, M., Witzmann, G., Vasely, M., Studnicka-Benke, A., Gal, A., Kunaver, M., Zenz, P., Smolen, JS. Cytokine production by synovial T-cells in rheumatoid arthritis. *Rheumatology*: 38: 202-213, 1999.

18. Kirkham, B., Portek, I., Lee, CS., Stavros, B., Lenarczyk, A., Lassere, M., Edmonds, J. Intra-articular variability of synovial membrane histology, immunohistology, and cytokine mRNA expression in patients with rheumatoid arthritis. *J Rheumatol.* 26(4): 777-784, 1999.

19. Firestein, GS., Alvaro-Gracia, JM., Maki, R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J. Immunol* , 144: 3347-53, 1990.

20. Buckley, CD. Treatment of rheumatoid arthritis. BMJ 315, 236-238, 1997

21. Sewell, KL., Trentham, DE., Pathogenesis of rheumatoid arthritis. *The Lancet*, 341, 283-286, 1993

22. Granelli-Piperno, A. In situ hybridisation for interleukin-2 and interleukin 2 receptor mRNA in T-cells activated in the presence or absence of cyclosporin A. *J Exp. Med* 168(5): 1649-58, 1988.

23. Tocci, MJ., Matkovich, DA., Collier, KA., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Sierkierka, JJ., Chin, J., Hutchinson, NI. The immune suppressant FK506 selectively inhibits expression of early T-cell activation genes. *J Immunol* 143(2): 718-726, 1989.

24. Herold, KC., Lancki, DW., Moldwin, RL., Fitch, FW. Immunosuppressive effects of cyclosporin A on clones T-cells. *J Immunol* 136(4): 1315-1321, 1986.

25. Ferraccioli, G., Falleti, E., De Vita, S., Di Poi, E., Damanto, R., Cassata, L., Salaffi, F. Circulating levels of interleukin 10 and other cytokines in rheumatoid arthritis treated with cyclosporin A or combination therapy. *J Rheumatol* 25(10): 1874-1979, 1998.

26. Moreland, LW. Inhibitors of tumor necrosis factor for rheumatoid arthritis. *J Rheumatol* 26 suppl 57: 7-15, 1999.

Chapter 8

SUMMARY AND GENERAL DISCUSSION

In this final section, a brief overview of the results is presented. The most important findings are discussed and the implications for the design of RA therapies are considered.

The first goal was: to optimize a technique that can be used to determine TCR V gene expression profiles in patient samples and to identify expanded TCR V gene elements.

Various approaches are available for the study of TCR V gene expression. Expensive real-time fully automated systems or two-step PCR based technologies with a distinct read-out method are described to quantify mRNA. The cell surface protein expression on the other hand, can be measured by flow-cytometry. In table 1, an overview is given of some quantification techniques, with their respective advantages/disadvantages.

We developed a PCR-ELISA to compare TCR V gene expression in peripheral blood and synovial samples of patients and controls, and demonstrated the applicability of the technique on two test systems (Chapter 3).
The PCR-ELISA is no 'absolute' quantification system: there are no internal or external quantification standards included (1). It is a semiquantitative technique that can be used to determine relative differences in the expression level of individual TCR V gene families in the total V gene expression profile.

In RA, synovial tissue biopsies are the best representative samples of the inflammatory disease site. Needle arthroscopy can be used to obtain synovial tissue samples. This is a non-invasive, safe and well tolerated approach (2). However, the samples are small and contain low amounts of cells. In the present study it was shown that the PCR-ELISA is a very sensitive method that can be used for repertoire analysis in blood and tissue samples containing as few as 50.000 lymphocytes (3). The technique is fast, sensitive, easy to use, and allows for a large scale study. As demonstrated in this thesis, PCR-ELISA combined with CDR3 region spectratyping (4), is an efficient tool to identify clonally expanded T-cell subsets in synovial samples of RA patients.

Using PCR-ELISA, TCR V gene expression profiles were studied in early and chronic RA patients (chapter 4 and 5) and V gene profiles were compared in paired blood and tissue samples of the patients. The TCR V gene expression is skewed in the synovial cavity samples of the

patients but not in their blood. A restricted TCR V gene expression in the synovial samples of RA patients possibly suggest an 'in vivo' antigen driven stimulation.

We also studied the TCR V gene expression in a 3y old boy with Acute disseminated encephalomyelitis (ADEM) (chapter 7). We found additional support for the contribution of a *Streptococcus pyogenes* infection to the dramatic demyelination in this patient. In this study PCR-ELISA was used as an efficient tool to identify expanded T-cell populations *in vivo* and after *in vitro* stimulation with superantigens that are potentially involved in this demyelinating disease.

Technique	*Advantages*	*Disadvantages*
A. PCR based quantification		
1. PCR amplification		
General properties	Fast, sensitive Large scale applicability Primer sequences covering the complete V gene repertoire available	non-functional messengers detected inter/intra-sample variation possible differences in amplification efficiency
Using internal/external standard	High quantitative value depending on read-out used	Labour intensive/time consuming
2. Read-out methods		
Ethidium bromide staining	Easy to perform low quantitative value	low sensitivity
Blotting and hybridisation	Sensitive labour intensive low quantitative value	radioactive probes used
QPCR 5000	Sensitive	labour intensive/time consuming expensive reagents
ELISA	Simple, sensitive, fast	
TaqMann, PCR LightCyler	Fast, sensitive, easy to use	Expensive instruments needed
B. Protein based systems		
Flow-cytometry	surface expressed protein level measured	limited panel of monoclonal antibodies available

The second goal was: to study possible differences in the TCR V gene expression profiles between early and chronic RA patients.

So far, TCR repertoire studies yielded conflicting results. Technological differences, genetic, environmental and clinical factors as well as differences in the manipulation of study material prior to analysis have to be taken into account. To avoid *in vitro* bias of the *in vivo* obtained properties, lymphocytes from blood and synovial fluid were used without any *in vitro* stimulation. In addition, since the TCR repertoire may change during disease progression, patients were grouped into early (<1y) and chronic (>1y) RA patients.

TCR V gene expression was studied with PCR-ELISA in early and chronic RA patients. Clonal analysis was performed by CDR3 region sequence analysis. Paired blood and tissue samples were handled simultaneously. Mononuclear cells were isolated and unstimulated, unselected T-cells were studied.

The TCR V gene profiles were heterogeneous in the blood of patients and controls studied, whereas a skewed TCR V gene expression was demonstrated in synovial samples of RA patients. The number of overrepresented V genes was significantly higher in the synovium of chronic (n=31) as compared to early RA patients (n=7). The TCR V gene restriction differed among patients, but was similar in both knees of patients with bilateral synovitis. TCR V gene skewing was observed in early RA patients, and to a lesser extent in chronic disease stages. CDR3 region clonal analysis revealed oligo-and poly-clonally expanded T-cells in a patient with recent disease onset and marked clonal expansions in a chronic RA patient.

These results possibly suggest an *in vivo* antigen driven clonal expansion of T-cells in the synovial cavity of RA patients. *In vivo* antigen driven clonal expansion in RA synovium was also reported by other groups, however, so far the pathogenic relevance of clonally expanded T-cells is not known (5,6,7,8,9,10,11).

Several important questions arise:

1. Are autoreactive T-cells activated in the blood or the synovium of the patients ?

Some of our findings indicate that activation of T-cells which accumulate in the joints occurs in the blood compartment.

