# Expression of interleukin-10 family cytokines in rheumatoid arthritis

Master's thesis
Institute of Medical Technology
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## TIIVISTELMÄ


**Tutkimuksen tausta ja tavoitteet:** Interleukiini-10 (IL-10) on anti-inflammatorinen sytokiini, jonka pitoisuudet ovat koholla nivelreumassa. Hiljattain on löydetty useita uusia sytokiineja, joista on geneettisen rakenteensa perusteella muodostettu IL-10-perhe. Näitä sytokiineja ovat IL-19, IL-20, IL-22, IL-24 ja IL-26. Tutkimustieto IL-10 perheen sytokiinien ilmentymisestä ja toiminnasta on toistaiseksi vähäistä. Tutkimuksen tavoitteena oli tutkia IL-10-perheen sytokiinien ilmentymistä nivelreumassa ja selvittää tarkemmin nivelreumassa koholla olevien sytokiinien ilmentymisprofiilia.

**Tutkimusmenetelmät:** Sytokiinien lähetti-RNA:n ilmentymistä tutkittiin valkosoluissa kvantitatiivisen käänteiskopiointi-PCR:n avulla (Q-RT-PCR). IL-19-proteiinin solun sisäistä ilmentymistä tutkittiin valkosoluissa virtaussytometrian avulla. IL-19:n vaikutusta muiden sytokiinien ilmentymiseen proteiinitasolla tutkittiin valkosoluissa ELISA:lla. Näytteinä käytettiin nivelreumapotilaiden veri-, nivelneste- ja nivelkudosnäytteitä, terveiden kontrollien verinäytteitä ja nivelrikkopotilaiden nivelkudosnäytteitä.

**Tutkimustulokset:** Nivelreumapotilaiden verenkierrossa IL-10, IL-22 ja IL-22R1 mRNA tasot olivat koholla normaalikontrolleihin verrattuna. Vastaavasti nivelreumapotilaiden nivelnestenäytteissä IL-10:n, IL-19:n ja IL-22-antagonistin määrät olivat lisääntyneet. IL-19:n mRNA-taso oli kohonnut myös nivelkudosnäytteissä. Makrofagit osoittautuivat IL-19:n ensisijaiseksi lähteeksi nivelissä. IL-19 proteiinin solunsisäiset tasot olivat myös koholla nivelreumapotilaiden näytteissä. IL-19 proteiinin ei kuitenkaan todettu muuttavan muiden sytokiinien ilmentymistä soluviljelmissä.

**Johtopäätökset:** Useimmat IL-10 perheen sytokiinit ja niiden reseptorit ilmentyvät nivelreumassa. IL-10:n lisäksi erityisesti IL-19:n pitoisuudet olivat lisääntyneet nivelreumapotilaiden nivelissä. IL-19:n toiminnallista tutkimista olisi jatkettava, jotta voitaisiin selvittää muuntuneen ilmentymisen merkitys nivelreuman patogeneesissä.

# MASTER'S THESIS


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## ABSTRACT


**Background and aims:** Interleukin-10 (IL-10) is an anti-inflammatory cytokine, and its expression is increased in rheumatoid arthritis (RA). A number of novel cytokines have been found recently, which have been designated as the IL-10 family based on their genetic characteristics. These cytokines include IL-19, IL-20, IL-22, IL-24 and IL-26. Information concerning the expression and function of these cytokines is restricted. The aim of this study was to investigate the expression of these cytokines in rheumatoid arthritis, and to characterize more specifically the expression patterns of cytokines that are elevated in rheumatoid joints.

**Methods:** mRNA expression of cytokines in leucocytes was studied by quantitative reverse transcriptase PCR (Q-RT-PCR). The levels of intracellular IL-19 protein were studied by flow cytometry. The effect of IL-19 protein on the expression of other cytokines was studied by ELISA. Peripheral blood, synovial fluid and synovial tissue samples from patients with RA, peripheral blood samples from healthy volunteers and synovial tissue samples from patients with osteoarthritis were studied.

**Results:** Elevated levels of IL-10, IL-22 and IL-22R1 were observed in the peripheral circulation of patients with RA. In addition, IL-10, IL-19 and IL-22BP were found to be upregulated in SFMC of patients with RA. Increased expression of IL-19 was also observed in RA synovial tissues. Macrophages were found as primary source for IL-19 expression in RA synovium. The expression of intracellular IL-19 protein was also upregulated in samples of patients with RA. However, IL-19 did not markedly change the expression pattern of other cytokines *in vitro*.

**Conclusions:** Most of the IL-10 family cytokines and their receptor subunits can be detected in RA. In addition to IL-10, IL-19 levels were increased in rheumatoid joints. Further functional studies are needed to determine the significance of the altered expression of IL-19 in the pathogenesis of RA.

**CONTENTS**

# ABBREVIATIONS

| | |
|---|---|
| **AP-1** | activator protein-1 |
| **BP** | binding protein |
| **cDNA** | complementary deoxyribonucleic acid |
| **C/EBP** | CCAAT/enhancer-binding protein |
| **ConA** | concanavalin A |
| **COX** | cyclo-oxygenase |
| **CRP** | C-reactive protein |
| **CSIF** | cytokine synthesis inhibitory factor |
| **Ct** | threshold value |
| **DMARD** | disease-modifying antirheumatic drug |
| **DNA** | deoxyribonucleic acid |
| **dNTP** | deoxynucleoside triphosphate |
| **EDTA** | ethylene diamine tetra-acetic acid |
| **ELISA** | enzyme-linked immunosorbent assay |
| **EST** | expressed sequence tag |
| **FACS** | fluorescence-activated cell sorter |
| **FBS** | fetal bovine serum |
| **FITC** | fluorescein isothiocyanate |
| **FRET** | fluorescence resonance energy transfer |
| **GAPDH** | glyseraldehyde-3-phosphate |
| **GM-CSF** | granulocyte-macrophage colony stimulating factor |
| **hBD-2** | human β-defensin-2 |
| **HBSS** | hank's balanced salt solution |
| **HLA** | human leucocyte antigen |
| **HPRT** | hypoxanthine-guanine phosphoribosyl transferase |
| **HRP** | horseradish peroxidase |
| **HSV** | herpesvirus saimiri |
| **HUVEC** | human umbilical vein endothelial cells |
| **IEC** | intestinal epithelial cell |
| **IL** | interleukin |
| **IL-1Ra** | interleukin 1 receptor antagonist |
| **IFN** | interferon |

| | |
|---|---|
| **LPS** | lipopolysaccharide |
| **MACS** | magnetic cell sorting |
| **MCP** | monocyte chemoattractant protein |
| **Mda-7** | melanoma differentiation associated gene-7 |
| **MHC** | major histocompatibility complex |
| **MMP** | matrix metalloproteinase |
| **M-MLV-RT** | moloney murine leukemia virus reverse transcriptase |
| **MS** | multiple sclerosis |
| **NA** | non applicable |
| **NK** | natural killer |
| **OA** | osteoarthritis |
| **PBMC** | peripheral blood mononuclear cell |
| **PBS** | phosphate buffered saline |
| **PE** | phycoeryhrin |
| **$PGE_2$** | prostaglandin |
| **Q-PCR** | quantitative polymerase chain reaction |
| **RA** | rheumatoid arthritis |
| **RAFS** | rheumatoid arthritis synovial fibroblast |
| **RF** | rheumatoid factor |
| **RNA** | ribonucleotide acid |
| **ROS** | reactive oxygen species |
| **RT** | room temperature |
| **RT-PCR** | reverse transcriptase polymerase chain reaction |
| **TBP** | TATA binding protein |
| **TNF** | tumor necrosis factor |
| **SFMC** | synovial fluid mononuclear cell |
| **SLE** | systemic lupus erythematosus |
| **STAT** | signal transducers and activator of transcription (protein) |

# 1. INTRODUCTION

In Finland, 40 000 – 50 000 patients suffer from rheumatoid arthritis (RA). The disease is a chronic autoinflammatory disease of the joints and it is highly disabling. It has been suggested that RA is a relatively new disease as first reliable descriptions are from 17$^{th}$ century and no reliable archaeological findings have been made. Alfred Garrod named the disease in 1858 to distinguish it from the other rheumatoid diseases.

The cause of RA is at present unknown. The importance of studying this disease can be easily justified: although RA accounts for only 8% of diagnosed musculoskeletal diseases, its treatment takes up as much as 40% of all the hospitalization days required for the treatment of musculoskeletal diseases in general hospitals. (Isomäki, 2002).

The immune and inflammatory responses are well-controlled processes. It is important for the immune system to engineer a proper response to any pathological condition. In addition, it is even more important to inhibit it in a timely manner since the response is destructive for the host immune system itself and to other tissues. There are several mechanisms for the regulation and balancing of these responses, and one of the mechanisms is the expression of anti-inflammatory cytokines possessing immunosuppressive activities. (Kotenko, 2002).

Interleukin (IL)-10 is one of the most important immunoregulative cytokines. It e.g. inhibits the inflammatory and the specific T cell response. The IL-10 family members, IL-19, IL-20, IL-22, IL-24 and IL-26 are recently identified cytokines whose amino acid sequences are up to 30% identical to that of IL-10. The genes are located in the human genome in two clusters. (Wolk et al., 2002). Receptors for these cytokines belong to the class II cytokine receptor family, and the receptors are IL-10R1, IL-10R2, IL-20R1, IL-20R2 and IL-22R1. In addition, a natural antagonist, IL-22BP (BP for binding protein), has been described for IL-22. (Pestka et al., 2004).

IL-10 has been shown to function as an anti-inflammatory cytokine in rheumatoid synovium (Isomäki et al., 1996). Its therapeutic possibilities in the treatment of RA have also been extensively studied. The biological functions of newly identified members of the IL-10 family may be similar to those of IL-10, and therefore it is relevant to study their expression profile in RA.

# 2. REVIEW OF LITERATURE

## 2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common human autoimmune disease. It is characterized by a chronic inflammation of the synovial joints and infiltration by blood-derived cells, mainly memory T cells, macrophages and plasma cells, all of which show signs of activation. (Feldmann, 1996). The disease is more common in women, especially among younger people, and this might implicate a role for sex hormones in the onset of RA. However, the disease is also more common in the elderly population, and this must also be taken into account when the onset of the disease is considered. (Akil&Amos, 1995).

RA usually presents as a polyarthritis of small joints or both small and large joints. Pain and other signs of inflammation express early stages of the disease and if the disease remains active additional joints will be affected and gradually irreversible tissue damage will occur. Damage to the larger weight bearing joints are the most disabling long-term effects. (Akil&Amos, 1995).

Approximately 80% of patients are seropositive for rheumatoid factor (RF). RF is an antibody that binds to the Fc portion of immunoglobulins. The finding of RF first led to the hypothesis that RA might be an autoimmune disease caused by self-reactive antibodies. (Firestein, 2003). Other molecules of the immune system that seem to be upregulated in RA include anti-inflammatory cytokines like IL-10 and pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), IL-1, IL-6 and IL-8. In the starting points of this cytokine cascade are TNF-α and IL-1. They recruit more cytokines to be activated. RA is an inflammatory disease, since the cytokine disequilibrium is tilted towards pro-inflammatory cytokines. Anti-inflammatory molecules remain in the minority (see figure 1). (Feldmann, 1996).

RA also has a genetic background as specific human leukocyte antigen (HLA)-DR-genes are associated with the disease. These genes reside in the major histocompatibility complex (MHC) and participate in antigen presentation. (Firestein, 2003). However, approximately 20 % of Caucasian rheumatoid patients do not express the suspected subtypes (DR1 and DR4) of the HLA-DR locus and thus do not share the same genetic background with those patients that do express these subtypes. (Feldmann, 1996).

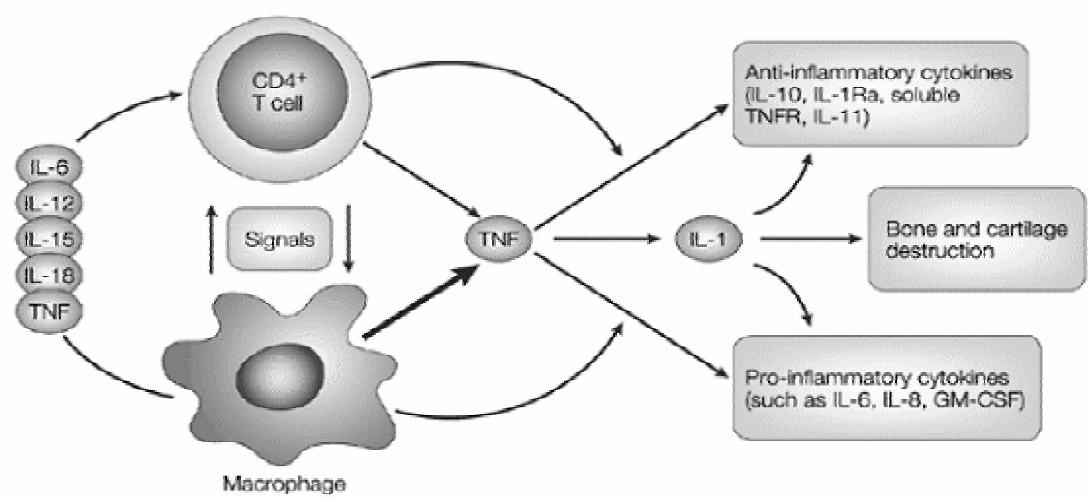



**Figure 1. The cytokine network in RA (Adopted from Feldmann et al., 2001).**

Many questions made concerning rheumatoid arthritis have been answered in the recent years, and this has led to enormous progress in how to treat patients with RA. However, many questions remain. There is still much to learn about the etiology, pathogenesis and therapy of rheumatoid arthritis.

### 2.1.1 The prevalence and etiology of rheumatoid arthritis

RA is a common disease affecting all racial groups worldwide. The prevalence in most cohorts is approximately 1%. Interestingly, the prevalence is higher in western industrialized countries when compared to e.g. nations habiting the subtropical or tropical areas. Incidence rates have been seen to be less than or the same as several decades ago. (O'Dell, 2005). Women are affected two to three times more often than men, but this female preponderance is less impressive when only those patients who are found to be serologically positive for RF and are found to have radiological evidence of erosive changes of joint are considered (Borigini&Paulus, 2001).

The etiology of RA is still unknown. At the moment, research has been concentrated on the following: genetic risk factors, the significance of autoimmunity in the onset of the disease and possible microbiologic agents in the etiology of RA. Many metabolic, psychological, social and work/environmental efforts to explain the etiology of RA have not been able to solve the problem. In addition, many viruses, bacteria, mycoplasmas and also prions have been suggested to cause the onset of the disease. Retroviruses, Epstein-Barr virus and intestinal flora are the ones that have been proposed recently. Risk factors that might predispose to RA are genetic (HLA-DRB1), fe-

male gender, the time right after childbirth or miscarriage, postmenopausal age and possibly also injuries in the joints and viral infections. (Isomäki, 2002).

### 2.1.2 The pathogenesis of rheumatoid arthritis

In RA, T cells, plasma cells, macrophages and dendritic cells are seen to accumulate in the joints. This migration into the joints is due to increased expression of several adhesion molecules on synovial endothelial cells and by action of chemotactic factors. The production of several cytokines, like IL-1, IL-6 and TNF-α, by activated macrophages and other cells in the synovium leads to the expression of cartilage-degrading matrix metalloproteinases (MMPs), including collagenase, and the activation of osteoclasts. These events result in the degradation of articular cartilage and the underlying bone (see figure 2). (Feldmann et al., 1996). The importance of many pro-inflammatory cytokines in the pathogenetic network has been confirmed by the successful use of neutralizing anti-cytokine antibodies in the treatment of RA (see table 1).

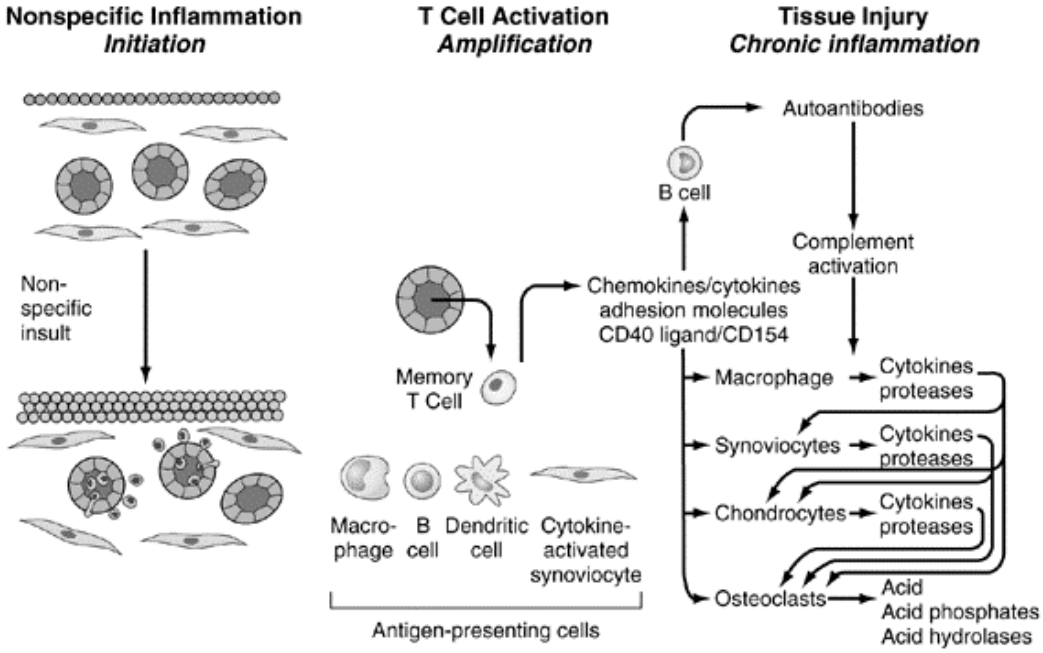



**Figure 2. The progression of rheumatoid arthritis (adopted from Lipsky, 2005).**

Approximately 30% of the cells infiltrating the synovium of patients with RA are T cells. They are mainly small noncycling CD4+ T cells that have markers of T cell activation. It is probable that these cells persist in the joints due to defective apoptosis. In addition, the T cell response in active RA patients is abnormally attenuated.

(Feldmann, 2001). It is likely that T cells are involved both in disease induction and disease maintenance, and play a major role in the pathogenesis of RA. The antigen(s) that drive T cell responses in RA is not known, although several have been suggested. These include collagen type II, other cartilage proteins and heat shock proteins (Feldmann et al., 1996).

Macrophages have been proposed to be the pathogenetic cells since they secrete high levels of proinflammatory cytokines (Firestein&Zvaifler, 1990). The activation of B cells is also typical of RA, and the recent findings on beneficial effects of depleting anti-CD20 antibody (rituximab) in patients with RA confirm the role of B cells in RA pathogenesis. Also several other cell types have been suspected to obtain pathogenetic characteristics in the onset of RA. However, the crucial evidence for the true pathogenesis of RA has not been shown and the area needs still more research.

### 2.1.3 The role of cytokines in rheumatoid arthritis

Cytokines are small soluble proteins with a size range of 6 to 60 kDa. They are produced by numerous cell types after their activation. Cytokines are important molecules in intercellular communication. They are of crucial importance in the reactions mediated by the immune system against pathogens. In addition, cytokines regulate hematopoiesis, wound healing, angiogenesis and physiological and pathological reorganization of tissues. (Wolk et al., 2006). Most cytokines act in a paracrine manner, although numerous examples of cytokines effecting as an autocrine or endocrine are also available (Fox, 2005). In RA, cytokines are involved in diverse pathogenic responses and maintain the chronic phase of inflammation and tissue destruction (Gabay, 2005). Also, many cytokines show pleiotropic actions and many different targets (Lubberts&van der Berg, 2003).

