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Gene expression in synovial fibroblasts from rheumatoid arthritis and osteoarthritis patients. Novel expression of IL-19 and IL-22 by fibroblasts

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Gene expression in synovial fibroblasts from rheumatoid arthritis and osteoarthritis patients. Novel expression of IL-19 and IL-22 by fibroblasts

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ABSTRACT

The role of the fibroblast like synoviocyte (FLS) in the pathogenesis of rheumatoid arthritis (RA) has been well documented. It has been established that activated FLS are vital in the process of degradation of cartilage and bone while also being key players in the formation of invasive pannus, the key feature of RA. They also are important sources of cytokines and other matrix degrading proteins. Studies of gene expression in FLS are important to determine if the FLS is capable of expressing any pro-inflammatory genes. Studies of gene expression in FLS have indicated that these cells can express genes for tumor necrosis factor (TNF) α, along with other pro-inflammatory genes.

Our objective was to study gene expression related to cytokines and cytokine receptors in FLS. We were looking for any previously unidentified cytokines and receptors that may play a role in the pathogenesis of RA. Using a microarray- based approach to identify any potential expressed cytokine genes we found that two pro-inflammatory cytokines belonging to the IL-10 family of cytokines, IL-19 and IL-22, are constitutively expressed in cultured FLS, and this expression was independent of length of culture. Additional analysis of IL-19 and IL-22 revealed that expression was not restricted to FLS but that dermal (foreskin) fibroblasts also strongly expressed these two cytokines at the mRNA level. This finding is unique due to the previous observation that IL-19 and IL-22 are produced in LPS-treated macrophages, and mitogen-stimulated T cells, respectively. The IL-10 family of cytokines was recently discovered based on structural homology and sequence identity between its members. The members of the IL-

10 family of cytokines are IL-19, IL-20, IL-22, IL-24, and IL-26, and knowledge of the biology of each member is limited at this time.

The other work in this thesis focused on identifying potential cytokine receptors expressed in FLS and other cells. From these studies, it was identified that FLS do not express membrane bound receptors for IL-19 or other IL-10 family members, but they are capable of expressing IL-22BP, a non membrane bound soluble form of the IL-22 receptor. This is the first indication that fibroblasts are capable of transcribing this gene.

The work presented in this thesis has addressed gene expression in FLS and the findings may prove to be important in understanding maintenance of inflammation in RA. The finding that FLS can transcribe mRNA for IL-19 and IL-22, may indicate that these cells are important sources of these pro-inflammatory cytokines *in vivo*. The receptor studies may also prove to be useful in expanding the knowledge of receptor expression in FLS.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACR	American College of Rheumatology
AP-1	Activator Protein-1
APC	Antigen Presenting Cells
ARA	American Rheumatology Association
bFGF	Basic Fibroblast Growth Factor
BP	Binding Protein
BSA	Bovine Serum Albumin
BSN	Bierrum and Shafer-Nielson
CAM	Cellular Adhesion Molecule
CD	Complementary Determinant
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curie
COX	Cyclooxygenase
CRF	cytokine receptor family
DEPC	Diethyl Pyrocarbonate
DF	Dermal Fibroblast
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunospecific Assay
EBV	Epstein-Barr Virus
EPA	Erythroid Potentiating Activity
FLS	Fibroblast-Like Synoviocyte
FN	Fibronectin
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HHV	Human Herpes Virus
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxydase
HTLV	Human T-cell Leukemia Virus
HVS	Herpes Virus Saimiri
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-TIF	IL-10-Related-T-cell-Derived-Inducible-Factor
IFN	Interferon
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MDA	Melanoma Differentiated Associate Gene
MHC	Major Histocompatibility Complex
MMP	Matrix Metallo-Proteinase
mRNA	Messenger Ribonucleic Acid
NK	Natural Killer

OA	Osteoarthritis
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phyto- Hemagglutinin
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SE	Shared Epitope
SF	Synovial Fibroblast
SLE	Systemic Lupus Erythematosus
ssRNA	Single-stranded ribonucleic acid
TCR	T Cell Receptor
TGF	Tumor Growth Factor
Th	T-helper
TIMP	Tissue Inhibitor of Metallo Proteinase
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