For instance, similar TCR V gene expression profiles and identical CDR3 region sequences observed in paired knees of a patient with bilateral synovitis (Chapter 4), suggest that identical T-cell clones are present in both knees of this patient.

In addition, only activated T-cells may have the potential to cross the endothelial cell wall and leave the peripheral circulation (12,13)

Once in the joint, in situ antigen stimulation can cause T-cell reactivation and clonal expansion. Peripheral activation, synovial reactivation and a subsequent clonal expansion were also found by others (14, 15, 16). So far, the nature of the peripheral antigens or the relevance of peripheral activated T-cell clones in the disease pathogenesis remains unknown.

2. Are antigens different in early and chronic RA ?

Synovial T-cells are thought to initiate the inflammatory response in the joints of RA patients (17). Dominant T-cell clones identified in later disease stages on the other hand might be involved in the disease progression (6). In this study, TCR V gene expression profiles revealed an increased number of overrepresented TCR V gene families in chronic as compared to early RA patients. This could suggest that the array of T-cell stimulating antigens is more diverse in a chronic disease stage as compared to early RA. Newly activated T-cells progressively infiltrate in the joint cavity and gradually decrease the proportion of the initiating T-cell clones in the synovial population. Our data therefore support the concept of determinant spreading, a process that has been shown to operate in experimental autoimmune encephalomyelitis (EAE) which is an animal model of multiple sclerosis (18, 19, 20). Determinant spreading could have been caused by the infiltration of irrelevant 'bystander' T-cells, or the influx of newly activated T-cells by locally sequestered antigens.

3. What may be the type of autoantigens involved ?

CDR3 region sequencing revealed some striking differences in the clonal composition of T-cells in an early and a chronic RA patient. In the early RA patient, synovial T-cells are oligo- or polyclonally expanded, whereas clear monoclonal expansions are demonstrated in the synovium of a chronic RA patient. These results are compatible with a superantigen-like stimulation pattern in the early RA patient, and a conventional antigen driven T-cell expansion in the chronic RA patient. So far, conventional antigen stimulations (21) and superantigen stimulations (22,23) are reported in RA synovium. Based on these data we hypothesize that different T-cell activation pathways may be operating in RA at different stages of the disease. In the early RA patient the TCR expression pattern is compatible with a T-cell activation process induced by microbial superantigens or different types of classical antigens. Perhaps, superantigen stimulates synovial T-cells, that subsequently cross-react with synovial autoantigens, become reactivated and overgrow the other synovial T-cells that are not reactivated by a cross-reactive epitope. In the chronic RA patient, only a few antigenic epitopes may have been responsible for the persistent expansion of T-cells in the joints.

The third goal was: to determine the TCR V gene profile in the synovium of early RA patients at two timepoints and to evaluate the effect of a CSA treatment on the TCR V gene profile.

Our and other reports demonstrate that in RA the TCR V gene profile broadens during disease progression, an observation also that was also made in other autoimmune diseases (17, 19, 20, 24). Broadening of the TCR V gene expression profile interferes with the characterization of disease relevant T-cells, that are potential targets for T-cell directed therapies. Indeed, it is possible that disease inducing T-cells are only present in the early phase of the disease process, while other irrelevant T-cells accumulate at later disease stages. We therefore studied TCR V gene expression in early RA patients, and compared the V gene profiles in the patient's blood and tissue samples. Untreated RA patients were studied to exclude potential treatment effects on the TCR V gene expression profile. TCR V gene repertoire changes were analyzed in synovial membrane biopsies over a 16 week period. Subsequently, the effect of CSA treatment (3 mg/kg/day) was studied on the TCR repertoire in the joints in a subgroup of the patients.

Synovial T-cells were found in all tissue biopsies studied at the first timepoint before any treatment. TCR V gene skewing was observed in synovial biopsies and some peripheral blood samples. TCR V gene families overrepresented in the peripheral blood of the patients were not overrepresented in synovial biopsies, but TCR V gene profiles at different sites in the same joint were highly identical. Identical T-cell clones occurring at different sites in the synovial cavity have been reported earlier (7), but were not found by others. This observation suggests a considerable heterogeneity of expanded T-cell clones at different sites in the diseased joints (25, 26, 27).

In a follow-up analysis we found dynamic clonotype changes in placebo control patients but persistence of overrepresented T-cell clones in CSA treated patients. We hypothesize that predominant clones are depleted in the synovial tissue through rapid apoptosis. However, as a result of the local or peripheral activation of new T-cells, other T-cell clones continuously accumulate in the joints of these patients. CSA is known to inhibit early events in T-cell activation but may also suppress apoptosis. It can be speculated that CSA treatment may have suppressed activation of new T-cell clones in the synovium of these patients, while clearance through apoptosis may have been reduced. This could result in a more stable TCR expression in the joints of the CSA treated patients. Persistence of T-cell clones was also demonstrated by others, and possibly suggests that these clones are actively involved in the disease progression (5, 6, 28). Moreover, no TCR mRNA was detected in synovial tissue of 3 out of 7 RA patients after CSA treatment, although this was also noted in 1 control patient. Although the expression of TCR mRNA may have been selectively inhibited in these samples, the most likely explanation for this observation is that these tissue samples contain few T-cells only.

These results suggest
1. TCR skewing in early RA synovial tissue.
2. that in situ reactivation of T-cells is reduced by CSA.
3. that dynamic clonotype changes occur during disease progression

The fourth goal was: to determine the cytokine profile in peripheral blood and synovial tissue samples of early RA patients and to study the effect of CSA on cytokine mRNA contents.

Cytokine profiles can be used to study the inflammatory micro-environment in blood and synovium. TNF-α, IFN-γ, IL-4 and IL-10 mRNA levels were determined in blood and tissue

samples of the early RA patients before and after treatment with CSA or placebo (see chapter 4). The levels of the cytokines studied were increased in blood of early RA patients as compared to the blood of healthy controls. Furthermore, TNF-α, IFN-γ, IL-4 and IL-10 cytokine mRNA levels were increased in synovial biopsies as compared to paired blood samples. The cytokine profiles show a high degree of inflammatory activity in synovium as compared to blood of the patients, and further suggest that T-cells and macrophages are involved in the (auto) immune response in the joints.

We also examined cytokine mRNA levels in blood and synovial tissue 16 weeks after treatment with CSA (6 patients) or placebo (4 patients). CSA is an immune suppressive drug that selectively acts on T-cells, it interferes with the production of IL-2 and IFN-γ on the transcriptional level (29). As compared with untreated RA patients, a clear decrease in the IFN-γ mRNA content was observed in synovial biopsy samples of CSA treated patients. During follow-up study, TNF-α mRNA levels were increased in synovial biopsy samples of 2/6 CSA treated and 3/4 control RA patients. This increase in TNF-α mRNA content could suggest that macrophage activity is not completely halted. Activated macrophages stimulate synoviocytes that subsequently irreversibly degrade bone and cartilage structures. These data show that CSA treatment inhibits T-cell but not macrophage activity in the joints of RA patients.

Final discussion

Some key findings of this study are discussed together with data from other groups to analyze their potential relevance for our current understanding of the pathogenesis in RA.