A large scale of cytokines has been implicated in the joints of RA patients. These include proinflammatory cytokines IL-1, IL-6, IL-15, IL-18, interferon (IFN)-γ, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF) (see table 1) and anti-inflammatory cytokines IL-4, IL-10 and IL-11. (Wong&Lord, 2004). Of these, IL-10 will be covered more thoroughly in section 2.2. Natural antagonists of proinflammatory cytokines, such as soluble TNF receptor and IL-1-receptor antagonist (IL-1Ra), are also present in the synovium. The level of inflammation is regulated by the balance of proinflammatory and anti-inflammatory cytokines. (Hale, 2005). It is

noteworthy that in RA for example the balance between IL-1Ra and IL-1 is disturbed since the level of IL-1 far exceeds the level of IL-1Ra. (Wong&Lord, 2004). However, it is good to point out that the function of cytokines in the maintenance of body homeostasis and in the pathogenic mechanisms of e.g. RA is complex and thus the separation between deleterious and protective cytokines is somewhat artificial. (Gabay, 2005).

**Table 1. Pro-inflammatory cytokines in RA (adopted from Fox, 2005).**

| Cytokine | Effect | Producing cells | | Structure of the drug under development | Phase of the treatment under development |
|---|---|---|---|---|---|
| | | T cells | Synovial macro-phages | | |
| TNF-α | Can activate inflammatory cells, involved in the destructive processes of RA | + | + + | Dimeric p75 TNF receptor and Ig fusion protein | Etanercept/on the market |
| | | | | Chimeric mAb to TNF | Infliximab/on the market |
| | | | | Human mAb to TNF | Adalimumab/on the market |
| IL-1 | Can activate inflammatory cells, involved in the destructive processes of RA | + | + + | Human IL-1 receptor antagonist | Anakinra/on the market |
| | | | | IL-1R1 + IL-1 accessory protein fused to human IgG Fc | IL-1 trap/phase II trials |
| IL-6 | Inflammatory properties | +/- | + | IL-6 receptor mAb | Tocilizumab, phase III trials ongoing |
| IL-15 | Stimulates T cell proliferation and B cell maturation | - | + | Human $IgG_1$ mAb directed against IL-15 | Anti-IL-15, phase II trials completed |
| IL-18 | Chemotactic and angiogenic activities | - | + | Recombinant form of natural IL-18BP | IL-18BP, results from animal models |
| IFN-γ | e.g. induces expression of class II MHC antigens in synovial cells | + | - | - | - |

TNF-α and IL-1 are the most critical cytokines involved in synovitis in RA (Wong&Lord, 2004). They can both activate a variety of cells of the immune system and accelerate the mechanisms for bone and cartilage breakdown ether directly or via activation of synovial fibroblasts (Fox, 2005). The tissue degradation is promoted by the upregulation of the expression of a series of proteases and the inhibition of the formation of extracellular matrix. These cytokines may also stimulate excessive matrix accumulation. (Gabay, 2005). IL-1 has a more important role in the activation of MMPs while TNF-α is considered to be more critical in the induction of expression of adhesion molecules. TNF-α also upregulates the synthesis of IL-1, and IL-1 and TNF-α both induce the production of IL-6, another proinflammatory cytokine. (Fox, 2005). Thus, there is a clear pattern of cytokines working in synergy and in cascades. IL-1 is in fact considered to be the secondary mediator that is responsible for the arthritic changes, and hence TNF-α alone would neither be arthritogenic or destructive towards joints. (Lubberts&van der Berg, 2003).

T cells from synovial tissue or fluid from patients with RA can be readily stimulated to produce IFN-γ, which is a prototypic $T_H1$ cytokine. The biologic effects of IFN-γ include induction of class II MHC expression on a variety of cell types. The other cytokines promoting $T_H1$ responses in RA include IL-12, IL-15 and IL-18. (Fox, 2005). IL-15 is involved in T cell proliferation and B cell maturation and isotype switching. It can also stimulate the differentiation of osteoclast progenitors into preosteoclasts. (Lubberts&van der Berg, 2003). IL-18 has also broad proinflammatory effects in e.g. chemotaxis and angiogenesis (Fox, 2005). The modulators of RA, such as IL-10 and IL-4, can inhibit $T_H1$ cell activity by suppressing IFN-γ expression. They also have direct inhibitory effects on the activity of macrophages in the synovium. Also, they may upregulate the natural inhibitors of IL-1 and TNF-α (IL-1Ra, soluble TNF receptor). (Lubberts&van der Berg, 2003).

### 2.2 IL-10 family of cytokines

IL-10 family of cytokines is a novel family of related cytokines. The family of IL-10-related cytokines contains several human members including IL-19, IL-20, IL-22, IL-24, IL-26 and several viral homolog's (see table 2). These cytokines have been identified in database searches for potential IL-10 homologs, and so far there is only a restricted amount of knowledge about their biological activities. (Oral et al., 2006). The expres-

sion pattern of these cytokines is discussed in detail in the following chapters, but the general pattern of expression and targeted cells is compiled in figure 4.

Genes encoding IL-10 family cytokines are located in the human genome in two clusters. The other one comprises the genes for IL-10, IL-19, IL-20 and IL-24 on chromosome 1q31-31, and the other the genes for IL-22 and IL-26 located on chromosome 12q15 (see figure 3). (Wolk et al., 2002).

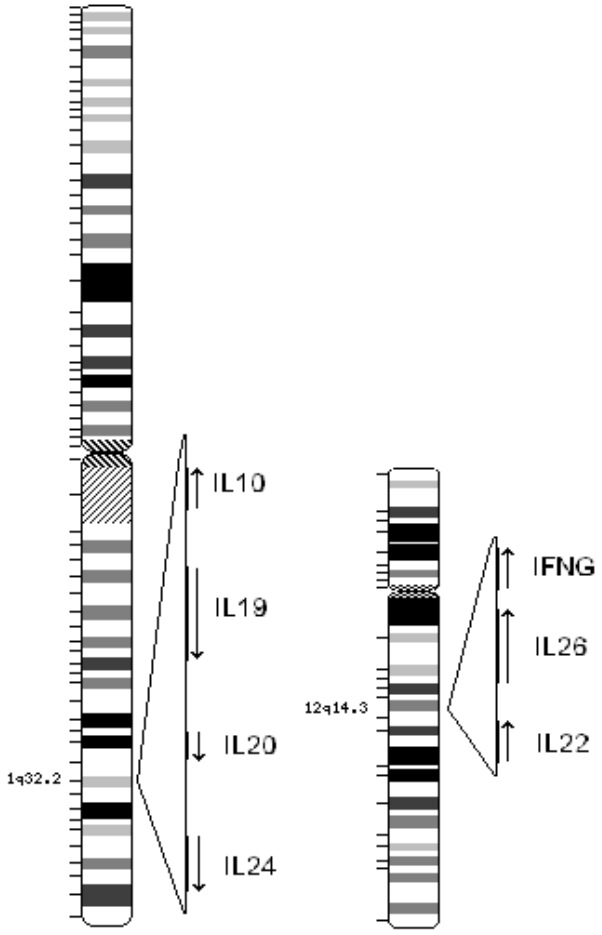



**Figure 3. The chromosomal localization of the genes encoding IL-10-related cytokines. Chromosome 1 on the left and chromosome 12 on the right. (Adopted from Kotenko, 2002).**

The receptors for IL-10 family cytokines along with IL-10 belong to the cytokine receptor family type 2, which are mostly transmembrane glycoproteins. The extracellular domain consists of a tandem fibronectin type III domain that is approximately 210 amino acids long. In addition, the receptor has several conserved amino acid positions that are important for the secondary structure. The functional receptor complex is formed after ligand binding as two particular receptor chains, R1 and R2, aggregate. (Wolk et al., 2002). An R1 type subunit binds ligand with high affinity, and it has a long intracellular domain that defines the specificity of signalling. The R2 type sub-

unit does not bind ligand on its own, and it possesses a short intracellular domain, which functions only in the initiation of the signal transduction event. (Kotenko, 2002).

**Table 2. The receptors and expression patterns of IL-10 family cytokines. (adopted from Pestka et al., 2004 and Nagalakshmi et al., 2004).**

| Cytokine | Receptor (R1+R2) | Expression of ligand (major cellular source indicated) | Expression of receptor | Function |
|---|---|---|---|---|
| IL-10 (CSIF) | IL-10R1 + IL-10R2 | Activated monocytes and T cells | IL-10R1 – all hematopoietic cells<br>IL-10R2 – ubiquitously in all cell types | Anti-inflammatory cytokine |
| IL-19 | IL-20R1+ IL-20R2 | Activated monocytes | IL-20R1, IL-20R2 – epithelial and stromal cells | Not clear |
| IL-20 | IL-20R1+ IL-20R2<br>IL-22R1+ IL-20R2 | Activated monocytes | IL-20R1, IL-20R2, IL-22R1 – epithelial and stromal cells | Potentially a role in skin biology and angiogenesis |
| IL-22 (IL-TIF) | IL-22R1+ IL-10R2<br>IL-22BP | Activated T cells | IL-22R1 – epithelial and stromal cells<br>IL-10R2 – ubiquitously in all cell types<br>IL-22BP – resting dendritic cells | Pro- and anti-inflammatory characteristics |
| IL-24 (Mda-7) | IL-20R1+ IL-20R2<br>IL-22R1+ IL-20R2 | Activated monocytes and T cells | IL-20R1, IL-20R2, IL-22R1 – epithelial and stromal cells | Immunomodulatory role, antitumour effects |
| IL-26 (AK155) | IL-20R1+ IL-10R2 | Activated monocytes and T cells | IL-20R1 – epithelial and stromal cells<br>IL-10R2 – ubiquitously in all cell types | Not clear |

The IL-10 family ligand-receptor pairing exhibits both cytokine pleiotropy and redundancy (Ozaki&Leonard, 2002). Several of the ligands in the IL-10 family bind to more than one functional receptor, while others seem to be very specific (Pestka et al., 2004, see table 2). The sharing of several receptor subunits suggests that these novel

IL-10 family cytokines could display similar as well as distinct functions (Oral et al., 2006). All major classes of vertebrates encode cytokine-receptor pairs with IL-10-like activities. It has been suggested that the class 2 cytokine receptor family evolved at the same time as the adaptive immune system, which was during the evolution of the vertebrates from invertebrate chordates. (Pestka et al., 2004).

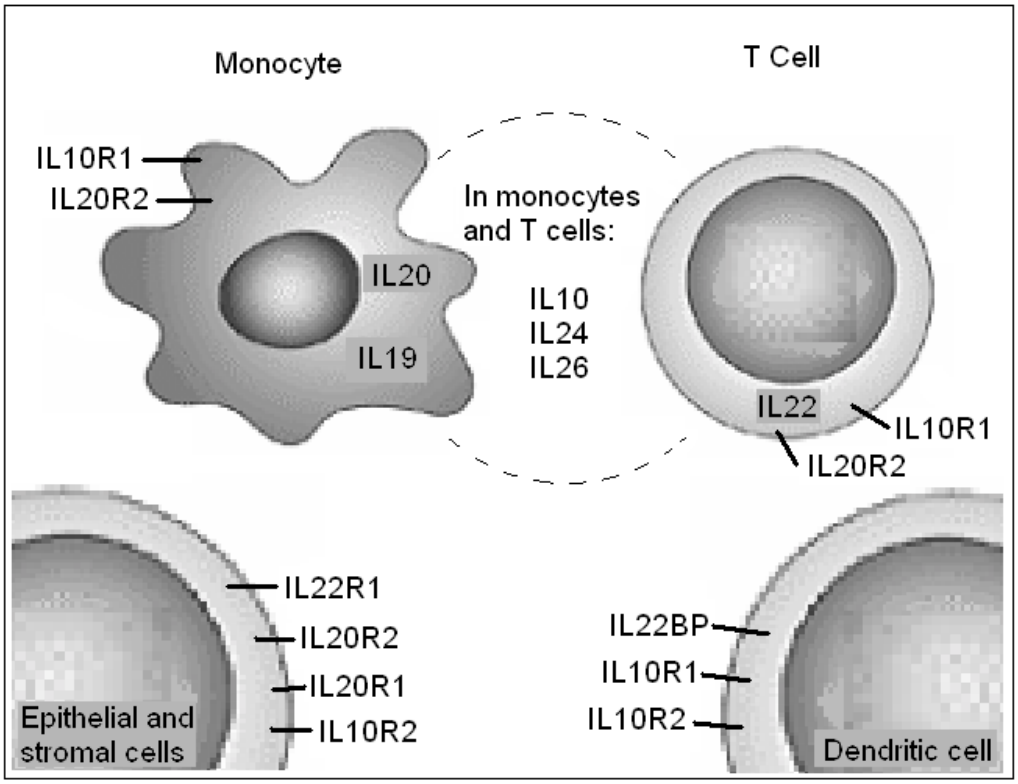



**Figure 4. Summary of general IL-10 family ligand and receptor expression pattern. Ligands are predominantly expressed by hematopoietic immune cells, whereas the novel homolog receptors are preferentially detected on epithelial and stromal cells of non-hematopoietic origin. (adopted from Nagalakshmi et al., 2004).**

### 2.2.1 IL-10

IL-10 was initially described as cytokine synthesis inhibitory factor (CSIF) in concavalin A (conA) stimulated T helper 2 cells. It was found to inhibit the production of many cytokines including IL-2, TNF-α, INF-γ and GM-CSF by $T_H1$ cells in response to antigens. The same effect was not seen in $T_H2$ cells. (Moore et al., 2001). IL-10 is produced from mature and immature T lymphocytes after mitogen activation or antigen binding to receptor membrane. In fact, $CD4^+$ T cells seem to be the principal producers among mature T cells. (Conti et al., 2003). However, monocytes and macrophages are the strongest producers of IL-10 (Wolk et al., 2002). IL-10 is a glycosylated secreted

protein, and it forms a homodimer (Conti et al., 2003). The functional receptor of IL-10 is composed of IL-10R1 and IL-10R2 subunits. (Pestka et al., 2004).

IL-10 is a pleiotropic, multifunctional cytokine that inhibits the inflammatory and specific T cell responses, mainly by affecting monocytic cells (Wolk et al., 2002). IL-10 is considered to be anti-inflammatory and immunosuppressive (Kotenko, 2002). IL-10 inhibits the antigen-presenting capacity of macrophages and dendritic cells. Furthermore, IL-10 has important role in blocking cytokine production and the expression of costimulatory molecules including CD80, CD86 and MHC class II molecules. All of these are molecules necessary for T cell survival. (Mosmann, 1994). Also, the secretion of chemokines is inhibited and the chemokine receptor expression is modified. However, β2-integrin ligand expression is increased along with radical generation. (Pestka et al., 2004). The production of IL-10 is tightly regulated as too massive levels of IL-10 lead to failure in controlling infectious pathogens and, on the other hand, insufficient amounts of IL-10 lead to pathologic processes secondary to tissue injury (Antoniv&Ivashkiv, 2006). It can be thought that the main function of IL-10 is to keep the inflammation under strict control by adjusting the intensity of the immune and inflammatory responses. The objective of anti-inflammatory cytokines such as IL-10 is to minimize damage to host tissues caused by either pathogen or immune system itself. (Kotenko, 2002).

IL-10 partially inhibits the induction of activities initiated by other cytokines, such as IFN-γ, IL-2, TNF-α and IL-4. In addition, IL-10 promotes development of immunoglobulin-producing B cells, enhances the survival of B cells and T cells, induces proliferation of mast cells and promotes the activity of natural killer (NK) cells by increasing cytotoxicity. IL-10 inhibits $CD4^+$ T cell chemotaxis towards IL-8 and T cell apoptosis by influencing to up-regulation of Bcl-2. The suppressor effect of IL-10 on T cells is directed towards blocking of the CD28 signaling cascade and subsequent phosphatidylinositol 3-kinase activation in T cells. (Pestka et al., 2004&Oral et al., 2006). Interestingly, IL-10 promotes the differentiation of dendritic cells to type 1 regulatory cells both in vitro and in vivo. (Pestka et al., 2004)

IL-10 deficient mice spontaneously develop enterocolitis and other symptoms that are similar to the chronic autoimmune disorder Crohn's disease (Kühn et al., 1993). These mice have also been shown to exhibit increased $T_H1$ responses, including en-

hanced clearance of bacterial, fungal and toxoplasmic infection. In addition, the mice have exaggerated asthmatic and allergic responses. (Pestka et al., 2004).

IL-10 has been shown to play a role in RA. IL-10 is produced by rheumatoid synovial fluid mononuclear cells (SFMC) and it functions as an immunoregulatory molecule in the rheumatoid synovium. Recombinant IL-10 decreases significantly the production of IL-1β, TNF-α and GM-CSF by SFMC and decreases spontaneous and IL-2-induced proliferation of SFMC. (Isomaki et al., 1996). Therapy based on IL-10 has been tested in the treatment of several diseases. Unfortunately, its use in Crohn's disease and psoriasis has led only to modest clinical responses. (Pestka et al., 2004). Refractoriness to exogenous IL-10 has raised questions about the anti-inflammatory potency of endogenous IL-10, which is consistently produced in e.g. inflamed rheumatoid synovium (Antoniv&Ivashkiv, 2006). On the other hand, in animal models of arthritis, IL-10 reduces joint swelling, cytokine production and cartilage degradation when administered to animals before or after the induction of the disease. (Moore et al., 2001). Also, neutralization of IL-10 in the treatment of systemic lupus erythematosus (SLE) has shown promise. (Pestka et al., 2004).

Immune and inflammatory processes can induce refractoriness to IL-10. The diminished IL-10 activity can then contribute to the inability to control and resolve inflammation in autoimmune diseases. Modulation of IL-10 signalling is one of the ways by which for example synovial macrophages become attenuated to the anti-inflammatory actions of IL-10. It has been shown that IFNs and immune complexes (the combination of an epitope with an antibody directed against that epitope) can modulate the signalling and bioactivity of IL-10 in primary human macrophages. The residual IL-10 response in synovial macrophages is similar to the reprogramming of IL-10 responses in IFN-preactivated macrophages. The altered response includes also a substantially smaller amount of induced genes when compared to control macrophages. In addition to losing anti-inflammatory functions, IL-10 can also acquire pro-inflammatory functions. (Antoniv&Ivashkiv, 2006).

### 2.2.2 IL-19

IL-19 is a secreted protein sharing 21% amino acid identity with IL-10. It was discovered by pursuing a focused searching for homologues of IL-10 from EST (Expressed Sequence Tag) databases. IL-19 gene is structurally similar to that of IL-10; it is com-

posed of seven exons and six introns of which five exons and four introns are within the coding region of IL-19 cDNA. (Gallagher et al., 2000 & Liao et al., 2002). Crystal structure for IL-19 has been solved, and it is a monomer constructed of seven amphipathic helices with three disulfide bridges making the framework of the protein rigid (Chang et al., 2003). IL-19 signals through a distinct receptor complex made up of IL-20R1 and IL-20R2 subunits. IL-19, IL-20 and IL-24 exhibit substantial sharing of receptor complexes as all three are capable of signaling through the IL-20R1/IL-20R2 receptor. In addition, IL-20 and IL-24 can signal through the IL-22R1/IL-20R2 receptor. This implies that it is the differences in ligand and receptor interactions and in signal transduction, which may lead to specificity and distinct biology for each cytokine. (Dumoutier et al., 2001 & Parrish-Novak et al. 2002).

The main source for human IL-19 mRNA are monocytes and additionally small amounts can be detected in B cells. Induction of IL-19 production is seen in monocytes 4 hours after lipopolysaccharide (LPS) activation, and pretreatment of monocytes with IL-4 or IL-13 further induces IL-19 mRNA. GM-CSF has also been found to directly increase IL-19 mRNA expression. (Gallagher et al., 2000 & Wolk et al., 2002). IL-19 itself also induces IL-19 production, and hence a positive feedback loop amplifies IL-19. In addition, IL-19 induces IL-10 production in cultured human peripheral blood mononuclear cells (PBMC), which acts as a down-regulator of IL-19 production. (Jordan et al., 2005). IL-19 has also been shown to increase the proliferation of T cells, the effect which has been monitored also in the presence of IL-20 and IL-24 in some experiments (Oral et al., 2006).