CHAPTER ONE

INTRODUCTION

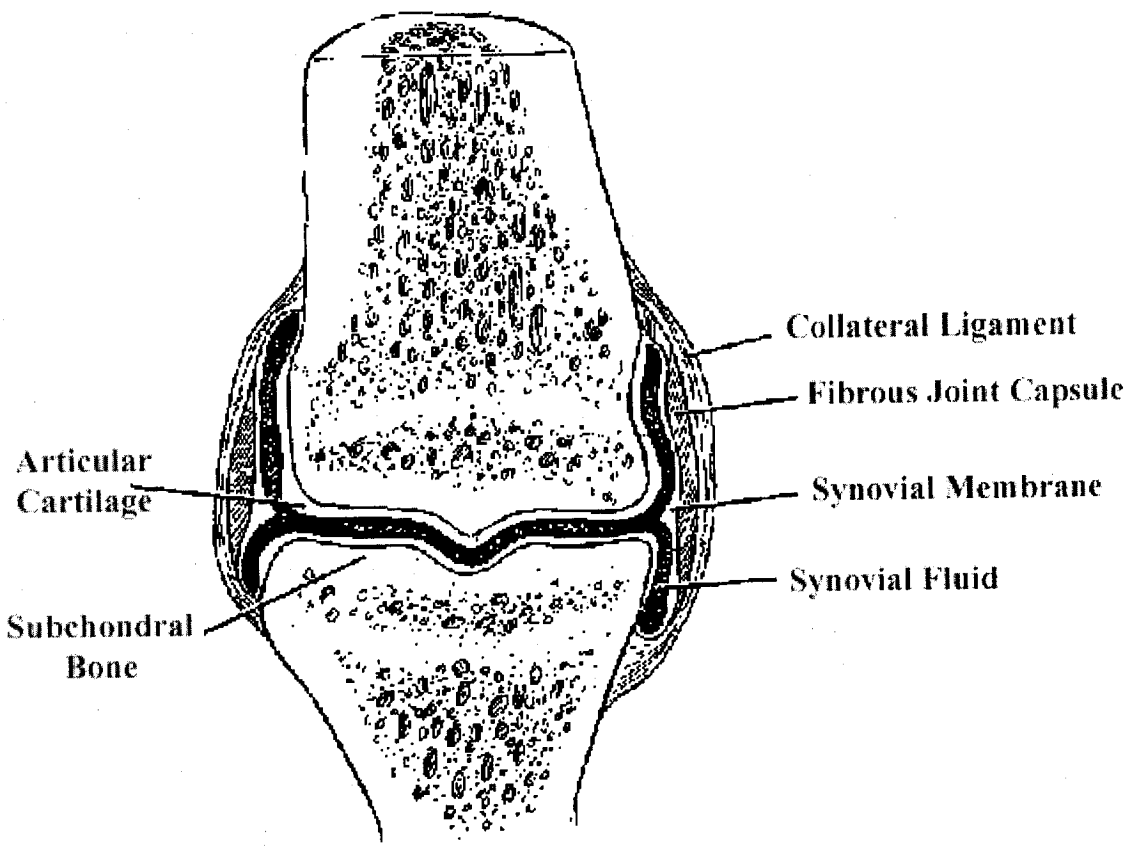
1.1 Overview

Arthritis is a disease of the synovial joints. There are over 100 different types of arthritis, and the two most common types are rheumatoid arthritis (RA) and osteoarthritis (OA). Together, both of these conditions affect close to 4 million Canadians annually. Arthritis is one of Canada's most common chronic conditions, ranking in the top three with hay fever/allergies and circulatory disorders. Typically, arthritis costs the Canadian economy nearly $18 billion each year (The Canadian Arthritis Society, 2004). Both RA and OA impair joint function, with OA being a degenerative disease, while RA is an inflammatory disease which causes joint swelling and synovitis (Conaghan et al., 1999).

1.2 Joint Structure

Understanding the anatomy of a joint is key to understanding arthritis and its associated symptoms. A joint is typically the area where two bones meet, otherwise known as articulation (Figure 1). Cartilage covers the ending of each bone, which provides a smooth surface for mobility. Cartilage is surrounded by a capsule composed of collagen, consisting of synovial sublining, typically 1-3 layers thick. The sublining is responsible for nourishing the joint and secreting lubricating fluid that allows movement.