The role of T-cells in RA

Our data and previous studies have shown that TCR profiles are highly skewed in the synovium of RA patients, suggesting that some T-cell populations are expanded in the joints. Unfortunately, there is only little information about antigen reactivity of these T-cells. It remains therefore unclear whether these T-cells play a pathogenic role in the disease process, or are unimportant by-products of the ongoing inflammatory process in the joints. Some of our findings are however compatible with a pathogenic role for T-cells in RA. For instance, expanded T-cell populations were already observed in patients with a disease duration of less than one year, identical T-cell clones were identified in two affected joints of patients with bilateral synovitis, and persisting clonal expansions were observed in a chronic RA patient. In addition, some T-cell depleting therapies are demonstrated to be clinically effective in RA. For instance, it has been shown that autologous stem-cell transplantation with T-cell depleted hemopoietic stem cells induced clinical remission in RA (31). It remains however possible that T-cells are only essential in the early phases of the disease, while other inflammatory subsets play a role in the later disease stages. It could be hypothesized that after an initial event triggered by synovial T-cells, synovitis is carried by synoviocytes and macrophages accumulating in the diseased joints (32, 33, 34, 35, 36). Subsequently, T-cells activated by locally sequestered antigens could be innocent bystanders with no active contribution to the disease progression. Further studies are necessary to determine whether autoreactive T-cells are essential in RA, or in a specific subgroup of patients, and whether T-cells are involved in all phases of the RA process or in disease initiation only.

Autoantigens and determinant spreading concept in RA

Which are the autoantigens involved in triggering activation of autoreactive T-cells in RA? Our observations indicate that the primary activation of autoreactive T-cells occurs in the blood compartment. In this scenario, autoreactive T-cells in the blood may become activated by recognition of a cross-reactive epitope of microbial origin, a process described as molecular mimicry (37). Autoreactive T-cells may become activated by microbial superantigens in a BV

gene-specific manner, which is in line with our observations of heterogeneous CDR3 region sequences among expanded BV gene families. Additional evidence for an *in vivo* superantigen stimulation in RA patients was provided in earlier reports: based on TCR V gene expression profiles (22, 23), and based on cross reactivity profiles of antigen specific T-cell clones from the blood of RA patients (37, 38)

TCR analysis of synovial T-cells showed oligo- or monoclonal expansions, suggesting a T-cell activation process induced by one or a few 'classical' antigens. Together, it seems that different antigens are involved in the activation of autoreactive T-cells in RA, and this may depend on the genetic background or the availability of relevant environmental triggers. An additional complicating factor may be the determinant spreading concept. Determinant spreading is a process characterized by the development of an immune response against antigens or antigen epitopes released during the progression of a chronic inflammatory response (39). We and others have shown that the TCR profile in the joints changes rapidly in early RA patients, and the TCR pattern becomes broader when disease progresses. This phenomenon may seriously complicate specific immune therapeutic strategies for the disease. However, in EAE studies it has been shown that determinant spreading can be efficiently prevented when T-cell activation is suppressed in the early steps of the determinant spreading cascade. (40)

Consequences for therapy design

The results presented in this thesis may have important consequences for the design of immunotherapies for RA. First, based on the heterogeneous TCR BV gene expression by expanded T-cells in the synovium, and the dynamic TCR clonotype changes in the joints of early RA patients, it seems unlikely that a TCR BV gene targeted approach such as TCR peptide vaccination will be effective in RA. In addition, due to the apparent heterogeneous antigen reactivity profiles of the potential pathogenic T-cells in RA, and the epitope spreading mechanism, no single autoantigen can be targeted by antigen specific immunotherapeutic strategies. Although, some elegant antigen-specific therapeutic designs, such as peptide ligand therapy are currently tested, it will be very difficult to interfere with all possible autoantigens involved in the disease process. In peptide ligand therapies, autoreactive T-cell activation is inhibited by *in vivo* administration of soluble MHC-binding competitors. As such, arthritogenic peptide presentation is blocked, and disease induction prevented (41, 42). Therapies which suppress activation of new autoreactive T-cell clones in an antigen non-specific manner may however be successful, especially when they are combined with a therapy that simultaneously eliminates previously activated T-cells in the joint. The data from our study suggest that CSA may be combined with, for instance, T-cell vaccination to bring about such an effect. Other antigen non-specific approaches such as anti-cytokine therapies are also good candidates. Some

successes were already reported for therapies targeting TNF-α, which is an important mediator of the inflammatory reactivity in RA joints (43, 44). Finally it may become worthwhile to study the effect of combination therapies in which two or more agents which interfere with different steps in the disease pathway are combined. Some of these combination therapies have yielded already promising results (45).

Mechanism of action of cyclosporin A

Cyclosporin A is a proven therapy for RA. Our study was the first to analyze the effects of CSA therapy on TCR expression and cytokine profiles in the joints of RA patients. Using needle arthroscopic biopsies and a sensitive PCR approach we were able to evaluate the effects of the therapy on the cells presented at the primary disease site: the synovial membrane. Our study has shown that CSA affects TCR expression profiles in the joints of the patients. The dynamic repertoire changes seen in placebo-treated patients could not be demonstrated in synovial tissue of the CSA treated patients. These observations could be the consequence of the suggested mechanism of action of CSA: suppression of T-cell activation (no new clones are activated), and a reduction of apoptosis (persistence of some clones in the synovial tissue) (46). In addition, a high proportion of CSA treated patients no longer had TCR mRNA in their synovial tissue, which could suggest a significant reduction of T-cells in the joints. CSA also suppresses T-cells cytokine secretion (47). Interestingly, the expression of IFN-γ was found to be highly reduced in the joints of the treated patients, whereas the TNF-α production, which was most likely primarily secreted by macrophages was not affected in all patients. This might be an important problem determining the clinical efficacy, since TNF-α is suggested to play an important pathogenic role in RA. The data therefore suggest that CSA treatment may be combined with another therapy that efficiently reduces the TNF-α production such as TNF inhibitors (48). Finally, the use of CSA as a therapy for RA should be focused on early RA patients since T-cells are most likely primarily important in the early phase of the disease process. The side effects of CSA may however complicate CSA therapy for these patients although the strict application of guidelines may prevent some adverse effects (49).

Future perspectives

Although the work presented in this thesis revealed some interesting aspects related to the RA pathogenesis, further studies are required to unravel the exact contribution of T-lymphocytes. Fortunately, new techniques are now available which will significantly facilitate the collection of information in this field of research. For instance as shown in our work new, fast and very accurate PCR based technologies are available to study TCR repertoires and to follow-up T-cell clones during disease progression, and to measure cytokine expression. New techniques such as tetramer-staining became available to measure antigen reactive T-cells (50), and new clinical approaches such as needle arthroscopy are available to provide study material (biopsies) from the diseased joints. These techniques allow efficient screening of T-cell activities in individual patients, and examination of the relevance of *in vivo* activated T-cells in relation to the clinical progression. In addition, the identification of genes that are essential for the development of the disease, may shed new light on the role of essential components of the inflammatory process such as cytokines, antigens, MHC elements and/or T-cell related molecules. Finally, the clinical effects of ongoing immunotherapeutic studies in RA may provide new clues for understanding the role of the targeted immune components in the pathogenesis.

References

1. Delassus, S. Quantification of cytokine transcripts using polymerase chain reaction. *Eur. Cyt. Netw.* 8(3): 239-244, 1997.

2. Baeten, D., Van den Bosh, F., Elewaut, D., Steur, A., Veys, E., De Keyser, F. Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. *Clin Rheumatol.* in press.

3. VanderBorght, A., Van der Aa, A., Geusens, P., Vandevyver, C., Raus, J., Stinissen, P. Identification of overrepresented T-cell receptor genes in blood and tissue biopsies by PCR-ELISA. *J. Immunol. Meth.* 223: 47-61, 1999.