IL-19 stimulates mouse splenic monocytes to produce IL-6 and TNF-α *in vitro*. However, pretreatment with IL-10 completely obstructs the induction of IL-6 and TNF-α by IL-19. IL-6 production can be partially restored to IL-19 treated mouse monocytes if IL-10 is added 2 hours after IL-19, but TNF-α induction is inhibited despite the delayed administration of IL-10. These differences in cytokine levels suggest that ether their production is induced in a different phase of IL-19 incubation or they are regulated through different mechanisms in signal transduction. Other effects in mouse monocytes that IL-19 has been shown to possess include induction of apoptosis and production of reactive oxygen species (ROS). (Liao et al., 2002). ROS are important toxic molecules of the immune system secreted by e.g. neutrophils and macrophages to control microbial pathogens and tumours (Bogdan et al., 2000). Apoptotic effect is likely due to the production of TNF-α and *in vivo* the mechanism could thus be to in-

duce apoptosis in a paracrine manner in neighboring tumour or infected cells. (Liao et al., 2002). However, these effects were not observed in human PBMC cultures, suggesting that the effects of IL-19 to murine splenic monocytes are different to human PB monocytes (Jordan et al., 2005). IL-19 along with IL-24 causes growth inhibition in certain cell line cultures (Conti et al., 2003).

IL-19 presumably has a role in the induction of $T_H2$ response. By inducing IL-4 and IL-10 production, IL-19 up-regulated the $T_R1/T_H2$ response. (Gallagher et al., 2004). Naïve $CD4^+$ cells will induce humoral response when activated to differentiate into $T_H2$ cells (Janeway, 2001) while $T_R1$ cells are induced regulatory $CD4^+$ T cells that have a crucial role in self-tolerance among other regulatory T cells (Lan et al., 2005). In addition, only the $T_H2$ cells contain both IL-20R1 and IL-20R2 subunits to form functional IL-19 receptor. (Gallagher et al., 2004). IL-19 also clearly modulates dendritic cell function by up-regulating IL-10 levels (Jordan et al., 2005); these IL-10 rich dendritic cells are found e.g. in chronic inflammatory events such as periodontal lesion (Jotwani et al.,2003). Besides increasing IL-4 and IL-10 production, IL-19 induces IL-13, IL-5 and IgE production in asthmatic mice: all of these are known factors in the pathogenesis of asthma. In addition to the murine asthma model, IL-19 has been found to be induced in the serum of patients with asthma. (Liao et al., 2004).

Besides IL-19, also IL-20 and IL-22 affect the $T_H1/T_H2$ balance by inhibiting IFN-γ and augmenting the response of IL-4 and IL-3. IFN-γ is a $T_H1$ specific and IL-4 and IL-3 are $T_H2$ specific cytokines. Stimulation of T cells with LPS leads to induction of IL-19, which can be inhibited by IFN-γ and induced by IL-4. (Oral et al., 2006).

### 2.2.3 IL-20

IL-20 is a member of the IL-10 family of cytokines. It was discovered by applying a bioinformatics algorithm designed to search helical cytokines to EST databases. Based on the amino acid identity, IL-20 gene has 28% similarity when compared to the IL-10 gene, and it shares even more similarity with IL-19 (40%) and IL-24 (33%). The receptor for IL-20 has been identified, and it comprises of IL-20R1 and IL-20R2 subunits. (Blumberg et al., 2001) In addition, IL-20 can signal through a receptor complex that consists of IL-22R1 and IL-20R2 (Dumoutier et al., 2001 ; Parrish-Novak et al. 2002). IL-20 protein is presumably a monomer like IL-19 (Blumberg et al., 2001).

IL-20 expression profiling has shown it to be expressed mainly in epithelial cells, myoepithelial cells, endothelial cells, macrophages and skeletal muscle cells (Hsing et al., 2006). Monocytes are the only immune cells that have been shown to produce IL-20 (Wolk et al., 2002). In addition, many cell types derived from the monocyte lineage have been identified to express IL-20. These cell types include e.g. microglial cells in brain tissue and Kupffer cells in the liver. It has been suggested that IL-20 could be important for inflammatory reactions in the brain and thus possibly also in other tissues. In connection to these findings, IL-20 has also shown to be a chemoattractive factor as it induces neurophil chemotaxis in vitro. (Hsing et al., 2006). IL-20 also selectively increases multipotential hematopoietic progenitors *in vivo* and *in vitro* (Liu et al., 2003).

The functional studies that have been done with IL-20 indicate involvement in skin biology. Transgenic overexpression studies in mice with mouse and human IL-20 show neonatal death and shiny appearance of skin. Changes observed in the skin include altered epidermal differentiation and hyper-proliferation including hyperkeratosis and thickened epidermis, which resembles human psoriatic skin. (Xu, 2004). IL-20 affects the $T_H1/T_H2$ balance similar to IL-19 by inhibiting IFN-γ ($T_H1$ cytokine) and augmenting IL-4 and IL-13 ($T_H2$ cytokines) production (Oral et al., 2006). IL-20 has been shown to be an angiogenetic factor in e.g. human umbilical vein endothelial cells (HUVECs). It induces proliferation, migration and vascular tube formation on endothelial cells. On the other hand, IL-20 induces the expression of other angiogenetic factors and also MMPs, and induces tumour vascularization in vivo. It has thus many proangiogenetic properties. However, IL-20 has been shown to downregulate the expression of cyclo-oxygenase 2 (COX-2) and prostaglandin ($PGE_2$), which promote angiogenesis. These pro- and antiangiogenetic properties of IL-20 can be explained by signaling through different IL-20 receptors and due to different microenvironment of different tissues. (Hsieh et al., 2006).

Psoriasis is an autoimmune disorder that manifests as an inflammatory dermatosis - T cells and cytokines have been indicated in its pathophysiology. IL-20 has been shown to be overexpressed along with its receptor subunits IL-20R1 and IL-20R2 in the keratinocytes of lesional psoriatic skin. This upregulation alters the interactions of endothelial cells, immune cells and keratinocytes and proposedly leads to dysregulation of proliferation and differentiation. In addition, IL-20 induces the expression of TNF-α in $CD8^+$ T cells, which are important in the pathogenesis of psoriasis. (Wei et al., 2006).

IL-20 has interestingly also been shown to be a proatherogenic cytokine (Chen et al., 2006).

IL-20 has been found to be possibly involved in the pathogenesis of RA. IL-20 expression was found to be upregulated in the synovial fluid of patients with RA compared to patients with osteoarthritis (OA) or gout. In addition, IL-20 and its receptor subunits were found to be consistently expressed in the synovial membranes of RA patients. It has been proposed that macrophages and synovial fibroblast would be the source of IL-20 and that IL-20 would act on synovial fibroblasts to induce the expression of chemoattractive factors and cytokines such as monocyte chemoattractant protein (MCP)-1, IL-6 and IL-8. IL-20 has also been demonstrated to be involved in angiogenesis. This process of new blood vessel formation from pre-existing vessels is important for the hypertrophic synovium in RA in order to be supplied with sufficient amount of nutrients and oxygen. IL-20 has thus many possible roles in the pathogenesis of RA and it is a regulator of a number of the crucial molecules. (Hsu et al., 2006).

### 2.2.4 IL-22

IL-22 was first identified in murine T cells as a gene that was specifically induced by IL-9. IL-22 shares 22% identity with IL-10 including 179 amino acids and a potential signal peptide. (Pestka et al., 2004). It is a glycosylated secreted α helical protein (Wolk et al., 2006). IL-22 has been found to be induced by IL-9 in thymic lymphoma cells, T helper cell clone TS2 and MC9 mast cells. IL-9 and conA, which is a lectin protein, induce IL-22 expression in freshly isolated splenocytes. In addition, LPS injection induces IL-22 production in various tissues. IL-22 is constitutively expressed in the thymus and brain. (Kotenko, 2002). The main producers of IL-22 are activated T cells; highest expression is in $CD4^+$ memory cells. Furthermore, lower levels are produced in NK cells (Wolk et al., 2002 and 2006). The active IL-22 receptor is composed of IL-22R1 and IL-10R1 subunits (Petska et al., 2004). T cell maturation towards $T_H1$ increases the activation induced IL-22 expression, while maturation towards $T_H2$ reduces it (Brand et al., 2006). IL-22 acts both locally and systemically (Wolk&Sabat, 2006).

Stimulation of human hepatoma cells with IL-22 has been found to lead to upregulation in the production of acute phase reactants such as serum amyloid A, α1-antichymotrypsin and haptoglobin. In addition, the production of ROS is induced in

resting B lymphocytes. (Pestka et al., 2004). IL-22 inducible genes in hepatocytes include also chemokines. Interestingly, IL-22 has been suggested to act as a protective factor in hepatocellular injuries. IL-22 may be involved in wound healing, as it promotes at least intestinal epithelial cell migration. (Brand et al., 2006).

Unlike IL-10, IL-22 seems to possess proinflammatory characteristics. IL-22 has been shown to be upregulated in intestinal inflammation such as Crohn's disease, which is a chronic inflammatory disorder of the gastrointestinal tract. *In vitro,* it promotes the intestinal barrier integrity through stimulation of intestinal epithelial cell (IEC) migration and the expression of a defensin, human β-defensin-2 (hBD-2). IL-22 activates proinflammatory functions in the IEC by activation of e.g. MAP kinases, Akt/PKB (= protein kinase B, a serine/threonine kinase belonging to the Rac subfamily of the Rho family) and STAT proteins and by inducing the expression of proinflammatory cytokines such as TNF-α and IL-8. IL-22 induces hBD-2 expression also in keratinocytes. Thus, IL-22 can be considered to be a positive regulator of the innate immunity in epithelial cells. (Brand et al., 2006)

Moreover, IL-22 induces the pancreatitis-associated protein-1 in pancreas acinar cells (Wolk et al., 2005). IL-22 is expressed as well in the rheumatoid synovium by the synovial fibroblasts and macrophages in patients with RA. IL-22R1 has been showed to be expressed only by rheumatoid synovial fibroblasts. In vitro, synovial fibroblasts (RAFS) express both IL-22 and IL-22R1 and stimulation of RAFS by recombinant IL-22 induces proliferation and expression of MCP-1. Thus, IL-22 may promote inflammatory responses in RA synovial tissues by inducing proliferation and the production of chemokines in the synovial fibroblasts. (Ikeuchi et al., 2005).

On the other hand, IL-22 has anti-inflammatory properties. It induces the production of antimicrobial proteins such as β-defensin-2, β-defensin-3 and psoriasin (Wolk, et al., 2006). Anti-inflammatory properties in the gut include IL-22-induced expression of follistatin and IL-11, which are protective acting proteins (Wolk&Sabat, 2006).

IL-22 is not a typical interleukin, since it seems that it does not influence immune cells. Resting or activated immune cells do not express the other subunit of the IL-22R, IL-22R1. Instead its targets are e.g. epidermal cells, and cells from the digestive and respiratory systems. Inflammation further increases the sensitivity of these cells to IL-22. It has been suggested that at least in epithelial cells the function of IL-22 is the defence against microbes, the inhibition of differentiation and intensifying cell mobility.

IL-22 regulated genes include three types of genes: antimicrobial proteins, differentiation-associated proteins and mobility-and-migration-regulated proteins. These all occur in psoriatic lesions, and in effect psoriatic patients have been showed to have vastly elevated IL-22 plasma levels, which correlated with the disease severity. Also, IL-22 transgenic mice exhibit skin thickening without immune cell infiltration, much the same way as IL-20 transgenic mice. (Wolk et al., 2006).

Further, it appears that cells, such as keratinocytes, hepatocytes and renal carcinoma cells that express a functional IL-22R1 are unresponsive to IL-10. In contrast, hematopoietic cells such as B cells and macrophages that express a functional IL-10R are unresponsive to IL-22. This may be of interesting biological relevance. (Boniface et al., 2005).

Systemic regulation of the activation of IL-22 is overtaken by IL-22 binding protein (IL-22BP). It has 34% identity to the IL-22R1 extracellular domain. Interestingly, it has higher affinity to IL-22 than the membrane bound receptor and can thus completely abolish the actions of IL-22. The protein seems to be glycosylated and it binds specifically to IL-22, not the other members of the IL-10 family. (Petska et al., 2004). The production of IL-22BP is suppressed by $T_H1$ and $T_H2$ cytokines (Oral et al., 2006). For example in the gut, IECs may regulate the intensity of IL-22 signalling by differential expression of IL-22BP and the ability to secrete anti-inflammatory IL-10 (Brand et al., 2006).

### 2.2.5 IL-24

IL-24 is the first homolog of IL-10 to be discovered although its nature as a four-helix bundle cytokine molecule was discovered later. IL-24 was cloned by subtraction hybridization in 1995 as a protein whose expression is elevated in terminally differentiated human melanoma cells, and given a name mda-7 for melanoma differentiation associated gene-7. (Kotenko, 2002). IL-10 and IL-24 share only 23% sequence identity, but the presence of an IL-10 signal sequence in IL-24 along with its genomic location indicates it belongs to the IL-10 subfamily (Petska et al., 2004). IL-24 is a glycosylated protein (Kotenko, 2002), and IL-24 proteins across species are highly homologues in amino acid sequence (Wang&Liang, 2005). However, IL-24 can activate overlapping but distinct signal transduction pathways in different species (Kotenko, 2002). IL-24 can use both IL-20R1/IL-20R2 or IL-22R1/IL-20R2 subunits as a functional receptor.

IL-24 expression is predominantly regulated by two transcription factors, namely activator protein-1 (AP-1) and CCAAT/enhancer-binding proteins (C/EBP). (Pestka et al., 2004).

IL-24 has two modes of action. It can ether function through its cell-surface receptor like classical cytokines or intracellularly in a non-receptor-mediated manner as a cytotoxic agent. The latter has gained more published data as a promising new gene therapy for treating a number of cancer types. IL-24 functions as a cytokine in physiological, low concentrations and overexpression of IL-24 leads to apoptosis selectively in cancer cells but not in normal cells. The function of IL-24 in effect differs from that of IL-10; IL-10 acts as a suppressor of immune response and inflammation, where as IL-24 has more of an immunomodulatory role and. (Pestka et al., 2004).

IL-24 is expressed in tissues associated with the immune system, e.g. spleen, thymus, PB leucocytes and normal melanocytes (Kotenko, 2002). Under physiological conditions, the major source for IL-24 seems to be activated monocytes and $T_H2$ cells (Wang&Liang, 2005). IL-24 is induced in PBMC after stimulation with LPS or PHA (phytohaemagglutanin), which are polyclonal activators. IL-24 is upregulated in human monocytes after LPS stimulus and in CD4+ naïve T cells and memory cells after anti-CD3 antibody stimulus. In addition, IL-24 is induced in rat alveolar macrophages after LPS or IL-4 treatment. (Pestka et al., 2004). IL-24 is also expressed in $CD19^+$ and $CD56^+$ cells late during a mitogen-driven response. CD19 is B-lymphocyte surface antigen B4 and CD56 is neutral cell adhesion molecule (NCAM), which is a marker for natural killer cells and some T hymphocytes. It has also been shown that expression of IL-24 in human PBMC results from proinflammatory cytokine stimulation and the response is regulated at the post-transcriptional level through stabilization of IL-24 mRNA. (Polndexter et al., 2005).

IL-24 induces the expression of several cytokines, including IL-6, IFN-γ, TNF-α, IL-1β, IL-12 and GM-CSF. This effect can be blocked with administration of IL-10. It has been suggested that the secondary cytokines induced by IL-24 activate antigen presenting cells to present tumour antigens and would thus mediate an antitumour immune response. On the other hand, IL-24 expression could be induced in human monocytes by infection with influenza A virus which would imply a role in antiviral immune response. (Pestka et al., 2004). In addition, IL-24 and its receptor can be induced by ras

oncogenes. It can be stated that IL-24 is a member of a complex cascade of cytokines involved in inflammation. (Wang&Liang, 2005).

Major target tissues of IL-24 are non-haematopoietic in origin and include e.g. skin, lung and reproductive tissues. Keratinocytes both express the IL-24 receptor and can also be activated by IL-24. IL-24 has been shown to be involved in wound healing. (Wang&Liang, 2005). Increased expression of normal human skin fibroblasts has been shown in correlation with increased levels of endogenous IL-24 (Kotenko, 2002). Overexpression of IL-24 has been shown in the epidermis of psoriatic skin, similar to IL-20. Thus there is a potential link between the over-activation of IL-24 and IL-20 signalling pathways and disease. Different cell types in psoriasis express IL-24 and IL-20. IL-20 is expressed in the keratinocytes themselves, where as infiltrating monocytes are the source of IL-24. (Wang&Liang, 2005). Thereby it is plausible that IL-24 acts as a proinflammatory paracrine factor and contributes to short-range signalling and performs immune related functions in skin (Gupta et al., 2006).

IL-24 expression can be induced in human melanomas after treatment with IFN-β and the protein kinase C activator mezerein. IL-24 can also be seen to translocate from the cytosol to the nucleus of differentiated cells; in normal melanocytes and overexpressing melanoma cells IL-24 has a diffuse cytoplasmic localization. In cells undergoing mitosis IL-24 has been associated with chromatin and could thus be involved in chromatin remodelling. In normal cells IL-24 has less profound effects, whereas one of the functions of IL-24 has been linked to suppression of the growth and colony formation of diverse human tumour cells. Promotion of apoptosis in cancer cells by IL-24 can be correlated with upregulation of Bcl-2-associated X protein (BAX) expression and G2/M cell cycle arrest. (Kotenko, 2002). Inhibition of IL-24 facilitates the progression to invasive and metastatic stages (Conti et al., 2003).

IL-24 as a suppressor and apoptosis-inducing gene displays a significant primary antitumour effect but also a potent “bystander antitumour effect” – this means that it does not only affect cells that directly receive this genetic agent but also exerts activity on tumour cells at a distance. Intratumoural injection of adenovirus-administered IL-24 has been shown to produce objective clinical responses and has as such potential as a gene therapy for diverse cancers. IL-24 has also the ability to radiosensitize tumour cells and inhibit tumour angiogenesis. In addition, release of IL-24 by normal cells has been indicated to inhibit anchorage-independent growth and tumour cell invasion. In addition, there is decreased cell survival and induction of tumour cell apoptosis. In order to

achieve these functions, the tumour cells have to have a complete set of functional receptors for IL-24; otherwise the therapy is useless. (Su et al., 2005)

### 2.2.6 IL-26

IL-26 was originally named AK155. It was cloned by subtraction hybridization and representational difference analysis as a protein expressed by herpesvirus saimiri (HSV) transformed T cells. HSV is a T cell tumour virus of New World monkeys and in human's infection with this virus leads to T cell lymphomas. IL-26 has also been found to be expressed at low levels in several other T cell lines and in freshly isolated PBMC from healthy donors. (Kotenko, 2002). IL-26 has 47% amino acid similarity to IL-10 (Pestka et al., 2004). IL-26 protein is likely to form a homodimer, and it seems that IL-26 is not glycosylated to a significant extent. (Kotenko, 2002). IL-26 signals through a specific and unique receptor complex, which is composed of IL-20R1 and IL-10R2 (Oral et al., 2006).

Expression of IL-26 is rather restricted to T cells. Expression pattern is similar to IL-22 in that it is induced in activated T cells and NK cells and the expression level increases in time. (Wolk et al., 2002). Expression can be induced with IL-2 and IL-12 or anti-CD3 antibodies; stimulus activates IL-26 specifically in activated memory cells and during polarization toward type 1 helper cells (Pestka et al., 2004). The target genes of IL-26 include at least IL-8, IL-10 and CD54 (ICAM-1). (Hör et al., 2004).

IL-26 protein is characterized by an unusual number of positively charged amino acids, which are mainly positioned in helix B. This characteristic of IL-26 allows it to efficiently interact with negatively charged heparin. Heparin is a glycosaminoglycan that is rich in negatively charged sulphate groups. Much more IL-26 is detectable in the presence of heparin. Heparin is similar to the proteoglycans that surround the cell membranes. Thus, the affinity of IL-26 to heparin could result in an immediate binding of secreted IL-26 to the surface of the IL-26 secreting or of adjacent cells. The cell surface bound IL-26 could further cause local or contact-dependent effects based on cell-type specific pattern of proteoglycans. Glycosaminoglycan moieties of proteoglycans could serve as coreceptors that recruit and enrich the cytokine in question at the cell surface, before the specific cytokine receptors are assigned in close local vicinity. (Hör et al., 2004).