Figure 1: Stucture of a normal joint.
(http://equinescience.colostate.edu/graduate/orthopedics/questions/anatomyjoint .html#)


Collateral Ligament
Fibrous Joint Capsule
Articular Cartilage
Synovial Membrane
Synovial Fluid
Subchondral Bone

1.3 Osteoarthritis

OA, or degenerative joint disease, is one of the oldest known forms of arthritis and is the single greatest cause of disability of the elderly in the United Kingdom (Brazier et al., 1999). OA is generally a non-inflammatory condition characterized by the initial breakdown of joint cartilage. This condition is typically observed from the 4^{th} decade onward in people and causes pain/discomfort due to bones rubbing against one another in the absence of cartilage. OA often arises in joints of the hands and in weight-bearing joints such as the hip, knees, or spinal column, and can manifest itself as monoarthritis or polyarthritis (Martin et al., 1994). Other factors that contribute to OA are obesity, joint injuries, and genetics. In more severe cases, joint damage occurs which may require surgery to reduce pain, and/or deformity and repair the disability.

1.4 Rheumatoid Arthritis

Rheumatic diseases were first recognized by Hippocrates in the fourth century BC, and the term rheumatology was introduced in 1949 by Joseph L. Hollander (Parish, 1963). RA may have existed in North America up to 3000 years ago, based on the appearance and distribution of lesions in ancient skeletons (Goemaere et al., 1990). Hippocrates used the term *rheuma* to indicate a flow of pain through the body, and today, RA is known as an autoimmune disease characterized by persistent inflammatory synovitis, cartilage destruction, and joint damage. RA is the most common form of inflammatory arthritis and has a major impact on individuals and societies through effects based on cost of treatment, disability of affected individuals, and loss of productivity of

the individual. RA is a disease that can occur at any age, but arises most commonly in women under 40 years of age (Jacobsen, 2004) and has a prevalence of about 1% in the general population. The female/male ratio of people affected with RA is 2.5/1 (Lee et al., 2001). There are also regional differences in RA prevalence, as regions of rural Sub-Saharan Africa have a lower prevalence of developing the disease while Prima Indians of the USA are at a greater risk to developing RA (Silman et al., 1993). These differences, are most likely genetically linked.

1.4.1 Clinical Symptoms in RA Patients

The first clinical description of RA is credited to Augustin-Jacob Landre-Beauvais's PhD thesis in 1800. The degree and severity in which RA presents in patients can be quite variable, and several months or longer can pass by before the disease is correctly diagnosed. The most common symptoms presented by patients include pain, stiffness, and swelling of peripheral joints (Lee et al., 2001). Based on these symptoms, the most widely used method to clinically diagnose RA was based on the criteria proposed in 1958 by the American Rheumatology Association (ARA) (Ropes et al, 1958). These criteria were modified in 1987 to define RA based on 7 different criterion which can be viewed in Table 1(Arnett et al., 1988). RA is defined by the presence of 4 or more criteria, and no further qualifications or list of exclusions are required. The diseases most common in the differential diagnosis of RA include systemic lupus erythematosus (SLE), psoriatic arthritis and rheumatoid factor (RF)-negative spondylarthropathies. However, to accurately assess the severity of RA, numerous standardized tests that can assess the quality of life of RA patients and the extent of joint destruction are performed. These are

the American College of Rheumatology (ACR) response (Felson et al., 1995; Van der Heijde 1993), and the Larsen (Larsen, 1975) or Sharp (Sharp, 1989) scores, respectively. The Larsen and Sharp scores use radiographs to accurately estimate the progression of RA in patients.

1.5 Pathophysiology of RA

1.5.1 Genetic Factors in RA

Human leukocyte antigen (HLA) – genes located within the major histocompatibility complex (MHC) on chromosome 6p have been shown to correlate with the onset or incidence of RA (Stastny, 1978). Individuals with genotypes HLA-DR4 and HLA-DR1 have been shown to be at a higher risk of developing RA. HLA-DR4 is common in North American and European Caucasians with RA, while HLA-DR1 is more common in Israeli Jews, Asian Indians, and Yakima Indians. Based on this information, it has been assumed that shared epitopes determine susceptibility to RA (Walport et al, 1992). This is also due to the finding for many HLA-DRB1 alleles including (DRB*0101, *0102,*0401, *0404, *0405, *0408, *1001, and *1002) are associated with RA (Ollier et al., 1992).