4. Pannetier, C., Even, J., Kourilsky, P. T-cell receptor diversity and clonal expansion in normal and clinical samples. *Immunol Today* 16(4): 176-181, 1995.

5. Kato, T., Kurokawa, M., Masusko-Hongo, K., Sasakawa, H., Sekine, T., Ueda, S., Yamamoto, K., Nishioka, K. T-cell clonality in synovial fluid of a patient with Rheumatoid Arthritis: persistent but fluctuant oligoclonal T-cell expansions. *J. Immunol.* 159: 5143-5149, 1997.

6. Alam, A., Lambert, N. Lulé, J., Coppin, H., Masieres, B., de Préval, C., Cantagrel, A. Persistence of dominant T-cell clones in synovial tissue during Rheumatoid Arthritis. *J Immunol.* 156: 3480-3485, 1996.

7. Ikeda, Y., Masuko, K., Nakai, Y., Kato, T., Hasanuma, T., Yoshino, SI., Nishioka, K?-., Yamamoto, K., High frequencies of identical T-cell clonotypes in synovial tissues of rheumatoid arthritis patients suggest the occurrence of common antigen driven responses. *Arthritis Rheum.* 39(3): 446-453, 1996.

8. Celis, L., Vandevyver, C., Geusens, P., Dequeker, J., Raus, J., Zhang, J. Clonal expansion of mycobacterial heat-shock protein reactive T-lymphocytes in synovial fluid and blood of rheumatoid arthritis patients. *Arthritis Rheum.* 40: 510-519, 1997.

9. Miltenburg, AMM., van Laar, JM., Daha, MR., De Vries, RRP., van den Elsen, PJ., Breedveld, FC. Dominant T-cell receptor b chain gene rearrangements indicate clonal expansion in the rheumatoid joint. Scand. *J. Immunol.* 31: 121-125, 1990.

10. Rittner, HL., Zettl, A., Jendro, MC., Bartz-Bassanella, P., Goronzy, JJ., Weyand, CM. Multiple mechanisms support oligoclonal T-cell expansion in rheumatoid synovitis. *Mol. Med.* 3(7): 452-465, 1997.

11. Borgato, L., Beri, R., Biasi, D., Testoni, R., Cugola, L., Ceru, S., De Sandre , G., Lunardi, C. Analysis of T-cell receptor repertoire in Rheumatoid Arthritis. *Clin. Exp. Rheumatol.* 15(5): 475-479, 1997.

12. Stinissen, P., Medaer, R., Raus, J. Myelin reactive T-cells in the autoimmune pathogenesis of multiple sclerosis. *Multiple Sclerosis* 4, 203-211, 1998.

13. Laffon, A., Garcia-Vicuna, R., Humbria, A., Postigo, AA., Corbi, AL., de Landaruzi, MO., Sanchez-Madrid, F. Up regulated expression and function of VLA-4 fibronectin receptors on human activated T-cells in rheumatoid arthritis. *J. Clin. Invest.* 88(2): 546-552, 1991.

14. Goronzy, JJ., Bartz-Bazzanella, P., Hu, W., Jendro, MC., Walser-Kuntz, DR., Weyand, CM. Dominant clonotypes in the repertoire of peripheral CD4+ T-cells in rheumatoid arthritis. *J. Clin. Invest.* 94(5): 2068-2076, 1994.

15. Hall, FC., Thomson, K., Procter, J., McMichael, AJ., Wordsworth, BP. TCR β spectratyping in RA: evidence of clonal expansions in peripheral blood lymphocytes. *Ann. Rheum. Dis.* 57: 319-322, 1998.

16. Striebich, CC., Falta, MT., Wang, Y., Bill, J., Kotzin, BL. Selective accumulation of related CD4+ T-cell clones in the synovial fluid of patients with rheumatoid arthritis. *J. Immunol.* 161: 4428-4436, 1998.

17. Fisher, DC., Opalka, B., Hoffmann, A., Mayr, W., Haubeck, HD. Limited heterogeneity of rearranged T-cell receptor Vα and Vβ transcripts in synovial fluid T-cells in early stages of rheumatoid arthritis. *Arthritis Rheum.* 39(3): 454-462, 1996.

18. Lehmann, PV., Sercarz, EE., Forsthuber, T., Dayan, CM., Gammon, G. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today* 14(5): 203-208, 1993.

19. Bucht, A., Oksenberg, JR., Lindblad, S., Gronberg, A., Steinman, L., Klareskog, L. Characterization of T-cell receptor αβ repertoire in synovial tissue from different temporal phases of rheumatoid arthritis. Scand. *J. Immunol.* 35: 159-165, 1992.

20. Elewaut, D., De Keyser, F., Van den Bosch, F., Verbruggen, G., Veys, E. Clonal analysis of T-cell lines expanded from rheumatoid synovium and subcutaneous nodules broadening of the clonal spectrum in rheumatoid synovium in relation to disease duration. Submitted.

21. Bröker, B., Korthäuer, U., Heppt, P., Weseloh, G., De La Camp, R., Kroczek, R., Emmrich, F. Biased T-cell receptor V gene usage in rheumatoid arthritis. *Arthritis Rheum.* 36: 1234-1243, 1993.

22. Howell, MD., Diveley, JP., Lundeen, KA., Esty, A., Winters, ST., Carlo, DJ., Brostoff, W. Limited T-cell receptor β chain heterogeneity among interleukin-2 receptor positive synovial T-cells suggests a role for superantigen in Rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA* 88: 10921-10925, 1991.

23. Palliard, X., West, SG., Lafferty, JA., Clements, JR., Kappler, JW., Marrack, P., Kotzin, B. Evidence for the effect of a superantigen in rheumatoid arthritis. *Science* 253: 325-329, 1991.

24. Lim, A., Toubert, A., Pannetier, C., Dougados, M., Charron, D., Kourilsky, P., Even, J. Spread of clonal T-cell expansions in rheumatoid arthritis patients. Hum Immunol. 48(1-2): 77-83, 1996.

25. Struyk, L. Hawes, GE., Mekkers, HMM., Tak, PP., Breedveld, FC., van den Elsen, P. Molecular analysis of the T-cell β chain repertoire in early rheumatoid arthritis : heterogeneous TCR BV gene usage with shared amino acid profiles in CDR3 regions of T-lymphocytes in multiple synovial tissue needle biopsies from the same joint. *Eur. J. Clin. Invest.* 26: 1092-1102, 1996.

26. van Laar, JM., Miltenburg, AM., Verdonk, MJ., Daha, MR., de Vries, RR., van den Elsen, P., Breedveld, FC. T-cell receptor beta-chain gene rearrangements of T-cell populations expanded from multiple sites of synovial tissue obtained from a patient with rheumatoid arthritis. *Scand. J. Immunol.* 35(2): 187-194, 1992.

27. Dolhain, R., Ter Haar, N., De Kuiper, R., Nieuwenhuis, I., Zwinderman, A., Breedveld, F., Miltenburg, A. Distribution of T-cells and signs of T-cell activation in the rheumatoid joint: implications for semiquantitative comparative histology. *Br. J. Rheumatol.* 37: 324-330, 1998.

28. Masuko-Hongo, K., Sekine, T., Ueda, S., Kobata, T., Yamamoto, K., Nishioka, K., Kato, T. Long-term persistent accumulation of CD8+ T-cells in synovial fluid of rheumatoid arthritis. *Ann. Rheum. Dis.* 56(10): 613-621, 1997.