The function of IL-26 could be to play the role of an autocrine growth factor causing uncontrolled proliferation of HSV-infected T cells. In addition, IL-26 could be involved in regulating the immune response to HSV infection. (Kotenko, 2002). On the other hand, it can be speculated that IL-26 would have more of an immunoprotective role and function against viral infections (Pestka et al., 2004). It has been suggested that IL-26 could play a role in the interaction of epithelial cells and lymphocytes that is typical for e.g. γ-herpesvirus pathogenesis and epithelium-associated virus-induced lymphomas and in cutaneous and mucosal immunity (Hör et al., 2004).

Genetic locus of IL-26 is situated at a region susceptible for autoimmune and allergic diseases (Conti et al., 2003). Also, other diseases have been investigated in regard to the IL-26 locus. For example, it has been thought that accumulated and epistatic effects of multiple genes may predispose to RA. A plausible candidate that has recently attracted attention in susceptibility to RA is IFN-γ, but this has not been confirmed. However, polymorphisms in the IFN-γ/IL-26 gene region are likely to be associated with susceptibility to auto-immune diseases such as RA and multiple sclerosis (MS) in a sex-specific manner. The linkage disequilibrium occurs closer to the IL-26 gene than to the IFN-γ gene. This region is involved in male versus female differential susceptibility to the inflammatory disease MS, and multiple-marker haplotype associated with RA in women. (Vandenbroeck et al., 2003).

## 2.3 Methodological considerations – quantitative/real time RT-PCR

### 2.3.1 Principle of real time RT-PCR

RT-PCR based assays are the most common methods for characterizing or comparing the expression patterns of a gene or a family of genes and examining steady-state mRNA levels in different sample populations (Bustin, 2002). The benefits of real time PCR to conventional PCR include sensitivity, large dynamic range and the potential for high throughout and accurate quantification (Huggett et al., 2005). The technique is very simply based on the ability to detect the nascent PCR product with the help of fluorescent tags. The increase in fluorescence emission during the PCR reaction is due to the growing amount of the target gene product and can be detected in real time by a modified thermocycler. (Anya et al., 2005). This technique is especially useful in immunological samples, where the target is to identify the expression of different splice vari-

ants of the original transcript. Also, the method is handy when detecting cytokines and transcription factor that are found in very low abundance. (Huggett et al., 2005).

There are two distinct chemistries that are used to create the measurable fluorescent signal. Fluorescent dyes that are specific for double stranded DNA can be used. In addition, there is the possibility to deploy many different kinds of sequence specific oligonucleotide probes. SYBR green I is a frequently used dsDNA-specific dye, which has 1000-fold higher fluorescence in the bound state when compared to free dye. It is an asymmetric cyanine dye that binds to the minor groove of dsDNA sequence. (Wilhelm&Pingoud, 2003). When using dsDNA specific dyes quasitemplate specific data can be produced if DNA melt curves are used to identify specific amplification products. SYBR green and analogous dyes are much cheaper than sequence-specific dyes. However, they can easily be incorporated into optimized and long-established protocols. Drawbacks of ds-DNA specific dyes include non-specific detection of primer dimers as a result of which the use of melting curves is obligatory. Also, amplification of a longer product will generate more signal than a shorter one. (Bustin&Nolan, 2004).

Sequence-specific dye requires the design and synthesis of one or more custom-made fluorescent probes for each PCR assay. The fluorescent signal is generated only if the amplicon-specific probe hybridizes to its complementary target. (Bustin&Nolan, 2004). The intensity of the signal can be related to the amount of PCR product by a decrease of the quench of a reporter fluorophor or by an increase of the fluorescence resonance energy transfer (FRET) from a donor to an acceptor fluorophor. The quench and the FRET are both dependent on the distance of the fluorophors and thus the PCR-product-dependent change in the distance between fluorophors is used to generate the sequence-specific signals. There are several different formats that have been generated based on this theory, and they include hybridization probes, TaqMan probes, molecular beacons, Sunrise primers and Scorpion primers. Most of them are based on a decrease of quench (see figure 5). (Wilhelm&Pingoud, 2003). The probes can be labelled with different, distinguishable reporter dyes, which enables the detection of amplification products from several distinct sequences in a single PCR reaction. This is referred to as multiplex PCR. The disadvantages of sequence-specific probes include the high price of the probes and the fact that artefacts that interfere with amplification efficiency cannot be detected. (Bustin&Nolan, 2004). Interestingly, SYBR green I has been reported to yield results to similar precision to those from fluorogenic probes or

even with higher precision. On the other hand, sequence-specific probes allow easy detection of point mutations. (Wilhelm&Pingoud, 2003).

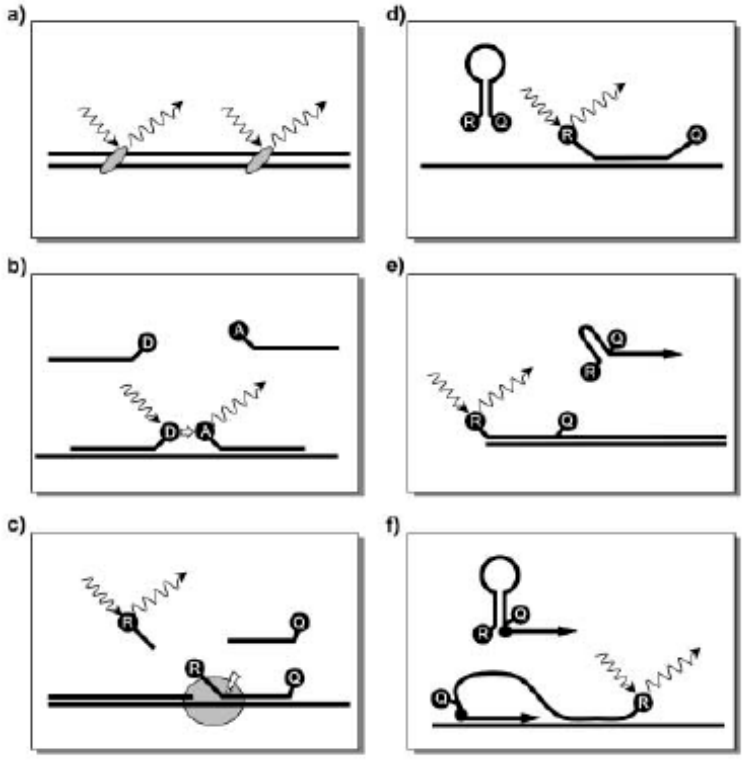



**Figure 5. a) SYBR green I b) hybridization probes c) TaqMan probes d) molecular beacon e) sunrise primer f) Scorpion primers. D=donor A=acceptor Q=quencher. The grey circle in c) indicates the Taq polymerase hydrolysing the TaqMan probe. (adopted from Wilhelm&Pingoud, 2003).**

### 2.3.2 Quantitative analysis

The basis of the quantitative analysis in real time PCR, in addition to the fluorescent dyes, is the software on the computer. Thereby, an amplification plot is constructed by means of the fluorescent emission data that is collected during PCR amplification (see figure 6). As can be seen in the figure, the amplification can be divided into three distinct phases: an initial lag phase where the accumulation of product is undetectable, a following exponential phase and finally a plateau phase. (Wilhelm&Pingoud, 2003) The amplification of the PCR product is dependent on the efficiency of amplification, which on the other hand depends on many factors including the length and sequence of the amplified DNA, primer design, the concentrations of the reaction buffers and the temperature and duration of each step in the PCR cycle. During the plateau phase of amplification the efficacy approaches zero and thus the multiplication of the product ceases. (Ding&Canton, 2004).

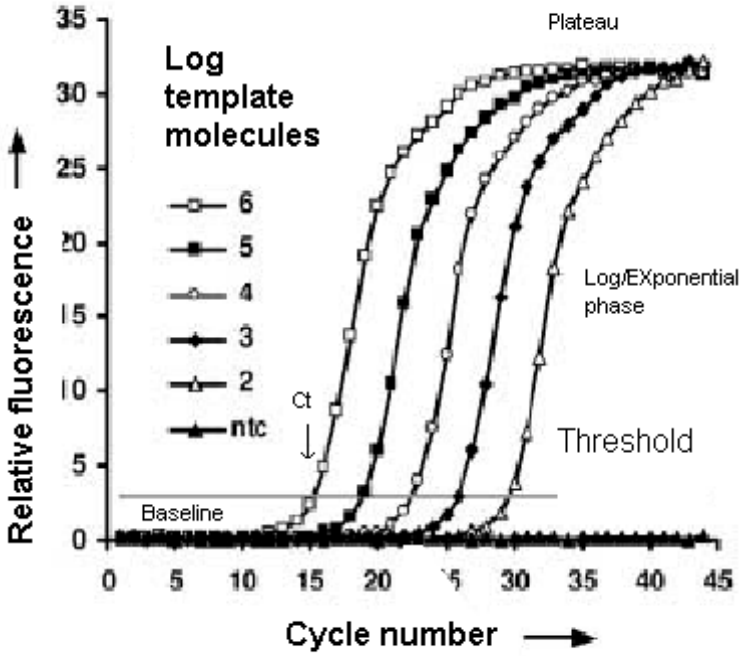



**Figure 6. amplification curves for increasing amount of template ($10^2$ – $10^6$). The observable exponential phase shifted towards higher cycle numbers for samples containing fewer target molecules. NTC = no template control. (adopted from Wilhelm&Pingoud, 2003).**

To determine the starting amount of the template, certain values must be ascertained. Baseline is defined from the PCR cycles during which the fluorescent signal is accumulating but still undetectable. Threshold (Ct), on the other hand, is an arbitrary coefficient that is chosen based on the variability of the baseline fluorescence. It is calculated from the standard deviation of the average signal of the baseline fluorescence so that the standard deviation is multiplied by ten. In addition, Ct is defined on the basis of the threshold, which is the fractional PCR cycle number when the fluorescent signal reaches a value higher than the threshold. Ct is used in the determination of the initial amount of template. This can be done in many ways, but using a series of dilutions of a standard is common: log of known or chosen initial copy numbers of the diluted standard can be plotted against the measured Ct values to form a strait line. Quantification is accomplished by determining the Ct values for the 'unknown' samples and by using the generated standard curve for attaining starting copy number. (Arya et al., 2005, Wilhelm&Pingoud, 2003). Normalization will be considered in paragraph 2.3.3.

### 2.3.3 Problems associated with real time RT-PCR

Real time RT-PCR is a very sensitive technique for quantification of expression levels in different kinds of samples. However, the sensitivity and specificity of the technique also raises several issues that should be taken into consideration when performing real time RT-PCR. These include for example the standardization of the used RT-PCR protocols. In addition, attention and consistency should be taken with regards to used reagents, assay design, template preparation and the analytical methods including analysis, reporting and interpretation. (Bustin&Nolan, 2004). Inherent variability of RNA, variability of extraction protocols that may also copurify inhibitors and different reverse transcription and PCR efficiencies may also be problematic (Huggett et al., 2005). It should be also noted that in real time RT-PCR, the single most likely source of data variation is probably due to the variability introduced by the person carrying out the experiment (Bustin, 2002).

Beginning from RNA, the starting material, samples should be handled with care. RNA must be of highest quality, free of DNA or nucleases and no copurification of inhibitors of the RT step or PCR should be allowed. There must be consistency when collecting, transporting and storing samples. Appropriate use of protocols and storage is crucial. (Bustin&Nolan, 2004). If tissue biopsies are used for template preparation, it should be noted that this results in the averaging of the expression profile. Thus, a specific cell type may me masked, lost or dismissed because of the bulk of the surrounding cells. RNA levels are not necessarily directly proportional to the protein produced by the cell, since many regulatory events take place at the post-transcriptional stage. (Bustin, 2002).

It is also noteworthy that the use of the different techniques to prime cDNA (random primers, oligo-dT, target-specific primers) gives different DNA yield and variability. Target-specific primers synthesize the most specific cDNA and it is thus the most sensitive option. However, since separate priming reactions are required to each target, it is wasteful. On the other hand, using random primers is non-specific and yields the most cDNA with least bias. Reverse transcription is achieved at multiple points along the transcript and so more than one cDNA transcript is produced per original target. cDNA synthesis using oligo-dT is more specific to mRNA than random priming since no rRNA will be transcribed. However, oligo-dT can struggle to generate transcripts from mRNAs with significant secondary structure and RNAs that lack a polyA-tail will not be transcribed at all. (Bustin&Nolan, 2004).

Normalization of different samples is important for producing reliable and comparable results. This begins with the use of the same sample size, for example similar tissue volume or weight. (Huggett et al., 2005) In order to use the same amount of RNA for each sample, accurate quantification of RNA must be performed by e.g. measuring $OD_{260}$ in spectrophotometer (Bustin, 2002). Reference genes have been used for a long time in the normalization of samples, but there are some problems associated with it. A relatively new method is to incorporate artificial molecules in the samples at the extraction stage at defined concentrations. The positive effect of the latter method is that they will be subjected to almost all the experimental errors and thus do not create false results. (Huggett et al., 2005).

When using reference genes for normalization, so-called house keeping genes are employed. These are genes that have traditionally been thought to be expressed at a relatively constant level that is not dependent on the cell type. Commonly used genes include for example β-actin, GAPDH (glyseraldehyde-3-phosphate), HPRT (hypoxanthine-guanine phosphoribosyl transferase) and 18S rRNA. Ideally, when using these genes all the steps in the Q-PCR measurement and also different input amounts of the RNA used in the RT step would be controlled for. However, all the classic reference genes have been shown to be regulated following certain signals. Thus, it seems reasonable to assume that genes, in general, are regulated and this can lead to unpredictable differences in expression patterns. If reference genes are used for normalization, they must be validated by measuring variation in the cycle threshold and it is advisable to use more than one gene. (Bustin&Nolan, 2004&Huggett et al., 2005).

In data-analysis, it may be necessary to adjust the baseline cycles in order to include the lowest point of the amplification plot (Bustin&Nolan, 2004). The starting quantity of template sequence can only be extrapolated back during the exponential phase of the PCR reaction (Arya et al., 2005). Importantly, the highest dilution of the standard curve to report consistent Ct values defines the lowest copy number that can be quantified with confidence. Thus, if the Ct values that have been recorded by any unknown translate into copy numbers lower than this benchmark, they should be recorded as qualitative (yes/no) results. (Bustin&Nolan, 2004). When planning gene expression studies it should be kept in mind that RT-PCR data is uninformative about the activity of the protein or possible mutations. Complete and biologically relevant analysis should be done after accompanying biochemical assays. (Bustin, 2002).

# 3. AIMS OF THE STUDY

The specific aims of the study were:

1. To identify suitable positive controls for the members of the IL-10 family, their receptors and IL-22BP for RT-PCR analysis.
2. To study the relative mRNA expression of IL-10 family cytokines, their receptors and IL-22BP in the synovial tissue, PB and SF samples from patients with RA and OA and from healthy volunteers
3. For cytokines that are found to be upregulated in RA patients, additional analysis determining their expression at protein level and the principle cell type expressing the cytokine will be performed.

# 4. MATERIALS AND METHODS

## 4.1 Patients samples

In order to study the expression of IL-10 family cytokines in RA, samples including synovial tissue, peripheral blood (PB) and synovial fluid (SF) were obtained. Synovial tissue samples from the hip, knee or wrist joints were obtained from ten patients with RA and ten patients with OA during orthopaedic surgery. To ensure stabilization of RNA and thus secure the reliability of the forthcoming results from e.g. RT-PCR analysis, the samples were immediately placed into tubes containing RNA*later* RNA stabilization reagent (Qiagen Inc, Valencia, CA) and stored at -20°C. The obtained body fluids were collected to 5-10ml heparinized tubes in order to inhibit clotting. PB samples were collected from 26 patients with active RA. Similarly, SF samples were obtained from 14 patients with RA by needle aspiration from inflamed knee joints. PB samples were collected also from 31 healthy blood donors (Finnish Red Cross Blood Transfusion Service, Tampere). To assure the good quality and stability of the PB/SF samples, further processing of the samples was initiated instantly.

**Table 3. Demographic and clinical characteristics of patients with rheumatoid arthritis (RA) and osteoarthritis (OA)**

| **Study group** | **RA n = 26** | **RA n = 10** | **OA n = 10** |
|---|---|---|---|
| **Sample obtained** | PB/SF | synovial tissue | synovial tissue |
| **Female/male** | 16/10 | 10/0 | 9/1 |
| **Age, years (median, range)** | 61,5 (32-87) | 66 (39-74) | 69 (60-77) |
| **Disease duration, years (median, range)** | 14 (0,25-49) | 21 (8-49) | NA |
| **RF positivity, n** | 18 | 6 | NA |
| **CRP, mg/l (mean±SD)** | 32±22 | 13±13* | 3±3* |
| **Medication, n** | | | |
| **-Steroids** | 20 | 7 | 0 |
| **-Conventional DMARDs** | 22 | 7 | 0 |
| **-Biological therapy** | 8 | 1 | 0 |
| **Operation, n** | | | |
| **-Hip arthroplasty** | NA | 5 | 2 |
| **-Knee arthroplasty** | | 4 | 8 |
| **-Wrist arthroplasty** | | 1 | 0 |
| **Macroscopic synovitis score (mean)**** | NA | 1,6 | 1,8 |

* Measured 1 to 4 weeks prior to operation

** The extent of synovitis was macroscopically scored by the operating surgeon from 0 to 3

The demographic and clinical characteristics of the patients are presented in table 3. Patients with RA met the 1987 American College of Rheumatology criteria for RA (Arnett et al., 1988), and patients with OA were classified as having primary OA according to the ACR criteria (Altman et al., 1986; Altman et al., 1991). Informed consent was obtained from all patients, and this study was approved by the medical ethics committee of Tampere University Hospital.

## 4.2 Cell preparations

### 4.2.1 Ficoll-Paque Plus density gradient centrifugation

PBMC and SFMC were isolated by a Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, Great Britain) density gradient centrifugation. Ficoll-Paque plus is a separation media consisting of a mixture of Ficoll 400 and an iodinated density gradient medium (sodium diatrizoate) used for purifying human lymphocytes. Ficoll 400 is a synthetic high molecular weight polymer of sucrose and epichlorohydrin, which has a low intrinsic viscosity and forms solutions of low osmotic pressures. The use of sodium diatrizoate in Ficoll-Paque plus provides optimal density and osmolarity for efficient removal of other cells.

The principle of Ficoll-Paque plus procedure is that defibrinated or anticoagulant-treated blood or synovial fluid is carefully layered on the Ficoll-Paque plus solution and centrifuged for a sufficient amount of time. Cells in the sample come in contact with the body fluid/Ficoll-Paque plus interface upon centrifugation and distinct cell populations form isolated layers based on differential migration in the gradient. Erythrocytes cluster as a pellet at the bottom for they efficiently form aggregates when they come in contact with Ficoll 400 at room temperature (RT); this leads to increased rate of sedimentation and complete migration through the Ficoll-Paque plus. In addition, granulocytes sediment to the bottom of the separation media immediately above erythrocytes since contact with slightly hypertonic Ficoll-Paque plus causes an increase in granulocyte density and contributes to their ideally complete sedimentation through the Ficoll-Paque plus. However, lymphocytes, monocytes and platelets remain as a concentrated band in the interface. These cell types do not attain a density great enough to be able to penetrate through the Ficoll-Paque plus and instead collect in the interface as a concentrated band. Highly purified lymphocytes and monocytes are recovered from the interface after subsequent washing and centrifugation steps, which remove platelets and any contaminating Ficoll-

Paque plus and plasma. (Ficoll-Paque plus for *in vitro* isolation of lymphocytes, Handbook Amersham Biosciences)

Before application to Ficoll-Paque plus, blood samples were diluted 1:1 in hank's balanced salt solution (HBSS). Dilution with a balanced salt solution increases the yield of lymphocytes since it reduces the amount of lymphocytes trapped in the aggregating clumps of red blood cells. Similarly to increase the lymphocyte yield, pelleted cells from the SF were suspended to an appropriate amount of phosphate buffered saline (PBS) depending on the size of the pellet (typically to a volume of 10-40ml). PB/SF samples were then centrifuged with Ficoll-Paque plus according to manufacturer's instructions. The collected lymphocyte fraction was washed twice with PBS containing 2 mM ethylene diamine tetra-acetic acid (EDTA) at 4°C.