Table 1: The 1987 revised criteria for RA (Arnett et al., 1988)

Criterion	Comment
Morning Stiffness	Duration > 1 hour lasting > 6 weeks
Arthritis of at least three areas	Soft tissue swelling or exudation lasting > 6 weeks
Symmetrical arthritis	At least one area, lasting >6 weeks
Rheumatoid nodules	Observed by a physician
Serum Rheumatoid factor	Assessed by a method positive <5% of control subjects
Arthritis of hand joints	Wrist metacapophalangeal (MCP), proximal interphalangeal (PIP) lasting > 6 weeks
Radiographic changes	Seen on anteroposterior films of wrists and hands

* The presence of 4 or more of these criteria are necessary to properly diagnose RA

HLA-DRB1 alleles encoding a highly conserved amino acid motif known as the shared epitope (SE), which is expressed in the third hypervariable region of different HLA-DR βchains, are associated with RA susceptibility (Gregersen et al., 1987). This SE has the amino acid sequence EQK/RRAA and is present in HLA-DR1/4 molecules (Ebringer et al., 2003). It is this seqeunce that is thought to attribute to MHC complex affinity for a self-arthritogenic peptide antigen, and cross-reactivity between a pathogen and the shared epitopes. Sequence homology between the shared epitope and prokaryotic sequences from Epstein-Barr virus (EBV) and *Escherichia coli* has been established (Ollier et al, 1997). The information linking HLA type to development of RA is an important factor in the pathophysiology of the disease as specific HLA types can contribute to the severity and progression of RA.

1.5.2 Gender factors in RA

With a higher incidence of RA in females than males, reproductive and hormonal factors are thought to play some role in the pathophysiology of RA. The highest incidence of RA is in women that fall in the pre-menopausal age group (Krishnan, 2003). The risk for developing RA during pregnancy is reduced, and this rises during the post-partum period (Symmons et al., 1994). Several studies have shown that exposure to the oral contraceptive pill can reduce the risk of developing severe RA or delay the onset of RA (Spector et al., 1990). Both menopause and the peak age for developing RA is getting later in women, but not in men, which is in keeping with the theory that the oral contraceptive pill delays onset of RA.

1.5.3 Histological Changes in RA

In a normal joint, the articular spaces and tendons are surrounded by the synovial membrane, which leaves the cartilage unattached. This membrane consists of synoviocytes with a thickness of 1-2 cells. In RA, the synovium becomes inflamed, which is key to pathophysiology of the disease. Inflammation is defined as 'a response of body tissues to injury or irritation, characterized by pain and swelling, redness, and heat'. Inflammation of a synovial joint results in an increase in numbers of inflammatory leukocytes, cellular hyperplasia, enhanced expression of adhesion molecule receptors, and an increase in the concentrations of many pro-inflammatory cytokines in the joint region (Kramer et al., 2003).

The formation of pannus is vital to the pathology of RA. Early in RA, edema can be seen in the cells of the synovium and this can result in multiplication of the synovial lining can occur. With disease progression, the synovial lining can continue to increase its size, eventually forming tissue known as pannus. This tissue is very destructive as it is known to attack and destroy articular cartliage and the soft subchondral bone (Gravallese, 2002).

The pathophysiology of RA includes the expansion of the RA synovial membrane on the surface of cartilage which ultimately leads to cartilage degradation through the release of many different enzymes such as collagenases and matrix metalloproteinases (MMP) (Gravallese, 2002). The synovial membrane, which consists of activated macrophage-like syonoviocytes (type A synoviocytes), and fibroblast-like synoviocytes (Type B synoviocytes) becomes thickened. Whereas the synovial lining is typically 1-2 cells thick in a normal joint, this can become up to 10-12 cells thick in a rheumatoid joint

(Keyszer et al., 1994). Neovascularization also allows an influx of of peripheral blood mononuclear cells (PBMC) into synovium, which further enhances the inflammation associated with RA.

1.6 Cellular Involvement in RA Pathogenesis

As mentioned previously, the hallmark of RA is inflammation. Inflammation is caused, and maintained by, cells foreign and native to the joint. Foreign cells, such as PBMC, invade the joint and influence the local environment so that it becomes pathogenic. The cells that are involved in the pathogenesis of RA include the cells of the immune system (macrophages, monocytes, B cells, T cells), and synovial fibroblasts.