29. Sevach, EM., The effects of cyclosporin A on the immune system. *Ann. Rev. Immunol.* 3: 397-342, 1985.

30. van Roon, J., Verhoef, CM., van Roy, J., Gmelig-Meyling, F., Hiber-Bruning, O., Lafeber, F., Bijlsma, J. Decrease in peripheral type I over type 2 T-cell cytokine production in patients with rheumatoid arthritis correlates with an increased severity of disease. *Ann Rheum* Dis. 56: 656-660, 1997.

31. Durez, P., Toungouz, M., Schandene, L., Lambermont, M., Goldman,M. Remission and immune reconstitution after T-cell depleted stem-cell transplantation for rheumatoid arthritis. *The lancet* 352, 881, 1998.

32. Howe, HS. Rheumatoid arthritis: a review. *Ann. Acad. Med. Singapore*, 27(1): 83-88, 1998

33. Panayi, GS. T-cell dependent pathways in rheumatoid arthritis. *Curr Opin Rheumatol* 9(3): 236-240, 1997.

34. Zhang, H., Phang, D., Laxer, R., Silverman, E., Sueihua, P., Doherty, PJ. Evolution of the T cell receptor β repertoire from synovial fluid T-cells of patients with juvenile onset rheumatoid arthritis. *J Rheumatol*. 24: 1396-1402, 1997.

35. Fisher, DC., Opalka, B., Hoffmann, A., Mayr, W., Haubeck, HD. Limited heterogeneity of rearranged T-cell receptor Vα and Vβ transcripts in synovial fluid T-cells in early stages of rheumatoid arthritis. *Arthritis Rheum*. 39(3): 454-462, 1996.

36. Bresnihan, B. Pathogenesis of joint damage in rheumatoid arthritis. *J Rheumatol* 26(3): 717-719, 1999.

37. Wilson, KB., Quale, AJ., Suleyman, S., Kjeldsen-Kragh, J., Forre, O., Natvig, J., Capra, J. Heterogeneity of TCR repertoire in synovial fluid T-lymphocytes responding to BCG in a patient with early rheumatoid arthritis. *Scand J Immunol* 38: 102-112, 1993.

38. Gaston, H., Life, P., Bailey, LC., Bacon, P. In vitro responses to a 65-kilodalton mycobacterial protein by synovial T-cells from inflammatory arthritis patients. *J immunol* 143(8): 2494-2500, 1989.

39. Vanderlugt, CJ., Miller, SD. Epitope spreading. *Curr Opin. Immunol*. 8(6): 831-836, 1996.

40. Tuohy, VK., Yu, M., Yin, L., Kawczak, JA., Johnson, JM., Mathisen, PM., Weinstock-Guttman, B., Kinkel, RP. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* 164: 93-100, 1998.

41. Hanson, GJ. DR (MHC class II) ligands: an approach to rheumatoid arthritis therapeutics. *Curr Pharm Des* 4(5): 397-402, 1998.

42. Kingsley, GS. Class II MHC binding peptides: a feasible treatment for arthritis ? *Clin Exp Rheumatol* 10(3): 297-300, 1992.

43. Rankin, ECC., Chay, EHS., Kassimos, D. The therapeutic effects of an engineered human anti-tumor necrosis factor antibody (CDP571) in rheumatoid arthritis. *Br J Rheumatol* 34: 334-342, 1995.

44. Moreland, LW., Baumgartner, S., Schiff, M. Treatment of rheumatoid arthritis with recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N Engl J Med* 337: 141-147, 1997.

45. Mottonen, T., Hannonen, P., Leirisalo-Repo, M., Nissila, M., Kautiainen, H., Korpela, M., Laasonen, L. et al. Comparison of combination therapy with single-drug therapy in early rheumatoid arthritis: a randomised trial. Lancet: 353(9164): 1568-1573, 1999.

46. Brunner, T., Yoo, NJ., LaFace, D., Ware, CF., Green, DR. Activation-induced cell death in murine T-cell hybridomas. Differential regulation of Fas(CD95) versus Fas ligand expression by cyclosporin A and FK506. *Int Immunol*. 8(7): 1017-1026, 1996.

47. Bentin, J. Mechanism of action of cyclosporin in rheumatoid arthritis. *Clin Rheumatol* 14(2): 22-25, 1995.

48. Moreland, LW. Inhibitors of tumor necrosis factor for rheumatoid arthritis. *J Rheumatol* 26 S57: 7-15, 1999.

49. Van den Borne, B., Landewe, R., The, H., Breedveld, F., Dijkmans; B., Cyclosporin A therapy in rheumatoid arthritis: only strict application of the guidelines for safe use can prevent irreversible renal function loss. *Br J Rheumatol*. 38(3): 254-259, 1999.

50. Hickling, JK., Measuring human T-lymphocyte function. *Expert reviews in Molecular Medicine*, accession number : txt001jhc, 1998.

NEDERLANDSE SAMENVATTING

Samenvatting

Rheumatoide artritis is een autoimmune aandoening die gekenmerkt wordt door chronische ontsteking van de gewrichten. Een groot percentage van de witte bloedcellen die aangetroffen wordt in de aangetaste gewrichten van patiënten zijn T-lymfocyten. In het verleden werd aangetoond dat T-cellen in de ontstoken gewrichten geactiveerd zijn en dus vermoedelijk actief zijn in het ziekteproces. Onder normale omstandigheden zijn T-lymfocyten verantwoordelijk voor de afweer van lichaamsvreemd materiaal zoals virussen, bacteriën en schimmels. Bij RA daarentegen veroorzaken T-cellen vermoedelijk een immuunrespons tegen het lichaamseigen materiaal van de gewrichten. Er wordt verondersteld dat deze 'auto-reactieve' T-cel respons het kraakbeen en het bot van de gewrichten aantast, en er een onomkeerbare schade veroorzaakt. De antigenen die in deze 'autoimmune' respons betrokken zijn, zijn echter nog niet gekend.

T-cellen worden gekarakteriseerd door een T-cel receptor (TCR) die op het oppervlak van de lymfocyten tot expressie wordt gebracht. De TCR bestaat uit een α en een β keten, die een willekeurige combinatie van een variabel (V), koppelings (J), diversiteits (D) en constant (C) gengebied bevatten. Wanneer T-cellen door antigenen worden geactiveerd en daardoor selectief gaan vermenigvuldigen treden er vermoedelijk veranderingen op in het TCR expressie profiel van deze populatie. Tengevolge van de T-cel activatie en proliferatie worden lymfocyten die een specifieke V gen familie tot expressie brengen selectief aangerijkt in de totale populatie.

In het verleden werden reeds verschillende pogingen ondernomen om de autoreactieve T-cellen in de gewrichten van RA patiënten te karakteriseren.

In deze studie werd aan de hand van TCR expressie analyse aangetoond dat T-cellen vermoedelijk actief betrokken zijn in de pathogenese van RA. TCR V gen profielen in bloed en synoviale stalen van vroege en chronische RA patiënten werden bestudeerd, expressie patronen onderling vergeleken, en mogelijke conclusies getrokken.