### 4.2.2 MACS (Magnetic cell separation)

For some studies T cells and monocytes/macrophages were purified from PBMC and SFMC using magnetic cell sorting technology (MACS; Miltenyi Biotec) according to manufacturer's instructions. The procedure has been first described in the beginning of 1990s and it is a popular means for cell separation. The technology is based on the use of small magnetic microparticles (particle ∅ $<<$0,5μm) conjugated to antibodies and a high-gradient magnetic field. Magnetic cell sorting is a flexible, fast and simple method for the separation of cells according to specific cell surface markers, and isolation of uncommon cells is less time consuming than with conventional applications. The procedure can be used for positive or negative selection of cells and cells can de labelled ether directly or indirectly. (Miltenyi et al., 1990). MACS Magnetic Cell Sorting Separator MidiMACS was used for high-gradient magnetic field and MACS buffer was filtered and it contained 0,5% bovine serum albumin (BSA) and 2mM EDTA in PBS.

$CD14^+$ PB monocytes and SF macrophages were isolated by positive selection with monoclonal anti-human CD14-coated microbeads from PBMC and SFMC, respectively (CD14 Microbeads; Miltenyi Biotec, Auburn, CA). CD14 glycoprotein is expressed in large quantities on most monocytes and macrophages and to a lesser degree in neutrophils, and as such is a suitable antigen for monocyte/macrophage separation of human cells. Manufacturer's LS columns (for the efficient isolation of max $10^8$ cells) were chosen for the isolation and in order to gain higher purity pre-separation filters were used to remove cell aggregates and other large particles for optimal separation re-

sults. Also application through a second column was used to ensure high purity of isolated $CD14^+$ cells. The overall principle is simple: Magnetic anti-CD14-coated microbeads attach to $CD14^+$ cells, and when labeled cell suspension is loaded on a column which is placed in the magnetic field of a MACS separator the $CD14^+$ cells are retained in the column as unlabeled cells run through. When removed from the magnetic field $CD14^+$ cells can be eluted as the positively selected cell fraction. (MACS CD14 MicroBeads Instructions).

$CD3^+$ T cells were purified by negative selection from $CD14^-$ cells using a cocktail of hapten-conjugated antibodies (Abs) specific for non-T cells and anti-hapten-coated microbeads (Pan T Cell Isolation Kit; Miltenyi Biotec). Similarly to $CD14^+$ cell isolation LS columns were used but pre-separation filters were considered unnecessary since the cell suspensions had already been handled through a pre-separation in the preceding $CD14^+$ purification step (see previous paragraph). First labeling step includes a incubation with a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and glycophorin A to label non-T cells, i.e. B cells, natural killer(NK) cells, dendritic cells, monocytes, granulocytes, and erythroid cells, from the cell suspension. There after, incubation with anti-biotin microbeads completes the magnetic labeling procedure, and loading to a column in the magnetic field of a MACS separator achieves complete depletion of magnetically labelled cells. Highly pure T cells pass through the column and thus $CD3^+$ T cells are purified as the negative cell fraction. (Pan T cell isolation kit Instructions).

**4.3 RNA isolation**

Total RNAs were isolated from 5-10 $x10^6$ PBMC, SFMC or isolated cell fractions ($CD14^+$ monocytes/macrophages and $CD3^+$ T cells) stored in -70ºC as cell pellets. In addition, total RNAs from 100-200 mg of synovial tissues stored in -20ºC in sufficient amount of stabilizing RNA*later* reagent were isolated. Before RNA isolation synovial tissue was homogenized first mechanically and subsequently by Ultra Turrax machine (Ultra Turrax –homogenizator, IKA). RNeasy MiniKit (Qiagen Inc.) was used for isolation and the procedure was carried out according to manufacturer's instructions. An additional 15 min incubation step in the presence of 27 Units DNAse I (Qiagen Inc.) was added to the protocol to digest any contaminating DNA. Isolated RNAs were stored in -70ºC.

### 4.4 Reverse transcription

For expression studies 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase M-MLV-RT (Invitrogen, Carlsbad, CA) and random hexamers as a primer (Amersham Biosciences). Random hexamers prime reverse transcription at multiple points along the transcript. First, 1 µg of total RNA was boiled in a total volume of 11,55 µl (volume adjusted with RNase-free $H_2O$ if necessary) for 4 minutes, cooled on ice for 2 minutes and subsequently spinned briefly. Next, 8,45 µl of a mixture containing 4 µl 5x First strand buffer (Invitrogen), 2 µl 100 mM DTT (dithiothreitol; Invitrogen), 1 µl 10 mM dNTPs mix (deoxynucleoside 5'-triphosphates; Finnzymes), 0,2 µl 0,125 U/ µl random hexamers (Amersham Pharmacia Biotech), 0,25 µl RNAguard Rnase inhibitor (Amersham Biosciences) and 1 µl M-MLV-RT (Invitrogen) was added to reach a final reaction volume of 20 µl. The reaction mixture was incubated at +37 ºC for 1 hour, boiled again for 4 minutes and chilled on ice for 2 minutes. cDNAs were stored in -20ºC.

### 4.5 Generation of positive controls for quantitative RT-PCR

Activated immune cells from isolated PBMC were defined as positive control candidates, since these cell types had previously been shown to express IL-10 family cytokines (Wolk et al., 2002). In the experiment of Wolk et al. all the studied cytokines (IL-19, IL-20, IL-22, IL-24 and IL-26) were expressed after typical cell-specific stimuli. The time span of the used stimulus ranged from 6 to 18 hours, and the upregulation of expression was seen in ether T cells or monocytes, and also in other immune cell subtypes. Based on these previous results LPS stimulated monocytes and PHA stimulated T cells were tested as suitable positive control for IL-10 family cytokines. Wolk et al. also showed that most of the receptors for IL-10 family members were expressed in human skin at significant quantities. Accordingly, human skin RNA (Stratagene, La Jolla, CA) was chosen as primary candidate for testing of positive controls for the receptors of IL-10 family and IL-22BP.

LPS stimulated positive controls were generated with the following procedure: Isolated PBMC from a healthy volunteer were cultured for 2, 6 or 18 hours in the presence of 0,1 µg/ml LPS (Sigma, St Louis, MO). Cell concentration was $1x10^6$ cells per 1 ml of medium, and typical cell number stimulated was $10x10^6$ cells. Culture medium was RPMI (Roswell Park Memorial Institute) 1640 medium (BioWhittaker) supplemented with 2mM L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; Bio-

chrom AG), 100 U/ml penicillin (BioWhittaker) and 100 µg/ml streptomycin (BioWhittaker). This culture medium was used in all cell cultures during this study. After stimulation, the cells were washed twice with PBS and total RNA was isolated with RNeasy Mini Kit protocol (Qiagen) (see chapter 4.3). LPS is a determinant of the cell wall of gram-negative bacteria. Monocytes and macrophages recognize LPS and thus become activated through LPS receptor, which is better known as CD14 antigen (Janeway, 2001).

PHA stimulated positive controls were generated using two alternative methods. First, similarly to LPS controls, isolated PBMC from a healthy volunteer were cultured for 2, 6 or 18 hours in the presence of 2 µg/ml PHA (Sigma). Cell concentration was $1x10^6$ cells per 1 ml of medium, and typical cell number stimulated was $10x10^6$ cells. After stimulation the cells were washed twice with PBS and total RNA was isolated with RNeasy Mini Kit protocol (Qiagen) (see chapter 4.3).

Alternatively, PHA stimulation was used to generate PHA blasts. PHA blasts were generated as follows: Isolated PBMC from a healthy volunteer were cultured at $1x10^6$ cells/ml in culture medium (see above) complemented with 2 µg/ml PHA for three days in +37 ºC. Next, the cells were washed twice with PBS to remove PHA stimulus and they were starved overnight in the absence of PHA in fresh culture medium. On the fifth day the cells were stimulated for 6 hours ether with 2 µg/ml PHA or with 1 µg/ml anti-CD3 Ab and 10 µg/ml anti-CD28 Ab in fresh culture medium in +37 ºC . Stimulated PHA blasts were washed twice with PBS and total RNA was isolated with RNeasy Mini Kit protocol (Qiagen) (see chapter 4.3). PHA is a polygonal T cell mitogen that induces nearly all T cells to proliferate in essentially the same way as an antigen and induces e.g. RNA expression (Janeway, 2001). Thus, altered RNA expression in PHA blasts generates strong candidates for positive controls as RNA amount is substantial. CD3 is part of the T cell receptor complex and CD28 is a costimulatory receptor on T cells. Simultaneous engagement of these receptors results in T cell activation.

### 4.6 Conventional RT-PCR

To initially test whether IL-10 family cytokines were expressed in RA conventional RT-PCR was used for evaluation. The 25-µl PCR reactions were performed in the PTC-200 apparatus (Bio-Rad Laboratories, inc., MA) and the reactions contained 17,5 µl PCR-grade $H_2O$, 1 µl (0,2 µM) of the relevant primers, 1 µl 10 mM deoxynucleoside triphosphate (dNTP) mix, 2,5 µl 10x polymerase buffer, 1 µl DyNAzyme polymerase

(1 U/25µl; Finnzymes), 1 µl aliquot of the desired positive control cDNA and PCR-grade $H_2O$ to adjust the volume to 25 µl. The forward and reverse primers recognizing separate exons of IL-10 family genes were designed using the Primer3 program (available at *http://www-genome.wi.mit.edu/cgi-bin/primer3.www.cgi*). Using primers that recognize separate exons prevents the amplification of unwanted genomic DNA. The sizes for PCR products ranged between 80 and 200 bp. The primers that were used for amplifying IL-10 family cytokines and receptors are presented in table 4.

**Table 4. Primers for IL-10 family cytokines**

| Cytokine /receptor | Sense primer | Antisense primer | Positive control |
|---|---|---|---|
| IL-10 | 5'-TCCCTGTGAA AACAAGAGCA-3' | 5'-CAGAGGTTTTAGC CTAGACC-3' | LPS stimulated PBMC |
| IL-19 | 5'-TACGTGGAC AGGGTGTTCAA-3' | 5'-TGGTTACGGTGGTC TCAGTA-3' | LPS stimulated PBMC |
| IL-20 | 5'-TTGCCTTCAG CCTTCTCTCT-3' | 5'-GTCACACGTTCGGT TTCTAC-3' | LPS stimulated PBMC |
| IL-22 | 5'-TGCAAAAGCC TGAAGGACACA-3' | 5'-TAATCTACGGGGTT TCGCTA-3' | α-CD3/ α-CD28 stimulated PHA blasts |
| IL-24 | 5'-GCTTTCGCCA ATTTAACACC-3' | 5'-AATGGGACGAAGA GACCTCG-3' | α-CD3/ α-CD28 stimulated PHA blasts |
| IL-26 | 5'-TGTCAATTTC AAGAACAGCTTC TG-3' | 5'-AAAGGACACGAAG TAGTCGA-3' | α-CD3/ α-CD28 stimulated PHA blasts |
| IL-10R1 | 5'-TCAGCCTCCT AACCTCTGGA-3' | 5'-AAGAAACGGAAAC AGGACGA-3' | Skin |
| IL-10R2 | 5'-CTTGCTGTGG TGCGTTTACA-3' | 5'-TTCTGAGACTCTCG CCGTTC-3' | Skin |
| IL-20R1 | 5'-TCTGGTATGT TTTGCCCGTA-3' | 5'-AAGAAACACGGAC GACTTTT-3' | Skin |

| Cytokine /receptor | Sense primer | Antisense primer | Positive control |
|---|---|---|---|
| IL-20R2 | 5'-TGTTGCCCCG TGGTGGT-3' | 5'-CTCCTCCCTTCTGC AGCTGAT-3' | Skin |
| IL-22R1 | 5'-GTTCTCCATG GGCTTCCTC-3' | 5'-GTGCAGGACTAGG GACAGAA-3' | Skin |
| IL-22BP | 5'-TTGCAACCAT GATGCCTAAA-3' | 5'-CGTGAATGACCGT TGTCGTC-3' | Skin |
| GAPDH | 5′-GATGACATC AAGAAGGTGGT G-3′ | 5′-GCTGTAGCCAAAT TCGTTGTC-3′ | - |
| TBP | 5'-GAATATAATC CCAAGCGGTTT-3' | 5'ACTTCACATCACAG CTCCCC-3' | - |

The RT-PCR conditions included the following steps:

1. Initial activation step 95ºC 2 min
2. Denaturation 94ºC 30 s
3. Annealing 58ºC 30s
4. Extension 72ºC 45s

-Steps 2-4 repeated for 40 cycles

5. Final extension 72ºC 5 min
6. Cooling 4ºC hold

The amount and purity of amplified PCR products were analyzed by 4% agarose gel electrophoresis. The size of the amplified products was determined using GeneRuler 50bp DNA Ladder (Fermentas) to confirm that the correct gene had been amplified.

### 4.7 Quantitative PCR (Q-PCR)

Quantitative RT-PCR was used for more quantitative analysis of the expression of IL-10 family cytokines in RA. For preparing the standard curves 5 µg of suitable total RNA was reverse transcribed in a reaction volume of 20 µl. IL-22, IL-24 and IL-26 were analyzed using PHA blasts activated with anti-CD3 Ab and anti-CD28, IL-10, IL-19 and IL-20 with PBMC cultured for 18 hours in the presence of LPS and IL-10 family receptors IL-10R1, IL-10R2, IL-20R1, IL-20R2, IL-22R1 and natural antagonist for

IL-22, IL-22BP, with human skin. Serial dilutions (one to five) of the standard cDNA were made to correspond to cDNA transcribed from 375, 75, 15, 3, 0.6 and 0.16 ng of total RNA.

The same forward and reverse primers were used for Q-PCR as for conventional RT-PCR (see table 4). The 15-µl real-time PCR reactions were performed in the LightCycler apparatus (Roche Diagnostics, Mannheim, Germany) using QuantiTect SYBR Green PCR kit (Qiagen Inc.) or LC-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) for IL-26 analysis. Reactions contained 1.5 µl aliquot of sample or standard cDNA, 0.5 µM of the relevant primers and 1 x ready-to-use reaction mixture containing *Taq* DNA polymerase, SYBR Green I fluorescent dye, dNTPs, 2.5 mM $MgCl_2$ and reaction buffer. Duplicate reactions were performed for each sample.

PCR amplification consisted of the following steps:

| | | |
|---|---|---|
| 1. Initial enzyme activation | 95°C | 15 min |
| 2. Denaturation | 95°C | 15s |
| 3. Annealing | 58°C | 20s |
| 4. Extension | 72°C | 15s |

- Steps 2-4 repeated for 45 cycles

| | | |
|---|---|---|
| 5. Melting curve analysis | 60°C → 95°C | |
| 6. Final cooling | 40°C | 30s |

PCR reaction of 45 cycles was used for GAPDH, IL-10, IL-22, IL-26 and for all the IL-family receptors and IL-22BP, while a 50 cycle reaction was used for IL-19 and IL-20. For certain primers an additional data-acquisition step was added after extension step to the end of each cycle to avoid detecting primer-dimers. This step was used to nullify the fluorescence caused by formation of primer-dimers so that the fluorescence of the amplified gene product was measured in a higher temperature after denaturation of primer-dimers. When using SYBR Green I fluorescent dye the produced fluorescence is not specific to a particular gene; the dye adheres to all double stranded DNA and thus the formation of primer-dimers may be problematic. Additional steps were used for IL-19 (78°C 5s), IL-20 (76°C 5s), IL-26 (79°C 5s) and IL-22R1 (80°C 5s).

The results were analyzed using the LightCycler Data Analysis software (version 3.5; Roche), and the mean IL-10 family expression values in relation to standard from duplicate samples were normalized by dividing them by the mean values obtained for GAPDH house-keeping gene (tissue, PBMC and SFMC samples) or TATA binding pro-

tein (TBP) house-keeping gene (cell fractions: T cells and monocyte/macrophages). The purity of PCR products was verified by analyzing melting curves of the products between 65°C and 95°C, and by agarose gel electrophoresis of the amplified products to determine their size.

### 4.8 Flow cytometry

#### 4.8.1 Purity analysis: cell fractions

Purity of isolated cell fractions (T cell and monocyte/macrophage cell populations) was assessed with flow cytometry. For this purpose an aliquot of 0,15 x$10^6$ cells from isolated cell populations were stained as follows: 0,05 x$10^6$ cells with FITC (fluorescein isothiocyanate)- conjugated IgG isotype control for control fluorescence, 0,05 x$10^6$ cells with FITC conjugated anti-CD3 mAb for analysing the purity of isolated T cell fraction and 0,05 x$10^6$ cells with FITC conjugated anti-CD14 mAb for analysing the purity of isolated monocyte/macrophage fraction. Both T cell and monocyte fractions were stained with all of the mentioned Abs. Cells ( á 0,05 x$10^6$) were pipeted in a volume of 200 µl to FACS (fluorescence-activated cell sorter) tubes and 10 µl of the appropriate antibodies were added to each tube. Then cells were incubated for 30 min and washed twice with 2 ml filtered FACS buffer (2% BSA and 0,01% $NaN_3$ in PBS) in 400g for 5 min. Finally cells were fixed in a solution containing 1% formaldehyde and PBS in a final volume of 400 µl. Cells were kept on ice and covered with aluminium foil during the procedure. Aluminium foil was used for protecting the fluorescent dye from fading. Stained cells were analyzed using FACS (fluorescence-activated cell sorter, Beckman Coulter). MACS-isolated monocyte and T cell populations contained >90% $CD14^+$ and $CD3^+$, respectively.

#### 4.8.2 Intracellular staining with anti-IL-19 antibodies

Samples used in the experiment were isolated PBMC and SFMC of patients with RA or PBMC of healthy donors. FACS staining procedure was as follows: 0,5 x$10^6$ cells were pipeted into each FACS tube and washed with 1 ml FACS buffer (2% BSA and 0,01% $NaN_3$ in PBS). Next, 10 µl of FITC conjugated antibodies were added for cell surface staining (see appendix for pipeting scheme) and incubated for 30 min in the dark. Cells were washed with 1 ml PBS and again with 1 ml FACS buffer, and then they were fixed with 10 min incubation in 500 µl of 4% paraformaldehyde at RT. The cells were then

washed twice with 2 ml PBS and once with 1 ml FACS buffer containing 0,1% saponin (permeabilization buffer; Sigma) to permeabilize the cell membrane. Next, 10 µl of phycoerythrin (PE)-conjugated anti-IL-19 mAb (R&R Systems) or mouse anti-$IgG_{2b}$ isotype control (R&R systems) were added to each tube and incubated in the dark. Finally, cells were washed twice with permeabilization buffer and once with FACS buffer and analyzed by FACS in a volume of 400 µl FACS buffer.

### 4.9 Stimulation of cells with IL-19

In order to study the effects of IL-19, isolated mononuclear cells were used. For the purposes of this study, PBMC and SFMC of patients with RA or PBMC of healthy donors were isolated. Depending on the amount of collected cells, 0,88 – 2 x $10^6$ cells were cultured over night in culture medium (see 4.5) in a 24-well plate. Cells were stimulated with 100 ng/ml recombinant human IL-19 (PeproTech, London, UK) in the presence or absence of 0,1-1 µg/ml LPS or cells were left untreated and used as a control. LPS is known to stimulate the expression of many cytokines in mononuclear cells and serves as a control for the possible changes induced by IL-19. By stimulating cells with both IL-19 and LPS, it is also possible to determine whether these stimuli potentate each other. Following incubation, culture medium was transferred to 1,5 ml tubes, centrifuged and the supernatants were stored in -70°C.

### 4.10 ELISA

ELISA (enzyme-linked immunosorbent assay) is used for rapid screening and quantification of a specific protein in a sample. The principle of the technique is to use an antibody to detect the protein of interest, and then a secondary antibody that has been linked to an enzyme that forms a coloured product. The intensity of the colour can be measured by a microtiter plate reader. The colour formation of each sample is directly proportional to the concentration of the protein of interest in the sample. Thus, by using a series of dilutions with known protein concentrations, it is possible to determine the concentration of the protein of interest in each sample. (Nelson&Cox, 2000).