The cells of the immune system are derived from a common pluripotent stem cell in the bone marrow. These pluripotent stem cells are divided into two types: myeloid progenitor (gives rise to granulocytes, eosinophils, basophils, dendritic cells, monocytes, macrophages, and mast cells), and lymphoid progenitor (gives rise to T cells, B Cells, and natural-killer (NK) cells). Cells derived from myeloid progenitor cells have a variety of functions usually are involved in innate immunity, while they also can act as antigen-presenting cells (APC) for cells involved in adaptive immunity.

Adaptive immunity is maintained by cells derived from lymphoid progenitor cells. T cells use their T cell receptor (TCR) complex to recognize intracelluar pathogens that are presented to T cells as peptides associated to a MHC protein present on APC. B cells recognize pathogens, such as bacteria, inhabiting extracellular fluid, with their immunoglobulin surface receptor.

1.6.1 T cells in RA

Alternative disease models suggests that RA is a T cell-dependent disease (Kotake et al., 2001), and that the activation of T cells is caused by the presence of some self-arthritic antigen (Weyand et al., 1997; Panayi et al., 1992). This model is supported by numerous findings, including the strong correlation of MHC class II molecules with development of RA, and that T cells in RA patients are abnormal as they contain oligoclonal populations that are deficient of CD28 expression, are defective in apoptosis, and exhibit autoreactivity (Schmidt et al., 1996; Schirmer et al., 1998). Further evidence supporting the claim that RA is a T cell-dependant disease has been found in animal models where specific T-cell clones were shown to transfer the disease (Klaresog et al., 1983, Taurog et al., 1983)

Arguments against RA being a T cell-dependent disease are based on the observation that lymphokines derived from T cells are absent in RA joints. More specifically, the levels of IL-2, and IFN-γ are relatively low (Feldmann et al., 1996), while lymphokines derived from macrophages, such as IL-1, IL-6, and TNFα are readily detectable in RA joints.

Taken either way, T cells play a very important role in development and maintenance of inflammation associated with RA. Their inherent ability to secrete cytokines and interact with other cells is the defining characteristic that allows them to participate in the pathogenesis of the disease. One of the major cellular manifestations that occurs as a result of T cell activity, is the activation of the synovial fibroblasts (SF), another cell type important in the maintenance of inflammation in RA (Cho et al., 2004).

1.6.5 Synoviocytes in RA

Recently, evidence has been growing that activated synovial fibroblasts (SF), in addition to macrophages and T cells play a major role in initiating and maintaining RA (Franz et al., 1998). In normal human synovium, there are typically two phenotypes of SF, those being type A and type B SF (Edwards, 2000). These were originally classified based on gene expression with type B fibroblasts known as fibroblast-like synoviocytes (FLS) (Barland et al., 1962), and type A are known as macrophage-like.

A striking feature of inflammatory arthritis is the hyperplasia of the SF in the lining layer (Qu et al., 1994). In culture these fibroblasts exhibit several novel properties including high proliferative rates, loss of contact inhibition, constitutive expression of cytokine mRNA and protein, and anchorage-independent cell growth (Ritchlin et al., 1994). It has been established that activated SF are vital in the process of degradation of cartilage and bone in the joints of patients with RA (Firestein, 1996; Kinne et al, 1995). RA-SF are a key player in the formation of invasive pannus, which is an altered form of synovial tissue and a characteristic feature of RA. This tissue is histologically different from other areas of the synovium and has various stages of progression. At early stages the synovial pannus is composed of mononuclear cells and fibroblasts (Shiozawa et al., 1983) that can infiltrate cartilage, while at later stages pannus can be replaced by fibrous pannus which is composed of a small vascular layer of pannus cells and collagen overlying cartilage (Lee et al., 2001).