Hoofdstuk 1: Inleiding

In het eerste hoofdstuk wordt een schematisch overzicht gegeven van de verschillende stappen die in het ontstaan en de progressie van de ontstekingsreactie bij RA betrokken zijn. De relevantie van T-cellen in het ziekteverloop wordt benadrukt, maar ook andere celtypes zoals macrofagen, synoviocyten en B-cellen die in de aangetaste gewrichten voorkomen worden kort voorgesteld. Een aantal eerder gepubliceerde studies over het TCR V gen profiel in bloed en synovium worden samengevat, en een aantal technieken die bruikbaar zijn voor TCR expressie studies worden kort beschreven. Algemeen blijkt dat TCR V gen profielen weinig uniform zijn, vermoedelijk patiënt specifiek zijn, en mogelijk beïnvloed worden door factoren zoals ziekteduur, behandeling en eventuele *in vitro* stimulaties uitgevoerd op het studiemateriaal.
Vervolgens worden een aantal therapieën voor RA beschreven; niet alleen klassieke therapieën die reeds op grote schaal worden toegepast, maar ook veelbelovende nieuwe therapieën die echter nog in een experimentele fase zijn, worden kort toegelicht.

Hoofdstuk 2: Materialen en methoden

In dit hoofdstuk worden alle gebruikte Materialen en Methoden beschreven. Aan de hand van een schematisch overzicht kan de lezer duidelijk onderscheiden hoe de TCR expressie in bloed en synoviale stalen in deze studie wordt bepaald.

Hoofdstuk 3: Identificatie van preferentieel gebruikte T cel receptor genen in bloed en synoviale gewrichtsbiopten met PCR-ELISA

Uit recente literatuur gegevens blijkt dat een groot aantal technieken beschikbaar zijn voor de studie van TCR V gen expressie. In tabel 8.1 van 'Summary and General discussion' werden een aantal technieken met hun respectievelijke voor- en nadelen voorgesteld.
In deze studie werd een PCR-ELISA ontwikkeld voor de studie van TCR V gen profielen in perifeer bloed en synoviale stalen van RA patiënten. In hoofdstuk 1 wordt beschreven hoe aan de hand van twee testsystemen: 'Toxic shock syndrome toxin' (TSST-1) gestimuleerde T-cellen en Jurkat T-cellen, de toepasbaarheid van de technologie werd geëvalueerd. T-cellen gestimuleerd met TSST-1 vertonen een selectieve expressie van de TCR BV 2 gen familie terwijl Jurkat T-cellen geïmmortaliseerde T-cellen zijn die selectief het TCR BV 8 gen op het T-cel oppervlak tot expressie brengen. Uit deze studie blijkt dat de PCR-ELISA een snelle, eenvoudige en gevoelige techniek is die bruikbaar is om relatieve verschillen in expressie van

individuele V gen families in het totale profiel te bepalen. De PCR-ELISA is echter geen 'absolute' kwantificatie techniek. Omdat geen interne of externe kwantificatie-standaarden worden gebruikt is de technologie semikwantitatief, en dus enkel bruikbaar om relatieve verschillen in TCR V gen expressie te bepalen.

De PCR-ELISA werd uitgevoerd op perifeer bloed, synoviaal vocht en synoviale membraanstalen van RA patiënten. Bij RA zijn synoviale membraanstalen vermoedelijk de meest representatieve stalen voor het ontstoken gewricht. Met behulp van naaldartroscopie kunnen kleine membraanbiopten worden bemonsterd. Naaldarthroscopie is bovendien een niet-invasieve en veilige techniek, maar de staaltjes zijn klein en bevatten maar zeer lage celaantallen. In dit deel van de studie wordt aangetoond dat PCR-ELISA bruikbaar is om V gen profielen te bepalen in stalen die niet meer dan 50.000 lymfocyten bevatten.
Preferentieel gebruikte TCR V genen worden aangetoond in synoviale vocht en membraanbiopten, maar niet in het perifere bloed van de patiënten. Bovendien blijkt dat PCR-ELISA in combinatie met CDR3 spectratyping een snelle en efficiënte methode is om de klonale samenstelling van preferentieel gebruikte V gen families te bepalen.

Hoofdstuk 4: Gerestricteerd TCR V gen gebruik in het synovium van vroege en chronische RA patiënten en persistentie van klonaal geëxpandeerde T-cellen in een chronische patiënt.

Uit resultaten van andere TCR studies bleek dat geen enkel TCR V gen kan worden teruggevonden in de gewrichten van alle patiënten. De resultaten van de TCR V genstudies zijn bovendien tegenstrijdig, en onderling zeer moeilijk vergelijkbaar. De variabiliteit is vermoedelijk te wijten aan genetische en omgevingsfactoren, maar ook technologische verschillen, en een verschillenende behandeling van de stalen voor de analyses worden uitgevoerd, zouden de resultaten kunnen beïnvloeden. In deze studie werden lymfocyten uit bloed en synoviaal vocht van RA patiënten bestudeerd zonder voorafgaande *in vitro* manipulaties om geen verstoring van de *in vivo* gegenereerde TCR profielen te veroorzaken.
TCR V genexpressie werd bestudeerd in vroege (N=7) en chronische (N=31) RA patiënten met een uniforme en vooraf goed gedefinieerde techniek, de PCR-ELISA. De klonale samenstelling van preferentieel gebruikte V gen families werd bepaald door CDR3-sequentie bepaling na clonering van de respectievelijke PCR amplicons in plasmiden.

De TCR V genexpressie bleek heterogeen te zijn in het bloed van patiënten en controles, maar gerestricteerd in de synoviale caviteitstalen. De TCR V gen patronen bleken patiëntafhankelijk te zijn, maar waren vergelijkbaar voor gepaarde gewrichten van patiënten met een bilaterale synovitis. TCR V genrestrictie werd aangetoond in de gewrichten van vroege en chronische RA patiënten, maar het aantal preferentieel gebruikte TCR V genen bij vroege RA patiënten bleek

significant lager dan bij chronische RA patiënten. Deze resultaten suggereren dat de betrokken antigenen op verschillende stadia van de aandoening misschien verschillen. Aan de hand van CDR3 sequentiebepalingen werden deze verschillen duidelijk, en kon zelfs de aard van het betrokken antigeen algemeen worden omschreven. CDR3 sequentiebepaling werd uitgevoerd voor één vroege en één chronische RA patiënt. Bij de vroege RA patiënt werden oligo- en polyklonale T-cellen gevonden, terwijl bij de chronische RA patiënt een duidelijke klonale expansie werd waargenomen.
Deze resultaten suggereren een *in vivo* antigeenstimulatie in de synoviale gewrichten van RA patiënten. Toch blijft de pathogenetische relevantie van de klonaal geëxpandeerde synoviale T-cellen nog steeds onbekend.

Een aantal belangrijke vragen kunnen in dit verband worden gesteld.

1. Worden de autoreactieve T-cellen in het bloed of in het synovium van de patiënten geactiveerd?

Een aantal gegevens uit deze studie laten vermoeden dat T-cellen in het bloed worden geactiveerd en nadien in het gewricht accumuleren. Niet alleen het voorkomen van vergelijkbare V gen profielen, maar ook de aanwezigheid van identieke CDR3-sequenties in beide gewrichten van een patiënt met bilaterale synovitis (hoofdstuk 4), laten sterk vermoeden dat dezelfde T-cel clonen in beide gewrichten van de patiënt voorkomen. Bovendien blijkt dat enkel geactiveerde T-cellen de endotheelwand van het bloedvat kunnen doorkruisen om de perifere circulatie te verlaten en in het gewricht terecht te komen. Wanneer de T-cellen het gewricht bereiken, kan een in situ antigeenstimulatie de T-cellen heractiveren en een klonale expansie veroorzaken. Perifere activatie, synoviale reactivatie en klonale expansie werden ook door andere onderzoeksgroepen beschreven. Toch blijven zowel de perifere antigenen als de relevantie van de perifeer geactiveerde T-cellen in de pathogenese nog steeds onbekend.