ELISAs for IL-1β, IL-6, IL-10 and TNF-α cytokines were performed for culture medium obtained from IL-19- and LPS-stimulated cultures described in chapter 4.9. PeliKine Compact™ human ELISA kits (Sanquin) were used according to manufacturer's instructions. The PeliKine Compact™ human ELISA kits are based on sandwich-type of enzyme immunoassay. First, a monoclonal anti human IL-1β, IL-6, IL-10 or TNF-α anti-

body is bound onto polystyrene microtiter wells of a 96-well plate. Further binding of unwanted protein on the surface of the well is prohibited by the use of dry milk powder containing blocking buffer. Next, human IL-1β, IL-6, IL-10 or TNF-α that is present in a sample or standard is captured by the antibody on the ELISA plate. Another biotinylated monoclonal antibody to human IL-1β, IL-6, IL-10 or TNF-α is added, and this antibody binds to the antibody-antigen complex present on the ELISA plate. In the following step, horseradish peroxidase (HRP) conjugated streptavidin is added to each well and it binds the biotinylated side of the formed sandwich. A coloured product is formed after addition of substrate for HRP, and the amount of coloured product is directly proportional to the amount of IL-1β, IL-6, IL-10 or TNF-α present in the sample or standard. The reaction has to be terminated by a stop solution containing 10% sulphur acid, and absorbance is measured in a microtiter plate reader. The concentration of IL-1β, IL-6, IL-10 or TNF-α can be determined by interpolation with the standard curve. Washing between each step is done using PBS or PBS containing 0,005% tween20. All the reagents should be at room temperature when applied to the procedure. The absorbance of the wells is read at 450 nm for IL-1β, IL-6, IL-10 and TNF-α in Multiscan Ascent microtiter plate reader (Thermo Labsystems)**.** Each cytokine was measured in a separate plate, but the procedure was done at the same time for each sample to minimize deviation in protein concentrations due to external sources of errors The detection limit is 0,8 pg/ml for IL-1β, 1,2 pg/ml for IL-6, 2,4 pg/ml for IL-10 and 2,8 pg/ml for TNF-α (Sanquin ELISA kit instruction).

### 4.11 Statistical analysis

Statistical analysis was performed using a non-parametric Mann-Whitney U test.

# 5. RESULTS

## 5.1 The majority of IL-10 family cytokines are expressed in RA samples

IL-10 is an important anti-inflammatory cytokine. Its expression pattern in RA has been studied in detail (e.g. Isomäki et al., 1996) and it is clearly up-regulated in RA. Since it possesses immunosuppressive properties, use of recombinant IL-10 as a therapy in RA has also been suggested though not pursuit forward at present (van Roon et al., 2003). However, the present knowledge on the expression of other members of the IL-10 family cytokines in RA is very limited. Efforts to understand the pathogenesis of RA includes knowledge on the expression levels of various cytokines in RA. We therefore wanted to examine whether IL-19, IL-20, IL-22, IL-24 and IL-26 can be detected in PB, SF and synovial tissue samples from patients with RA and whether their expression would be different to that in healthy volunteers. Since IL-10 is known to be upregulated in RA, it was included as a positive control.

RT-PCR analysis of three synovial tissue samples and paired PBMC and SFMC samples from three patients with RA was initially performed. Most of the tested

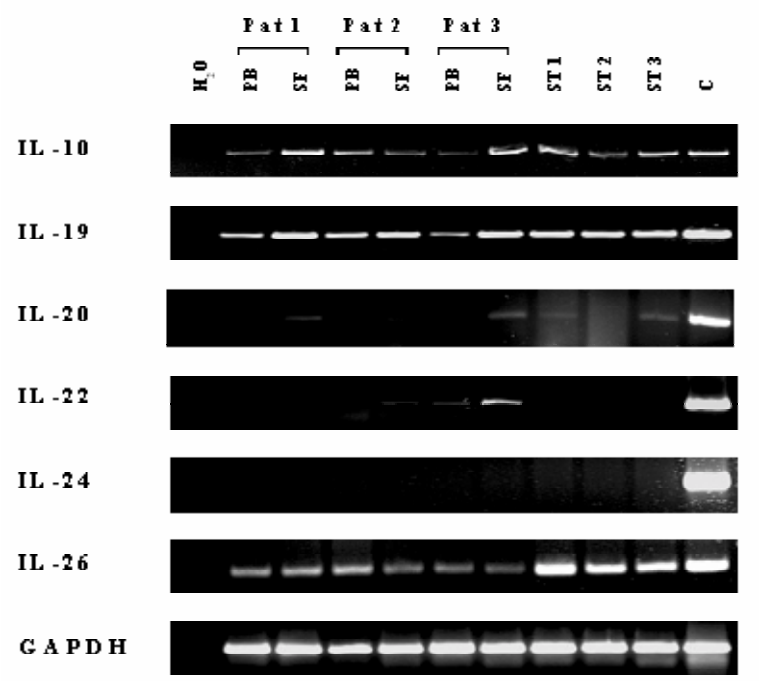



**Figure 7. RT-PCR test panel of three synovial tissue samples (ST) and three paired PBMC and SFMC samples (PB/SF) from patients with RA. $H_2O$ as a negative control and positive controls (C) are included in the panel.**

cytokines were detected in RA samples. IL-10 and IL-19 showed to be most prominently expressed and significant levels were detected in both PB, SF and synovial tissue samples (figure 7). Also, there was a tendency for higher expression of these cytokines in the synovial tissue and SF samples when compared with the PB samples. IL-20 was detectable only in a single SF sample. IL-22 was expressed at low levels in PB and SF samples, but not in the synovial tissues, whereas IL-26 was primarily expressed in the synovial tissue samples. IL-24 was negative in all of the tested samples, and therefore was excluded from further analysis. Together, these preliminary data imply that IL-10 and IL-19 are expressed at significant levels in RA patients, while the expression of IL-20, IL-22 and IL-26 is more restricted.

### 5.2 Elevated levels of IL-10 and IL-22 in the peripheral circulation of patients with RA

On the basis of the small-scale RT-PCR test panel results, a more detailed and quantitative analysis of the IL-10 family cytokines in RA was pursued by using real time RT-PCR. Immune cells, especially T cells and monocytes/macrophages have been thought to have a significant role in the induction and maintenance of RA (Tran et al., 2005). We therefore first investigated the expression profile of IL-10 family cytokines in isolated PBMC, which consists mostly of T cells and monocytes. The expression levels of cytokines in PBMC from patients with RA and healthy donors were compared.

IL-10 was the only cytokine that was expressed in all RA samples, and the expression levels were prominently higher in RA patients than in controls ($p= 0.0068$). A P-value below 0,05 is considered to be statistically meaningful. This finding is in accordance with previously published studies on IL-10 expression in RA (Isomäki et al., 1996). IL-19 and IL-20 were observed in seven and four out of ten patient samples studied, respectively (figure 8). IL-26 could only be detected in two patients. The mean levels of IL-19, IL-20 and IL-26 were not considerably different between RA patients and controls. The difference in IL-22 expression between RA samples and controls, however, was statistically significant ($p=0,0039$); IL-22 was detected in PBMC from eight patients with RA and only in one control PBMC. However, it is noteworthy that the mean level of IL-22 expression in RA PBMC was clearly lower than that of IL-10 or IL-19.

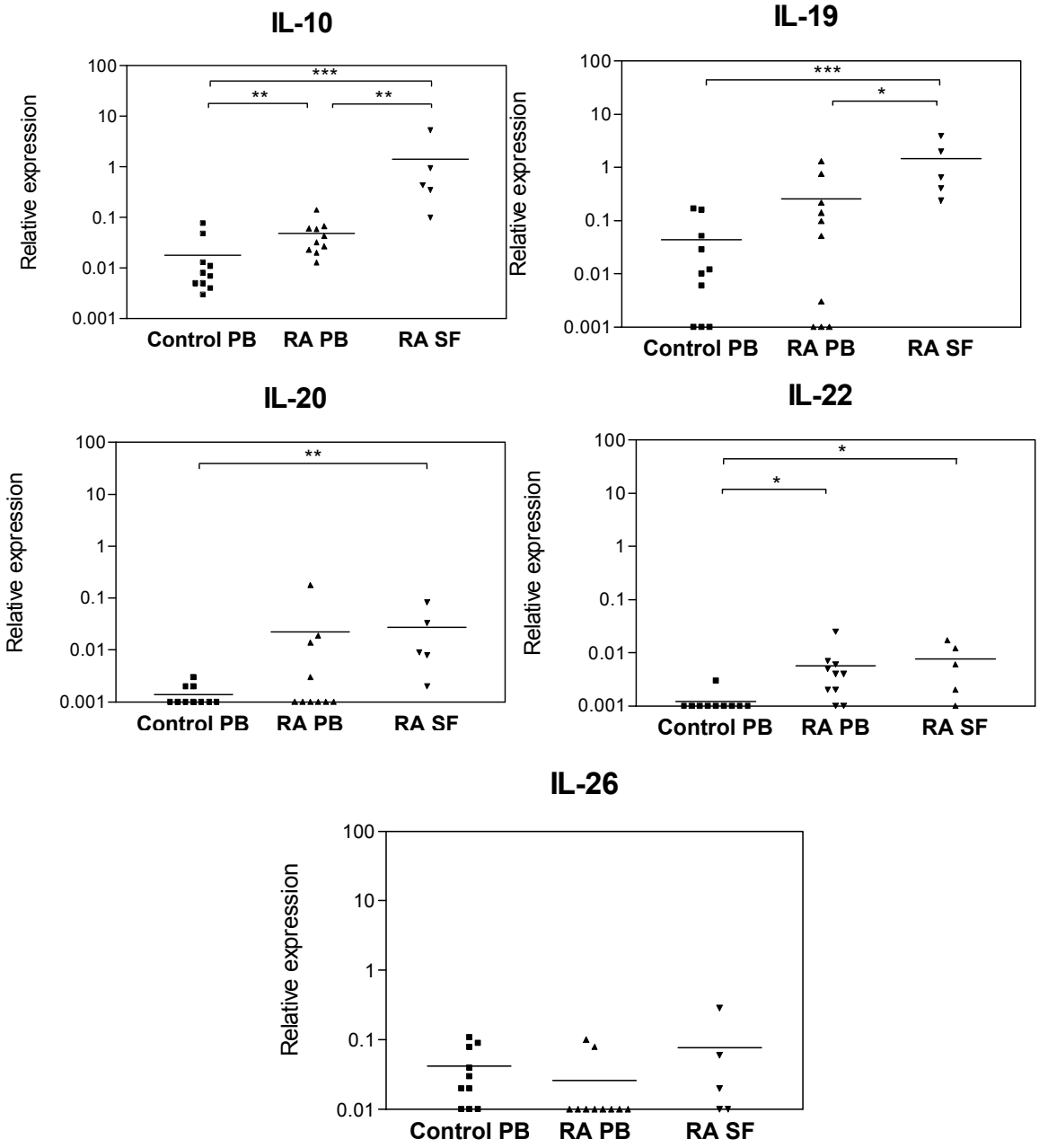



**Figure 8. Expression of IL-10 family cytokines in PBMC and SFMC samples.**

### 5.3 IL-10 and IL-19 are upregulated in SFMC

Next, we studied the expression of IL-10 family cytokines in SFMC derived from five RA patients. These results were compared with PBMC from RA patients and healthy volunteers. The expression levels of IL-10 were prominently higher in SFMC than in PBMC (fig. 8). Similar to IL-10, IL-19 levels were also significantly elevated in SFMC when compared to PBMC from ether RA patients or healthy volunteers (fig. 8). The levels of IL-20, IL-22 and IL-26 were not clearly different between PBMC and SFMC from RA patients. However, when compared with PBMC from healthy volunteers, SFMC from RA patients demonstrated elevated levels of IL-20 and IL-22 (see figure 8).

### 5.4 Increased expression of IL-19 in RA synovial tissues

Based on the results so far the presumption was that certain cytokines, such as IL-10 and IL-19, were expressed at a higher level in the synovium than in the peripheral circulation. Subsequently, to compare the synovial expression levels of IL-10 family cytokines between RA patients and patients with a non-inflammatory joint disease, their expression was studied in synovial tissue samples derived from ten patients with RA and OA. Synovial tissues had been obtained from the patients during ortopaedic operations as described in chapter 4.1.

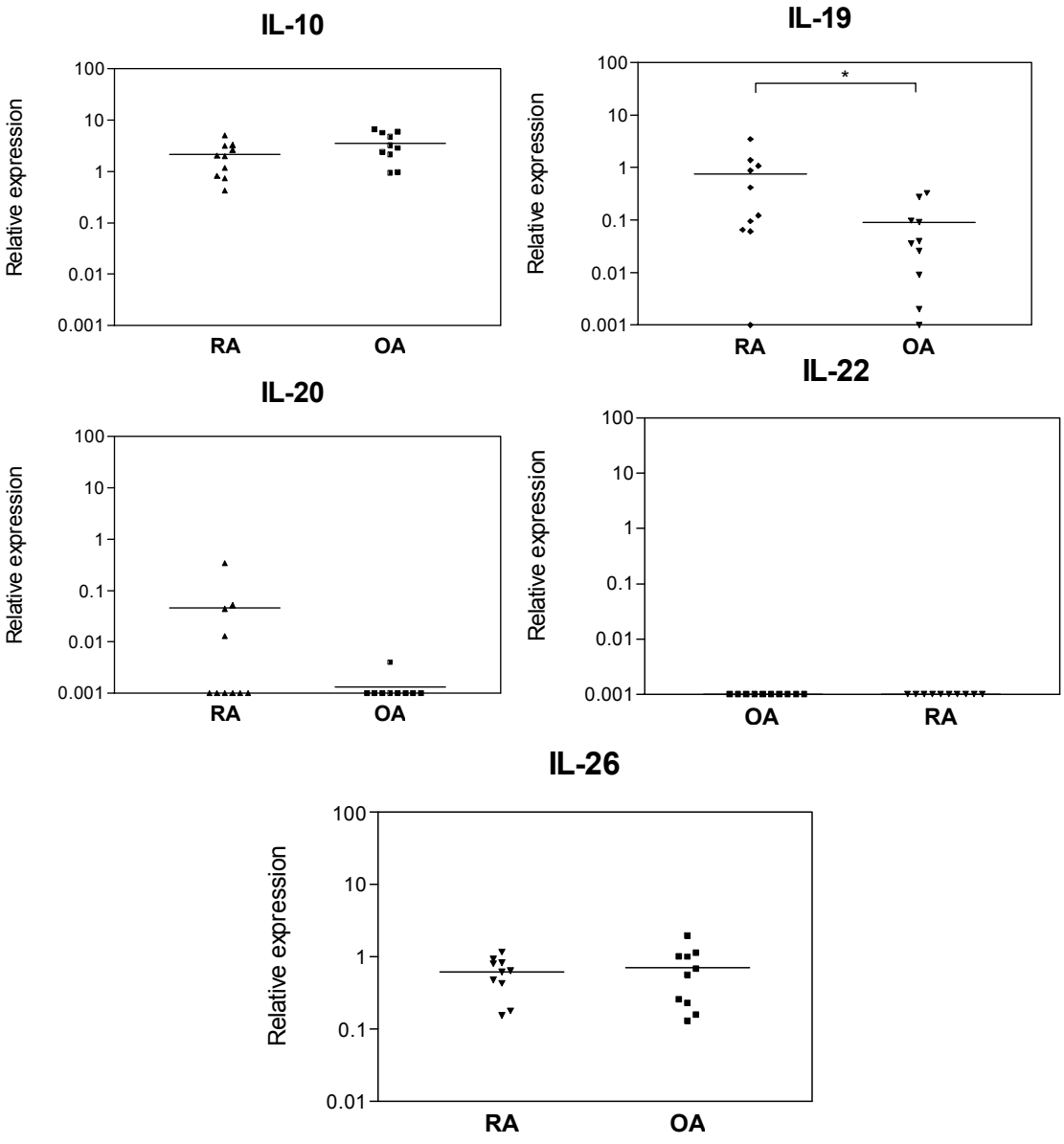



**Figure 9. Expression of IL-10 family cytokines in synovial tissue samples.**

Significant quantities of IL-10, IL-19 and IL-26 were present in the synovial tissues from patients with RA (fig. 9). This expression was not specific for RA, since these cytokines were also detected in the synovium of OA patients. However, the levels of IL-19 were significantly higher in RA than in OA synovium with a p-value of 0,036. IL-20 could be detected only in 4 out of 10 synovial tissue samples from patients with RA. IL-22 was not detected ether in the synovial samples from patients with RA or in those of OA patients, which is consistent with the initial results from RT-PCR analysis.

### 5.5 Macrophages as primary source for IL-19 expression in RA joints

Since IL-19 was clearly up-regulated in the synovium we wanted to see which immune cells were primarily responsible for the elevated expression of this cytokine. Thus we studied the expression of IL-19 in T cell and monocyte/macrophage cell fractions from RA patients and healthy volunteers. A variable amount of 5-9 samples from PB or SF T cells or monocytes/macrophages were obtained from patients with RA and the results from these samples were compared to those from eight PB T cell or monocyte samples from healthy volunteers. The rise in IL-19 mRNA production was most clearly seen in SF macrophages with a statistically significant difference compared to PB monocytes from RA patients ($p=0.0013$) or healthy donors ($p=0.0007$) (see figure 10). However, the expression of IL-19 was also upregulated in SF T cells when compared with PB T cells, but the expression level of IL-19 was clearly lower than in SF macrophages. The primary source for IL-19 in the synovium of RA patients therefore seems to be macrophages, which is consistent with previous findings (Wolk et al., 2002).

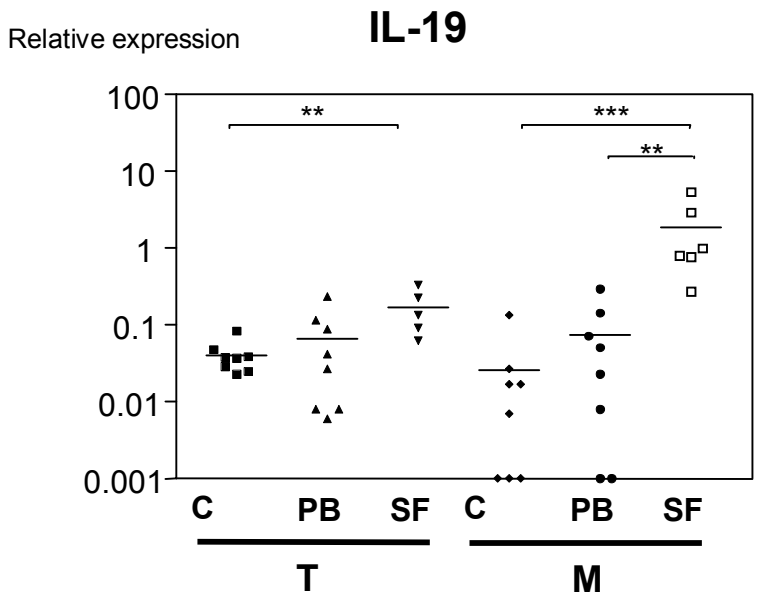



**Figure 10. Expression of IL-19 mRNA in cell fractions; T = T cells, M = monocytes/macrophages, C = healthy PB control, PB = RA PB, SF = RA SF**

### 5.6 Intracellular IL-19 protein expression

We found IL-19 mRNA to be upregulated in synovial samples. However, mRNA levels of cytokines do not always correlate with the protein levels of the same cytokines. Since protein expression is necessary for the biological actions of cytokines, we wanted to confirm that significant levels of IL-19 protein are produced in RA joints. We decided to use intracellular flow cytometry for these purposes. First, we studied the expression of IL-19 protein in PBMC from eight healthy volunteers. Due to methodological problems we were able to study IL-19 expression in CD14 positive cells in only five of these samples. Low level of IL-19 protein expression was detected, and there were no differences between T cells and monocytes (see figure 11). Next, the expression of IL-19 protein in T cells from seven RA patients and in monocytes from five RA patients was studied. The expression of IL-19 protein in RA differed highly between individual samples. However, IL-19 expression was elevated in both PB and SF T cells and macrophages from RA patients when compared to controls. The differences were statistically significant for all other comparisons except for IL-19 levels in PB monocytes between RA patients and controls (p=0,07).

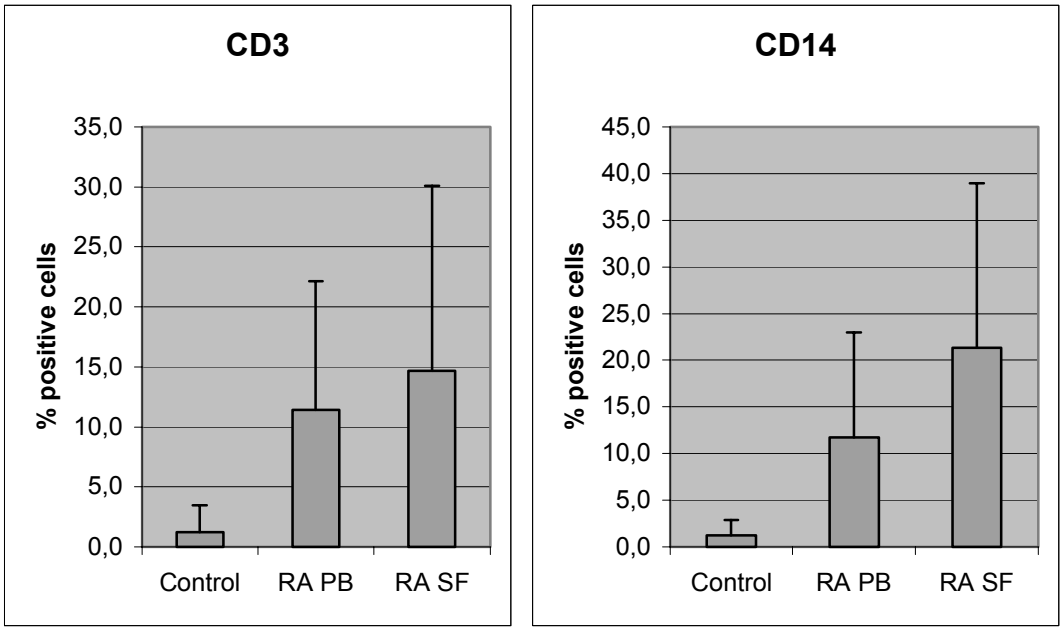



**Figure 11. IL-19 intracellular protein expression**

### 5.7 Stimulation of immune cells with IL-19

Since IL-19 was elevated in RA joints, we were interested to study the possible effects that IL-19 might have on the expression of other cytokines in the synovium of patients with RA. Thus, we stimulated PBMC and SFMC from patients with RA or PBMC from healthy volunteers with IL-19 *in vitro* and measured the amount of IL-1β, IL-6, IL-10 and TNF-α produced in these cultures by ELISA. Results obtained with SFMC expression are not shown, for we were able to collect only two samples and sample number must be further increased to attain reliable results. The sample number for normal PBMC was six and for RA PBMC three (for IL-1β, IL-10 and TNF-α) or four (for IL-6).

In RA patients, IL-19 increased the expression of IL-1, IL-6 and IL-10 slightly when compared to unstimulated controls, but there were high individual variations and the increase was not seen in all patients (see figure 12). In addition, cytokine levels following IL-19 stimulation were very low when compared to those induced by LPS. IL-19 did not markedly affect LPS-induced cytokine production when samples were stimulated by both LPS and IL-19. It thus seems that IL-19 does not have significant effects on the production of IL-1, IL-6, IL-10 or TNF-α by mononuclear cells.

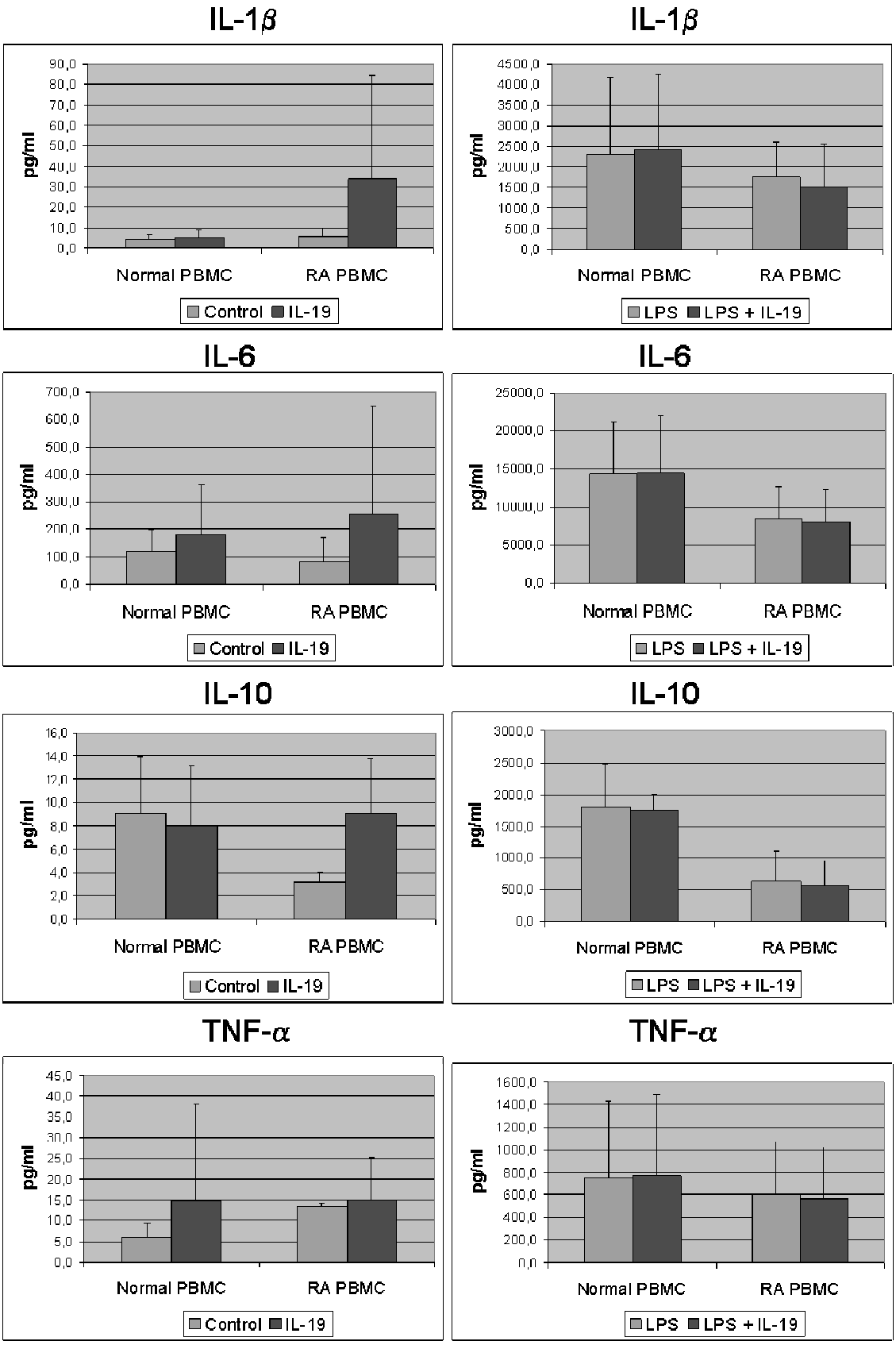



**Figure 12. Result from ELISA. Reader should notice the different scales of Y-axis on the right and left panels.**

### 5.8 The expression of receptors for IL-10 family cytokines in RA

The receptor subunits for IL-10 receptor, IL-10R1 and IL-10R2, have been found to be expressed by immune cells in considerable amounts. Nonetheless, the other receptor subunits for IL-10 family cytokines were for the most part not found in these cell types. (Wolk et al., 2002). However, inflammatory processes may alter the expression of receptor subunits and lead to upregulation of these receptors; this has been shown in RA for IL-22R1 (Ikeuchi et al., 2005). Therefore the expression of receptors for IL-10 family cytokines was studied using quantitative real-time PCR in PBMC, SFMC and synovial tissue samples from patients with RA using samples from healthy volunteers and patients with OA as controls. Initial small-scale testing was conducted using conventional RT-PCR to confirm that these gene products can be detected in RA samples. Since we were unable to show IL-20R1 expression with RT-PCR it was excluded from further studies.

As expected, the functional IL-10 receptor subunits IL-10R1 and IL-10R2 were expressed at significant numbers in all cellular compartments studied (figure 13). Also IL-20R2 and IL-22R1 were detected in all samples. The levels of expression between RA patients and controls (OA patients vs. RA patients; healthy volunteers vs. RA patients) were similar for IL-10R1, IL-10R2 and IL-20R2 receptor subunits. For IL-22R1, the expression level in synovial tissues was similar between patients with RA and OA. In contrast, IL-22R1 expression was upregulated in PBMC from patients with RA when compared with healthy donors. However, this upregulation was not observed in SFMC when compared with PBMC.

Since our results suggested that both IL-22 and the cytokine binding subunit of its receptor (IL-22R1) were elevated in PBMC from patients with RA, we decided to include also the naturally occurring inhibitor of IL-22, IL-22BP, in our expression studies. The levels of IL-22BP in PBMC from patients and healthy donors were undetectable, while SFMC showed clearly elevated levels of this antagonist. In the synovial tissues IL-22BP was found to be negative in the majority of samples, but rather high levels were detected in synovial samples from four RA patients (figure 14).

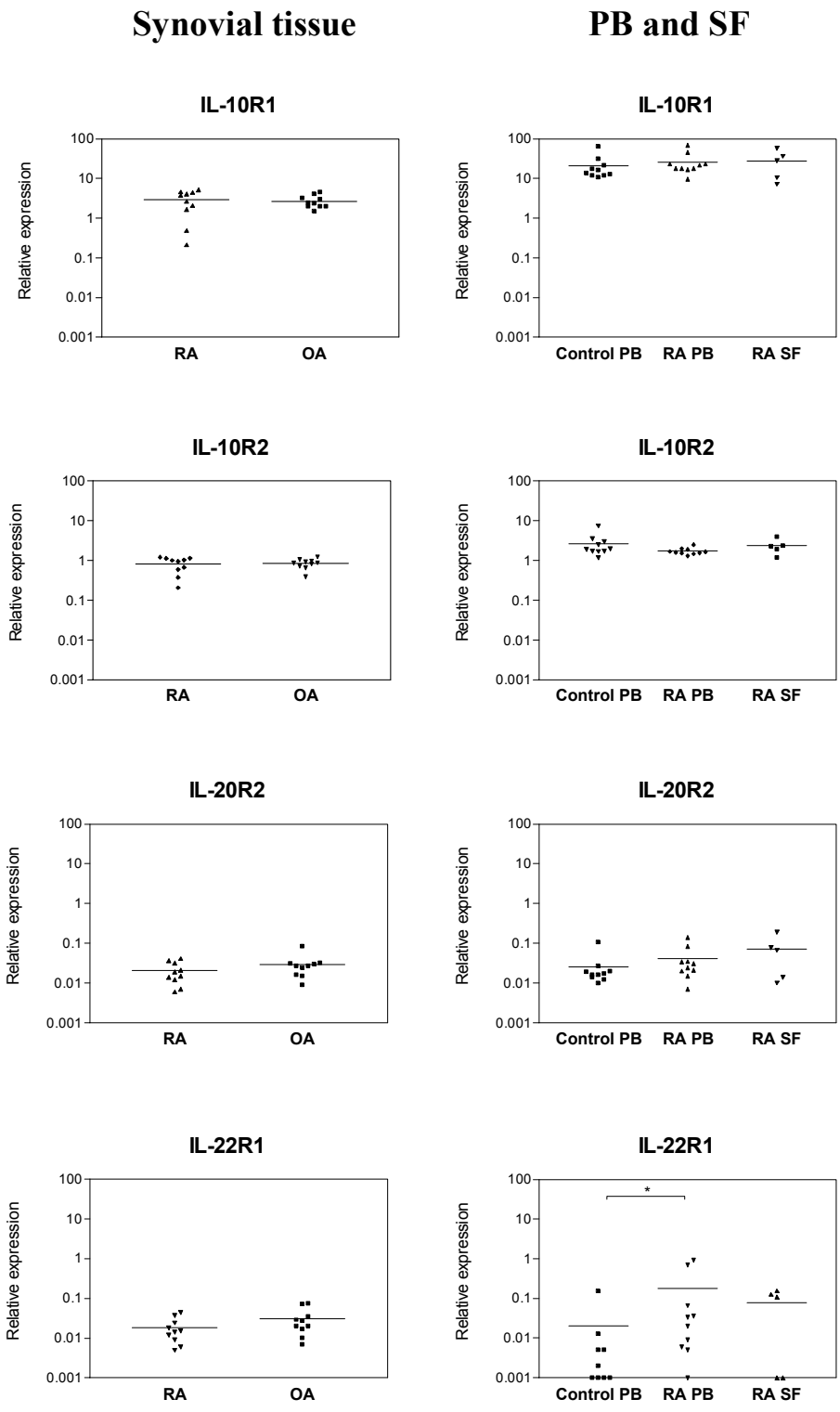
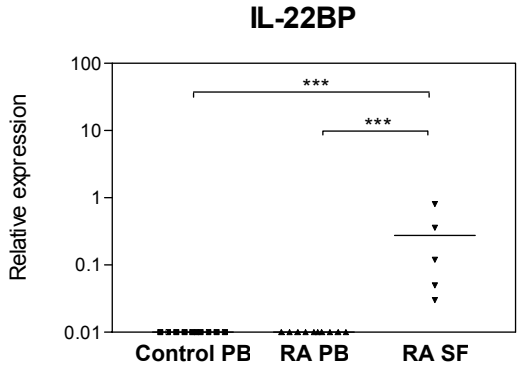



**Figure 13. Expression of receptors for IL-10 family cytokines.**

**PB and SF**




**Synovial tissue**

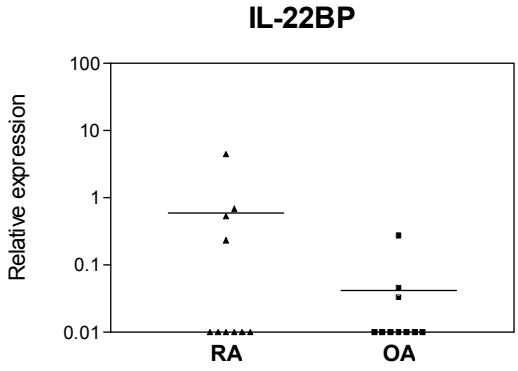



**Figure 14. IL-22BP expression**

# 6. DISCUSSION

IL-10-family of cytokines is a relatively newly characterized protein family. So far, the expression of certain individual members has been studied in RA. This is the first thorough study on the expression profile of all IL-10 family members and their representative receptor subunits in patients with RA.

Upregulation of IL-10 in RA has been previously shown in many studies. We could also clearly demonstrate this in our study. The expression of IL-10 was high in both peripheral circulation and the joints of patients with RA. However, the level of expression was similar in synovial tissues from RA and OA patients. OA is not an autoimmune disease, but some degree of inflammation is present in the joints of patients with OA. The characteristics of OA include progressive breakdown of articular cartilage that is associated with inflammation, and also osteophyte formation and joint deformity (Glass, 2006). This finding that the level of IL-10 is similar in both RA and OA is interesting. RA patients used in this study had long-lasting, rather inactive disease as suggested by low level of CRP in these patients (see table 3). In addition, RA patients were on disease-suppressing medication. Furthermore, even though the blood CRP levels of patients with OA were rather low, the mean macroscopic synovitis score determined by the operating surgeon was similar between RA and OA patients (table 3). Thus, the selection of patients may partly explain the finding that similar levels of IL-10 were observed in patients with RA and OA. Therefore, future studies utilizing synovial tissues from actively inflamed joints of RA patients are needed to examine whether more clear differences in IL-10 levels between patients with RA and OA can be observed.

We found IL-19 to be upregulated in SFMC and synovial tissues from patients with RA at transcriptional level. In addition, we found macrophages to be the principle producers of IL-19 in the RA joints. In contrast to these findings, we concluded that IL-19 is not upregulated in PBMC. According to our results, IL-19 mRNA levels were similar both in T cells and monocytes and between controls and patients with RA in peripheral circulation. At the protein level, IL-19 production was upregulated both in T cells and macrophages from the joints of patients with RA, even though macrophages were clearly the principle producers. In contrast to the findings obtained with IL-19 mRNA expression, IL-19 protein was upregulated also in the peripheral circulation of

RA patients. The level of IL-19 produced by healthy controls was insignificant. There were thus differences when comparing the findings at protein level or mRNA levels. This could imply that IL-19 is post-transcriptionally regulated.

IL-19 functional receptor is composed of IL-20R1 and IL-20R2 subunits. However, we did not detect IL-20R1 expression in our samples. Since cytokines generally function in a paracrine manner, it seems that there should be a functional IL-19 receptor in the RA synovium if IL-19 expression in RA is relevant *in vivo*. Other members of the IL-10 family cytokines have been shown to signal through more than one functional receptor. These include e.g. IL-20 and IL-24. However, the receptor complexes that these cytokines utilize have not been connected to IL-19 signaling. A possibility is that a so far unidentified receptor subunit or a completely new functional receptor is involved in IL-19 signaling in the RA synovium. It is also possible, that the IL-20R1/IL-20R2 receptor is expressed in a relatively small cell population and its expression cannot be detected in mixed cell populations.

According to a study by Jordan et al. (2005), IL-19 induces the production of anti-inflammatory IL-10 by human monocytes. Furthermore, Liao et al. have found IL-19 to stimulate the production of IL-6 and TNF-α in mouse splenic monocytes (2002). However, we could not reproduce these results in our stimulation studies: IL-19 did not induce the production of IL-1, IL-6, IL-10 or TNF-α by PBMC *in vitro*. We used 10 ng/ml of IL-19 in our experiment, which was around ten times higher than the concentration of IL-19 used by Jordan et al. in their experiment. However, we used PBMC and they used purified monocytes, suggesting that the initial response induced by IL-19 could be modified and downregulated by other cell types that were present in our cultures. On the other hand, in the experiment by Liao et al, the induction of the cytokines was clearly seen only after using a relatively high concentration of IL-19 (100 ng/ml for IL-6 and 25-100 ng/ml for TNF-α).The concentration we used is closer to the possible physiological concentrations of IL-19 in rheumatoid joint. In addition, Liao et al. used mouse splenic monocytes in the experiment, and it is clear that the effects of IL-19 may differ between species. For example, Liao et al showed IL-19 to induce apoptosis in murine monocytes, but this effect was not seen in human peripheral monocytes in the study by Jordan et al. (2005). Also, in support of our findings, Jordan et al. did not detect upregulation of TNF-α production in peripheral blood monocytes. Nevertheless, the possible role that IL-19 may play in the pathogenesis of RA is unknown at present, and further functional studies are needed to address this issue.

In our study, we detected increased amounts of IL-20 in SFMC of patients with RA, and in a proportion of PBMC and synovial tissue samples. Using our Q-RT-PCR, a significant proportion of samples remained below the detection limit. Whether these samples were true negative or whether the detection limit of PCR was too high for these purposes is not known. By fine-tuning the procedure, it might be possible to elicit more impressive results. However, this was not possible in the confinements of this master's thesis project. Previously, IL-20 protein has been shown to be upregulated in SF and synovial membrane of patients with RA by Hsu et al (2006). As a distinction from the results produced by Hsu et al. with ELISA and immunohistochemistry, we used Q-PCR to detect differences at transcriptional level instead of measuring the produced protein. Nevertheless, our present results are in accordance to those obtained by Hsu et al. Together, these results indicate upregulation of IL-20 production in RA joints, although the levels of IL-20 seem to be lower than those of IL-10 or IL-19.

As with IL-19, the possible role of IL-20 in RA pathogenesis is still unknown. Earlier findings have shown IL-20 to induce the expression of pro-inflammatory TNF-α in psoriasis (Wei et al., 2006). It has also been suggested that the pro-angiogenetic characteristics of IL-20 could provide the hypertrophic synovium in RA sufficient transport of oxygen and nutrients and a route for the inflammatory cells to reach the joint (Hsu et al., 2006). According to our results, IL-20 may signal via IL-22R1/IL-20R2 receptor complex in synovial cells. In contrast to our results, Hsu et al. could also show IL-20R1 expression in synovial membrane of patients with RA. It is therefore possible that IL-20 may signal via two distinct receptors in RA joints.