2. Zijn antigenen in vroege en chronische RA verschillend ?

Synoviale T-cellen zijn vermoedelijk betrokken in de initiatie van de ontstekingsreactie in de gewrichten van RA patiënten. Dominante T-cel clonen die in een later ziektestadium worden aangetroffen zouden eerder in het verdere verloop van de ziekte een belangrijke rol spelen. Uit onze data blijkt dat het aantal preferentieel gebruikte V genfamilies in chronische RA patiënten groter is dan in vroege RA patiënten. Dit zou kunnen betekenen dat het aantal antigenen dat in het ziekteproces betrokken is in een vroeg stadium klein is, en groter wordt naarmate de ontstekingsreactie langer duurt. Geleidelijk aan zullen nieuw geactiveerde T-cellen het gewricht infiltreren waardoor de bijdrage van de initiële T-celclonen in de synoviale populatie afneemt. Deze gegevens wijzen duidelijk in de richting van determinantspreiding, een proces dat ook werd aangetoond in experimentele autoimmune encephalomyelitis (EAE), het diermodel voor MS.

3. Wat is de aard van het antigeen betrokken bij T-cel activatie bij RA ?

Uit de CDR3 sequentie resultaten bleek dat de klonale samenstelling van T-cellen in vroege en chronische RA patiënten verschillend is. Bij een vroege RA patiënt werden oligo-of polyklonale T-cellen aangetoond, terwijl bij een chronische RA patiënt klonaal geëxpandeerde T-cel-populaties werden gevonden. Deze resultaten suggereren dat bij vroege RA de stimulatie door superantigenen of diverse klassieke antigenen gebeurt en dat klassieke antigeenstimulatie verantwoordelijk is voor T-cel activatie in een later stadium van de aandoening. Synoviale T-cellen worden vermoedelijk in het gewricht geactiveerd door kruisreactie met synoviale autoantigenen, nadien kunnen deze klonaal geexpandeerde T-cellen niet-geactiveerde synoviale T-cellen overgroeien.

Hoofdstuk 5: Dynamische T-cel receptor clonotype veranderingen in het synoviaal membraan van vroege RA patiënten worden beïnvloed door behandeling met Cyclosporin A (Neoral).

Onze voorgaande resultaten en bevindingen uit de literatuur suggereren dat het TCR V genexpressieprofiel in RA verbreedt naarmate de ziekte langer duurt. Hierdoor wordt het moeilijk om ziekte-relevante T-cellen te identificeren. Deze ziekte-relevante T-cellen zijn nochtans potentiële doelwitten voor T-cel gerichte therapieën zoals T-cel vaccinatie of TCR peptide vaccinatie. Het zou echter kunnen dat de ziekte-relevante T-cellen enkel in het beginstadium van de aandoening voorkomen en naarmate de ontsteking langer duurt overgroeid worden door niet relevante T-cellen. Uit dit alles blijkt dat, met het oog op de ontwikkeling van een T-cel therapie, TCR profielen best bij vroege RA patiënten worden bestudeerd. In deel 5

van de studie werden TCR V gen profielen bij vroege RA patiënten bestudeerd op 2 verschillende tijdstippen met een tussentijd van 16 weken. Een deel van de studiegroep werd behandeld met CSA (3mg/kg/dag) terwijl de overige patiënten als placebo controle groep in de studie werden opgenomen. Via PCR-ELISA en CDR3 spectratyping werd niet alleen de TCR V genexpressie, maar ook de klonale samenstelling van de preferentieel gebruikte V gen families bepaald.

Bij het begin van de studie werd in elk van de bestudeerde biopten T-cel receptor specifiek materiaal aangetoond. Het TCR V gengebruik was gerestricteerd in de synoviale biopten en zelfs in een aantal perifere bloed stalen. TCR BV genfamilies die preferentieel gebruikt werden in het bloed werden echter niet in verhoogde mate teruggevonden in de biopten van dezelfde patiënten. De TCR V genexpressie op verschillende plaatsen in het aangetaste gewricht bleek daarentegen zeer vergelijkbaar te zijn.
Tijdens de studie bleken in de placebocontrole groep zeer fundamentele veranderingen in de T-cel populatie te zijn opgetreden. Geen enkel van de T-celclonen aangetoond op het eerste tijdstip werd 16 weken later in de synoviale populatie teruggevonden. Deze dynamische clonotype veranderingen zijn vermoedelijk het gevolg van een continue lokale of perifere activatie die ervoor zorgt dat steeds nieuwe T-celclonen in het gewricht terecht komen, terwijl T-celclonen die reeds in het gewricht aanwezig zijn vermoedelijk door apoptose worden gedepleteerd. In de CSA behandelde patiënten daarentegen werden vóór en na de behandeling dezelfde T-celclonen aangetoond. CSA is een immuunsuppressief middel dat niet alleen T-cel activatie maar ook apoptose onderdrukt. Wij vermoeden dat tengevolge van de CSA behandeling niet alleen de infiltratie van nieuwe T-cellen in het gewricht werd onderdrukt, maar ook de 'opkuis' van die T-cel clonen die reeds in het gewricht aanwezig zijn, werd verminderd.
Deze veronderstellingen zouden de persistentie van T-celclonen in de CSA behandelde en de dynamische clonotype veranderingen in de placebo controle groep kunnen verklaren. Bovendien werd vastgesteld dat in 3 van de 7 CSA patiënten geen TCR mRNA kon worden waargenomen 16 weken na de start van de behandeling. Gezien dit ook in 1 van de 5 placebo controle patiënten het geval is, lijkt het meer waarschijnlijk om te stellen dat deze biopten 16 weken na de eerste staalname te weinig T-cellen bevatten om de TCR expressie profielen met onze techniek te bepalen.

Hoofdstuk 6: Kwantificatie van cytokine mRNA in de synoviale gewrichten van vroege RA patiënten: Effect van CSA behandeling.

In dit deel van de studie werden T-cel gemedieerde cytokineboodschappers gekwantificeerd in bloed en synovium van vroege RA patiënten, en werd het effect van CSA behandeling op cytokine mRNA niveau bestudeerd. De TNF-α, IFN-γ, IL-4 en IL-10 mRNA hoeveelheid werden bepaald via kwantitatieve PCR. De cytokine mRNA inhoud was hoger in het bloed van RA patiënten dan van gezonde controles. Bovendien bleek de TNF-α, IFN-γ, IL-4 en IL-10 mRNA inhoud in de biopten groter te zijn dan in het bloed van de patiënten, wat vermoedelijk wijst op een hoge graad van inflammatoire activiteit in de aangetaste gewrichten. In dit deel van de studie werd ook het effect van CSA op cytokine mRNA inhoud bestudeerd. CSA onderdrukt de produktie van IL-2 en IFN-γ op transcriptioneel niveau. Na 16 weken behandeling bleek inderdaad dat de IFN-γ mRNA inhoud in de synoviale biopten van de behandelde patiënten zeer sterk was gereduceerd, een daling die niet voorkwam in de biopten van de placebocontrole patiënten. De TNF-α mRNA inhoud nam toe in enkel CSA behandelde en niet-behandelde patiënten, wat zou kunnen suggereren dat CSA de T-cel activatie, maar niet de macrofaag onderdrukt. Geactiveerde macrofagen zullen vervolgens synoviocyten stimuleren, waardoor kraakbeen en botdegradaties aanhouden.