According to our results, IL-22 was also upregulated in PBMC and SFMC of patients with RA. In contrast, we did not detect IL-22 mRNA in the synovial tissues of patients with RA or OA. IL-22 has been previously shown to be upregulated in RA joints by Ikeuchi et al. (2005).They used conventional RT-PCR with PBMC from normal donors as a negative control to detect IL-22 in the SFMC and synovial tissues of patients with RA, but did not study the expression of IL-22 in PB. In complete contrast to our studies, Ikeuchi et al. demonstrate expression of IL-22 protein in the rheumatoid synovium by immunohistochemistry. According to them IL-22 is present in both the synovial lining and the sublining layers, and synovial fibroblasts and macrophages are the predominant producers of this cytokine. Although the expression of mRNA and protein are independently regulated, it is difficult to understand how significant amount of IL-22 protein

could be present in the synovial membranes in the absence of IL-22 mRNA. Therefore, further studies are clearly needed to clarify these differential results.

Functional studies that Ikeuchi et al. have conducted suggest increased proliferation and chemokine production by RA synovial fibroblasts in response to treatment with IL-22. Both their study and the present study show that IL-22R1 is expressed in RA joints. IL-22 could thus be involved in maintaining the hypertrophic state of the RA synovium that contributes to the progression of the disease. However, we found the natural antagonist of IL-22, IL-22BP, to be upregulated in the SF of patients with RA. The expression level of IL-22BP was slightly higher in the synovial tissues in RA when compared to OA, but the change was not statistically significant. Nevertheless, our results suggest that the effects of IL-22 in RA joints may be, at least in part, blocked by its naturally occurring antagonist.

We did not detect IL-24 expression in our early preliminary experiments with RT-PCR, so our conclusion was that IL-24 is not expressed in RA joints. According to some studies, IL-24 does induce the expression of several pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α (Pestka et al., 2004), which are also important in the pathogenesis of RA. On the basis of the present study, this cytokine does not seem to contribute to the progression of RA.

The expression of IL-26 was not altered between healthy controls or patients with RA or OA. In the synovial tissues, there was a rather high constitutive expression of IL-26 in both RA and OA. The expression level in PB and SF was lower. In fact, most of the PB samples from patients with RA were negative for IL-26, but there was no statistically meaningful difference between the different sample groups. The functional receptor for IL-26 is composed of IL-20R1 and IL-10R2 subunits, and we did not detect IL-20R1 in our samples. IL-26 functional receptor IL-20R1/IL-10R2 has been recently described (Hör et al., 2004), so it seems likely that it could also signal through some other unknown functional receptor. The characterization of the function of IL-26 is still incomplete, but it does seem to have a role in immune responses, especially when it comes to viral infections. Many viruses have been suspected to be involved in the onset of RA, so a connection between a cytokine involved in viral immunity and RA would be interesting. IL-26 gene has been shown to be situated in a region that has been suspected to be involved in autoimmune diseases, such as RA. However, out results do not seem to support this view, at least when it comes to possible alterations in IL-26 expression.

The relatively newly discovered IL-10 family cytokines IL-19, IL-20, IL-22, IL-24 and IL-26 are still poorly characterized both in regard to source, target cells and function. Many of the functions that so far have been attributed to these cytokines could potentially be important in the regulation of rheumatoid inflammation. Clearly more studies need to be conducted to assess the possible significance of these cytokines in the pathogenesis of RA.

# 7. CONCLUSIONS

We identified LPS- and PHA-stimulated PBMC as suitable positive controls for RT-PCR detecting IL-10 family cytokines. Also, we identified skin cDNA as a proper positive control for RT-PCR measuring the expression of the IL-10 family cytokine receptor subunits and the IL-22 antagonist IL-22BP.

Most of the IL-10 family cytokines and their receptor subunits can be detected in RA. In the peripheral circulation of patients with RA, IL-10 and IL-22 are upregulated. In the synovial fluid, IL-10, IL-19, IL-20 and IL-22 are increased. However, the expression levels of IL-10 and IL-19 are considerably higher when compared to those of IL-20 or IL-22. IL-19 is upregulated also in the synovial tissue of patients with RA.

The principle producers of IL-19 mRNA and protein are macrophages, although significant amounts of IL-19 protein are also produced by T cells. IL-19 does not induce the production of the major pro-inflammatory cytokines IL-1, IL-6 or TNF-α or the anti-inflammatory cytokine IL-10 by PBMC.

# 8. REFERENCES


Akil M, Amos RS. ABC of rheumatology Rheumatoid arthritis--I: Clinical features and diagnosis. BMJ 1995;310:587-590.

Antoniv TT, Ivashkiv LB. Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages. Arthritis Rheum 2006;54:2711-2721.

Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K, Brown C, Cooke TD, Daniel W, Feldman D. The American college of rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991;34:505-514.

Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American rheumatism association. Arthritis Rheum 1986;29:1039-1049.

Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS. The American rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-324.

Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HRH. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 2005;5:209-219.

Blumberg H, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S, Eagan M, Foster D, Haldeman BA, Hammond A, Haugen H, Jelinik L, Kelly JD, Madden K, Maurer MF, Parrish-Novak J, Prunkard D, Sexson S, Sprecher C, Waggie K, West J, Whitmore TE, Yao L, Kuechl MK, Dale BA, Chandrasekher YA. Interleukin 20: discovery, receptor identification, and role in epidermal function. Cell 2001;104:9-19.

Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 2000:12:64-76.

Boniface K, Bernard FX, Garcia M, Gurney JCL, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 2005;174:3695-3702.

Borigini MJ, Paulus HE. Rheumatoid arthritis. In: Weisman MH, Weinblatt ME, Louie JS (eds), Treatment of the rheumatic diseases: companion to Kelley's textbook of rheumatology, W.B. Saunders company, Philadelphia, 2001, pp. 217-235.

Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkühn T, Göke B, Auernhammer CJ, Dambacher J. IL-22 is increased in active Crohn's disease and promotes proimflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2006;290:827-838.

Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23-39.

Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004;15:155-166.

Chang C, Magracheva E, Kozlov S, Fong S, Tobin G, Kotenko S, Wlodawer A, Zdanov A. Crystal structure of interleukin-19 defines a new subfamily of helical cytokines. J Biol Chem 2003;278:3308-3313.

Chen WY, Cheng BC, Jiang MJ, Hsieh MY, Chang MS. IL-20 is expressed in atherosclerosis plaques and promotes atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2006;26:2090-2095.

Conti P, Kempuraj D, Frydas S, Kandere K, Boucher W, Letourneau R, Madhappan B, Sagimoto K, Christodoulou S, Theoharides TC. IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26. Immunol Lett 2003;88:171-174.

Ding C, Cantor CR. Quantitative analysis of nucleic acids – the last few years of progress. J Biochem Mol Biol 2004;37:1-10.

Dumoutier L, Leemans C, Lejeune D, Kotenko SV, Renauld JC. Cutting edge: STAT activation by Il-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. J Immunol 2001;167:3545-3549.

Feldmann M. Pathogenesis of arthritis: recent research progress. Nat Immunol 2001;2:771-773.

Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85:307-310.

Ficoll-Paque PLUS for *in vitro* isolation of lymphocytes, Handbook from Amersham biosciences, 1-18.

Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-361.

Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990;33:768-773.

Fox DA. Etiology and pathogenesis of rheumatoid arthritis. In: Koopman WJ, Moreland LW (eds), Arthritis and allied conditions: a textbook in rheumatology, Lippincott Williams&Wilkins, Philadelphia, 2005, pp. 1089-1115.

Gabay G. Cytokines and cytokine receptors. . In: Koopman WJ, Moreland LW (eds), Arthritis and allied conditions: a textbook in rheumatology, Lippincott Williams&Wilkins, Philadelphia, 2005, pp. 423-475.

Gallagher G, Dickensheets H, Eskdale J, Izotova LS, Mirochnitchenko OV, Peat JD, Vazquez N, Pestka S, Donnelly RP, Kotenko SV. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun 2000;2:151-155.

Gallagher G, Eskdale J, Jordan W, Peat J, Campbell J, Boniotto M, Lennon GP, Dickensheets H, Donnelly RP. Human interleukin-19 and its receptor: a potential role in the induction of Th2 responses. Int Immunopharmacol 2004;4:615-626.

Glass GG. Osteoarthritis Dis Mon 2006;52:343-362.

Gupta P, Su ZZ, Lebedeva IV, Sarkar D, Sauane M, Emdad L, Bachelor MA, Grant S, Curiel DT, Dent P, Fisher PB. mda-7/IL-24; multifunctional cancer-specific apoptosis-inducing cytokine. Pharmacology & Therapeutics 2006;111:596-628.

Hale LP. Pathology of rheumatoid arthritis and associated disorders. In: Koopman WJ, Moreland LW (eds), Arthritis and allied conditions: a textbook in rheumatology, Lippincott Williams&Wilkins, Philadelphia, 2005, pp. 1117-1040.

Hsieh MY, Chen WY, Jiang MJ, Cheng BC, Huang TY, Chang MS. Interleukin-20 promotes angiogenesis in a direct and indirect manner. Genes Immun 2006;7:234-242.

Hsing, CH, Ho CL, Chang LY, Lee YL, Chuang SS, Chang MS. Tissue microarray analysis of interleukin-20 expression. Cytokine 2006;35:44-52.

Hsu YH, Li HH, Hsieh MY, Liu MF, Huang KY, Chin LS, Chen PC, Cheng HH, Chang MS. Function of interleukin-20 as a proimflammatory molecule in rheumatoid arthritis and experimental arthritis. Arthritis Rheum 2006;54:2722-2733.

Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 2005;6:279-284.

Hör S, Pirzer H, Dumoutier L, Bauer F, Wittmann S, Sticht H, Renauld JC, de Waal Malefyt R, Fickenscher H. The T-cell lymphokine interleukin-26 targets epithelial cells through the interleukin-20 receptor 1 and interleukin-10 receptor 2 chains. J Biol Chem 2004;279:33343-33351.

Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, Nojima Y. Expressio of interleukin-22 in rheumatoid arthritis. Arthritis Rheum 2005;52:1037-1046.

Isomäki H. Nivelreuma. In: Leirisalo-Repo M, Hämäläinen M, Moilanen E (eds), Reumataudit, Duodecim, Rauma, 2002, pp. 152-166.

Isomäki P, Luukkainen R, Saario R, Toivanen P, Punnonen J. Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium. Arthritis Rheum 1996;35:611-619.

Janeway CA Jr. How the immune system protects the host from infection. Microbes Infect 2001;13:1167-1171.

Jordan WJ, Eskdale J, Boniotto M, Lennon GP, Peat J, Campbell JDM, Gallagher G. Human IL-19 regulates immunity through auto-induction of IL-19 and production of IL-10. Eur J Immunol 2005;35:1576-1582.

Jotwani R, Pulendran B, Agrawal S, Cutler CW. Human dendritic cells respond to porphyromonas gingivalis LPS by promoting a Th2 effector response in vitro. Eur J Immunol 2003;33:2980-2986.

Kotenko SV. The family of IL-10-related cytokines and their receptors: related, but to what extent? Cytokine Growth Factor Rev 2002;13:223-240

Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75:263-274.

Lan RY, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev 2005;6:351-363.

Liao SC, Cheng YC, Wang YC, Wang CW, Yang SM, Yu CK, Shieh CC, Cheng KC, Lee MF, Chiang SR, Shieh JM, Chang MS. IL-19 induced Th2 cytokines and was up-regulated in asthma patients. J Immunol 2004;173:6712-6718.

Liao YC, Liang WG, Chen FW, Hsu JH, Yang JJ, Chang MS. IL-19 induces production of IL-6 and TNF-α and results in cell apoptosis through TNF-α. J Immunol 2002;169:4288-4297.

Lipsky PE. Rheumatoid arthritis. In: Kasper DL, Fauci AS, Longo DL, Braunwald E, Hauser SL, Jameson JH, Harrison TR, Resnick WR, Wintrobe MN, Thorn GW, Adams RD, Beeson PB, Bennett IL, Isselbacker KJ, Petersdorf RG, Wilson JD, Martin JB, Root R, Kasper DL, Longo DL, Jameson JL (eds), Harrison's principles of internal medicine, McGraw-Hill, USA, 2005, pp. 1969-1977.

Liu L, Ding C, Zeng W, Heuer JG, Tetreault JW, Noblitt TW, Hangoc G, Cooper S, Brune KA, Sharma G, Fox N, Rowlison SW, Rogers DP, Witcher DR, Lambooy PK, Wroblewski VJ, Miller JR, Broxmeyer HE. Selective enhancement of multipotential hematopoietic progenitors in vitro and in vivo by IL-20. Blood 2003;102:3206-3209.

Lubberts E, van der Berg WB. Cytokines in the pathogenesis of rheumatoid arthritis and collagen-induced arthritis. In: Santamaria P (eds), Cytokines and chemokines in autoimmune disease, Kluwer academic/Plenum publishers, New York, 2003, pp.194-202.

MACS CD14 MicroBeads instructions, Miltenyi Biotech.

Miltenyi S, Müller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990;11:231-238.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and he interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

Mosmann TR. Properties and functions of interleukin-10. Adv. Immunol. 1994;56:1-26.

Nagalakshmi ML, Murphy E, McClanahan T, de Waal Malefyt R. Expression patterns of IL-10 ligand and receptor gene families provide leads for biological characterization. Int Immunopharmacol 2004;4:577-592.

Nelson DL, Cox MM. Protein function. In: Nelson DL, Cox MM (eds), Lehninger principles of biochemistry, Worth publishers, New York, 2000, pp. 203-242.

O'Dell JR. Rheumatoid arthritis: The clinical picture. In: Koopman WJ, Moreland LW (eds), Arthritis and allied conditions: a textbook in rheumatology, Lippincott Williams&Wilkins, Philadelphia, 2005, pp. 1165-1193.

Oral HB, Kotenko SV, Yilmaz M, Mani O, Zumkehr J, Blaser K, Akdis CA, Akdis M. Regulation of T cells and cytokines by the interleukin-10(IL-10)-family cytokines IL-19, IL-20, IL-22, IL-24 and IL-26. Eur J Immunol 2006;36:380-388.

Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. J Biol Chem 2002; 277:29355-293558.

Pan T Cell isolation kit II instructions, Miltenyi Biotech.

Parrish-Novak J, Wenfeng X, Brenders T, Yao L, Jones C, West J, Brandt C, Jelinek L, Madden K, McKernan PA, Foster DC, Jaspers S, Chandrasekhers YA. Interleukins 19, 20, and 24 signal through two distinct receptor complexes. J Biol Chem 2002;277:47517-47523.

PeliKine Compact™ human IL-1β ELISA kit instructions, Sanquin.

PeliKine Compact™ human IL-6 ELISA kit instructions, Sanquin.

PeliKine Compact™ human IL-10 ELISA kit instructions, Sanquin.

PeliKine Compact™ human TNFα ELISA kit instructions, Sanquin.

Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. Annu Rev Immunol 2004;22:929-979.

Polndexter NJ, Walch ET, Chada S, Grimm EA. Cytokine induction of interleukin-24 in human peripheral blood mononuclear cells. J Leukoc Biol 2005;78:745-752.

Su Z, Emdad L, Sauane M, Lebedeva I, Sarkar D, Gupta P, James CD, Randolp A, Valerie K, Walter MR, Dent P, Fisher PB. Unique aspects of mda-7/IL-24 antitumor bystander activity: establishing a role for secretion of MDA-7/IL-24 protein by normal cells. Oncogene 2005;51:7552-7566.

Tran CN, Lundy SK, Fox DA. Synovial biology and T cells in rheumatoid arthritis. Pathophysiology 2005;12:183-189.

Vanderbroeck K, Cunningham S, Goris A, Alloza I, Heggarty S, Graham C, Bell A, Rooney M. Polymorphisms in the interferon-γ/interleukin-26 gene region contribute to sex bias in susceptibility to rheumatoid arthritis. Arthritis Rheum 2003;48:2773-2778.

van Roon J, Wijngaarden S, Lafeber FP, Damen C, van de wwinkel J, Bijlsma JW. Interleukin 10 treatment of patients with rheumatoid arthritis enhances Fc gamma

receptor expression on monocytes and responsiveness to immune complex stimulation. J Rheumatol 2003;30:648-651.

Wang M, Liang P. Interleukin-24 and its receptor. Immunology 2005;114:166-170.

Wei CC, Hsu YH, Li HH, Wang YC, Hsieh MY, Chen WY, Hsing CH, Chang MS. IL-20: biological functions and clinical implications. J Biomed Sci 2006;13:601-612.

Wilhelm J, Pingoud A. Real-time polymerase chain reaction. ChemBioChem 2003;4:1120-1128.

Wolk K, Kunz S, Asadullah K, Sabat R. Cutting edge: immune cells as sources and targets of the IL-10 family members? J Immunol 2002;168:5397-5402

Wolk K, Sabat R. Interleukin-22: a novel T- and NK-cell derived cytokine that regulates the biology of tissue cells. Cytokine Growth Factor Rew 2006;17:367-380.

Wolk K, Witte E, Reineke U, Witte K, Friedrich M, Sterry W, Asadullah K, Volk HD, Sabat R. Genes Immun 2005;6:8-18.

Wolk K, Witte E, Wallace E, Döcke W-D, Kunz S, Asadullah K, Volk H-D, Sterry W, Sabat R. IL-22 regulates the expression of genes responsible for antimicrobial defence, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. Eur J Immunol 2006;36:1309-1323.

Wong H, Lord JM. Factors underlying chronic inflammation in rheumatoid arthritis. Arch Immunol Ther Exp 2004;52:379-388.

Xu W. Interleukin-20. Int Immunopharmacol 2004 ;4 :627-633.

# APPENDIX

## Solutions for cell culture

| | |
|---|---|
| 1X HBSS | 1,258 mM $CaCl_2 * 2H_2O$<br>5,365 mM KCl<br>0,4408 mM $KH_2PO_4$<br>0,492 mM $MgCl_2 * 6H_2O$<br>0,406 mM $MgSO_4 * 7H2O$<br>0,137 mM NaCl<br>4,166 mM $NaHCO_3$<br>0,338 mM $Na_2HPO_4 * 2H_2O$<br>5,55 mM d-glucose<br>0,01 g/l Phenol Red<br>-Sterilize by filtering |
| 10X PBS | 1,37 M NaCl<br>0,027 M KCl<br>0,101 M $Na_2HPO_4 * 2H_2O$<br>0,0176 M $KH_2PO_4$ |

## Solutions for ELISA

| | | |
|---|---|---|
| Coating buffer | 0,1 M $Na_2CO_3/NaHCO_3$ buffer (pH 9,6) | |
| Washing buffer | PBS with 0,005% TWEEN20<br>-storage up to one month at 2-8°C | |
| Substrate buffer | 0,11 M $CH_3COOH/CH_3COONa$ buffer (pH 5,5)<br>-storage up to 2 weeks at 2-8°C | |
| Substrate solution | 12 ml | substrate buffer |
| | 200 µl | TMB stock solution<br>- 30 mg 3,5,3',5'-tetramethylbenzidine (TMB)<br>- 5 ml dimethylsulfoxide (DMSO)<br>-storage RT up to 1 month<br>-protect against light |
| | 12 µl | 3% $H_2O_2$ |
| | -prepared just before use | |

**Table 1. Pipeting scheme for intracellular staining with anti-IL-19 antibodies.**

| Tube n:o | Cell type | FITC-conjugated antibody | PE-conjugated antibody |
|---|---|---|---|
| 1 | PBMC | IgG | IgG |
| 2 | PBMC | IgG | IL-19 |
| 3 | PBMC | CD3 | IgG |
| 4 | PBMC | CD3 | IL-19 |
| 5 | PBMC | CD14 | IgG |
| 6 | PBMC | CD14 | IL-19 |
| 7 | SFMC | IgG | IgG |
| 8 | SFMC | IgG | IL-19 |
| 9 | SFMC | CD3 | IgG |
| 10 | SFMC | CD3 | IL-19 |
| 11 | SFMC | CD14 | IgG |
| 12 | SFMC | CD14 | IL-19 |