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Curriculum Vitae

Ann VanderBorght werd geboren op 24 juni 1968 in het Brabantse Honsem (Boutersem). In 1986 behaalde ze het diploma middelbaar onderwijs aan het Heilig Hart Instituut te Heverlee, waarna ze begon aan een drie jaar durende opleiding voor gegradueerde in de industriële scheikunde aan de Katholieke Industriële Hogeschool Limburg te Diepenbeek. Als gegradueerde in de industriële scheikunde begon ze in 1989 aan het Limburgs Universitair Centrum op de eerste kandidatuur Scheikunde en in juni 1993 studeerde ze af als licentiaat Scheikunde-biochemie aan de Universitaire Instelling Antwerpen. Met het licentiaatsdiploma op zak, trok ze terug naar de Limburgse Hogeschool waar ze gedurende het academiejaar 93-94 en 94-95 als lesgever technische vakken werd tewerkgesteld. Ondertussen behaalde ze het aggregaatsdiploma hoger onderwijs aan de Universitaire Instelling Antwerpen en in april 1995 startte ze aan het Dr. L Willems-Instituut met de voorbereiding van een doctoraat. Ze kwam terecht in het reuma-onderzoek en concentreerde zich gedurende 4 jaar als LUC bursaal op de studie van T-cel receptor expressie bij reumatoïde artritis.

Bibliography

Articles

Identification of overrepresented T-cell receptor genes in blood and tissue biopsies by PCR-ELISA.
VanderBorght, A., Van der Aa, A., Geusens, P., Vandevyver, C., Raus, J., Stinissen, P *Journal of Immunological Methods*, 223; 47-61, 1999.

Skewed T-cell receptor variable gene usage in the synovium of early and chronic rheumatoid arthritis patients and persistence of clonally expanded T-cells in a chronic patient.
A.VanderBorght, P. Geusens, C. Vandevyver, J. Raus, P. Stinissen. *Submitted*

Dynamic T-cell receptor clonotype changes in synovial tissue of early rheumatoid arthritis patients are influenced with Cyclosporin A.
A. VanderBorght, F. De Keyser, P. Geusens, M. De Backer, M. Malaise, D. Baeten, E. Van Den Bosch, E. Veys, J. Raus, P. Stinissen. *Submitted*

Cytokine mRNA quantification in synovial tissue of early rheumatoid arthritis patients: Effect of Cyclosporin A (Neoral) treatment.
A. VanderBorght, F. De Keyser, P. Geusens, M. De Backer, E. Veys, J. Raus, P. Stinissen. *in preparation*

Encephalomyelitis associated anti-myelin autoreactivity induced by Streptococcal Pyrogenic exotoxins.
P. Jorens, **A. VanderBorght**, B. Ceulemans, H. Van Bever, L. Bossaert, M. Ieven, H. Goossens, P. Parizel, H. Van Dijk, J. Raus, P. Stinissen. *Submitted*

Published abstracts

VanderBorght, A., Stinissen, P., Geusens, P., Celis, L., Raus, J., Vandevyver, C. Evidence for clonal expansion of T-cells in synovial fluid of Rheumatoid Arthritis patients. *Archives of Physiology and Biochemistry*. 105, B39, 1997

VanderBorght, A., Geusens, P., De Keyser, F., De Backer, M., Malaise, M., Baeten, D., Van Den Bosch, E., Veys, E., Raus, J., Stinissen, P., TCR expression in the joints of early RA patients before and after Cyclosporin A microemulsion treatment. *Scandinavian Journal of Immunology*, 49(3), maart 1999.

Van Der Aa, A., **VanderBorght, A.**, Raus, J., Stinissen, P. Cerebrospinal fluid T-cells after in vitro stimulation with IL-2 and IL-4: Phenotypic, functional and T-cell receptor analysis. *Archives of Physiology and Biochemistry*, 107, B24, 1999.

VanderBorght, A., Geusens, P., De Keyser, F., De Backer, M., Malaise, M., Baeten, D., Van Den Bosch, E., Veys, E., Raus, J., Stinissen, P., TCR expression in the joints of early RA patients before and after Cyclosporin A microemulsion treatment. *Archives of Physiology and Biochemistry*, 107, B25, 1999.

Oral presentations

VanderBorght, A., Geusens, P., Raus, J., Stinissen, P. Restricted T-cell receptor variable gene usage in synovium of patients with rheumatoid arthritis. Genetic aspects of autoimmune diseases. Noorderwijkerhout, Nederland. 7-10 mei 1998

Posters and abstracts

VanderBorght, A., Celis, L., Geusens, P., Raus, J., Vandevyver, C. Semi-quantitative T-cell receptor V gene analysis in rheumatoid arthritis. Rheumatology in Europe, EULAR 96, IX Symposium, Madrid, 7-10 october 1996.

VanderBorght, A., Stinissen, P., Geusens, P., Celis, L., Raus, J., Vandevyver, C. Semiquantitative analysis of T-cell receptor V gene usage in rheumatoid arthritis. XIXthe ILAR Congress of Rheumatology, Singapore 8-13 juni 1997

VanderBorght, A., Geusens, P., Raus, J., Stinissen, P. Restricted T-cell receptor variable gene usage in synovium of patients with rheumatoid arthritis. Genetic aspects of autoimmune diseases. Noorderwijkerhout, Nederland: 7-10 mei 1998

VanderBorght, A., Geusens, P., De Keyser, F., De Backer, M., Malaise, M., Baeten, D., Van Den Bosch, E., Veys, E., Raus, J., Stinissen, P. TCR expression in the joints of early RA patients before and after Cyclosporin A microemulsion treatment. The 19th European Workshop for Rheumatology Research, Oslo, Noorwegen: 25-28 februari 1999

VanderBorght, A., Celis, L., Geusens, P., Raus, J., Vandevyver, C. Semiquantitative T-cell receptor V gene analysis in Rheumatoid Arthritis. Belgisch Biochemische vereniging, april 1997, Luik, België.

Stinissen, P., **VanderBorght, A.**, Geusens, P., Celis, L., Vandevyver, C., Raus, J. Study of autoreactive T-cells and T-cell receptor expression in rheumatoid arthritis. Meeting of the Belgian Cell Biology Society, Diepenbeek, Belgium, 25 october 1997.

VanderBorght, A., Geusens, P., Raus, J., Stinissen, P. TCR V gene analysis of synovial T-cells of rheumatoid arthritis patients reveals similar TCR patterns in both affected joints. 3rd Working Group Biotechnology, Aachen-Liege-Maastricht-Diepenbeek, Sart-Tilman, 28 mei, 1998

VanderBorght, A., Geusens, P., Raus, J., Stinissen, P. TCR V gene analysis of synovial T-cells of rheumatoid arthritis patients reveals similar TCR patterns in both affected joints. Meeting of the Belgian Immunological Society, UCL Brussels, 29/5/98.

VanderBorght, A., Geusens, P., Raus, J., Stinissen, P. TCR V gene analysis of synovial T-cells of rheumatoid arthritis patients reveals similar TCR patterns in both affected joints. Koninklijke Belgische vereniging van Reumatologie, Mons, 11/98