The altered state of activated SF may be due to specific changes in the transcription of disease-relevant genes and alterations in signaling pathways of the SF. A specific factor upregulated in RA-SF is AP1, which consists of the c-Fos and c-Jun

transcription elements. Upregulation of AP1 has been linked to degradation of bone and cartilage, while also being linked to increased MMP production (Pap et al., 2000; Benbow et al., 1997). Another factor upregulated in RA-SF that is relevant to RA pathogenesis is NF-κB. Upregulation of NF- κB has been detected in RA-SF and has been linked to prolonging the disease and to mediation of synovial inflammation (Miagkov et al., 1998; Han et al., 1998; Marok et al., 1996; Palombella et al., 1998). NF-κB is upregulated by pro-inflammatory cytokines, and in-turn upregulates the production of adhesion molecules and cytokines in RA-SF.

The synthesis of cytokines by SF are important in the pathology associated with RA. SF may contribute to the inflammatory milieu of the RA synovium through the production of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), TNFα, IL-6, IL-7, IL-8, and IL-15 (Ritchlin et al., 2000). Thus, there is increasing evidence that FLS can synthesize cytokines and that selective modulation of these factors may be key to the successful treatment of RA.

Another interesting feature of FLS is their inherent ability to remodel and degrade the extra cellular matrix (ECM). Degradation of the ECM leads to morbidity in RA due to the destruction of cartilage, bone, and soft tissues of the joint. FLS are known to produce matrix metalloproteinases (MMP's) (Stuhlmeier, 2003), which are a family of zinc binding endopeptidases with over 20 members. These proteins contribute to both normal and pathological tissue remodeling and are thought to be a main contributor to joint destruction in RA (Fowlkes et al., 2002). MMP's maybe considered regulatory molecules as they have been shown to regulate Fas-mediated apoptosis (Powell et al.,

1999), expression of cell surface molecules (Noe et al., 2001) and expression of pro-inflammatory cytokines such as TNFα (Black et al., 1997), and IL-1β (Ito et al., 1996).

MMP's are each encoded by separate genes and they all have various substrates. The are secreted as an inactivated form, and are activated following proteolytic processing (Fowlkes et al., 2002). Once activated, MMP's are tightly regulated by a family of proteins known as tissue inhibitors of metalloproteinase (TIMP). The activity of the MMP's is controlled by TIMP family members 1-4 (Nagase et al., 1999), which have been found to be produced in a variety of cells.

1.7 Cytokines and RA

Cytokines are locally acting protein mediators of various size and structure that are involved in many biological processes such as inflammation, cell growth, and immunity. Activated inflammatory cells are considered the major contributors to cytokine production, but other cells, such as fibroblasts and epithelial cells can also produce these proteins. Cytokines act on cells that express receptors specific for each protein. Once bound to their receptors, cytokines cause an intracellular signalling cascade which results in altered gene expression of the target cell.

Analysis for cytokine expression at the protein and mRNA level in the inflamed synoivum of RA and other disorders has indicated that there is an abundance of cytokines with various properties. Few T cell cytokines (such as IL-2, and IL-17) have been identified in RA synovium, with the majority of cytokines coming from other cellular sources. Pro-inflammatory cytokines that have been detected in RA synovium that are not T cell derived include TNFα, IL-1, IL-6, and IL-15 (Vervordeldonk and Tak, 2002).

Anti-inflammatory cytokines, such as IL-10, have also been detected (Lettesjo et al., 1998.

1.7.1 IL-1

IL-1 is produced by a variety of cells that include macrophages, monocytes, B cells, activated T-cells, fibroblasts, neutrophils, and synovial lining cells. This cytokine is a 17kDa protein that is derived from a 31 kDa protein, and has two forms, IL-1α and IL-1β. The molecule is important in host defence against mico-organisms such as *Listeria monocytogenes* (Denis et al., 1994).

IL-1 is a pro-inflammatory cytokine and is one of the most abundant cytokines in the rheumatoid joint. It can induce inflammation by activating synoviocytes, macrophages, and endothelial cells to produce a variety of cytokines, chemokines, and inflammatory mediators (Feldmann et al., 1996). The production of IL-6, TNFα, and cyclooxygenase (COX)-2 is also upregulated by IL-1 (Dinarello, 1996). Ultimately this leads to the migration of inflammatory cells into inflammatory sites such as rheumatoid joints. Synoviocyte growth, and enhanced synthesis of metalloproteinases and collagenases are caused by IL-1. These factors are involved in the degradation and destruction of joint bone and cartilage (DeMarco et al., 1997).

1.7.2 IL-6

IL-6 is a pleiotropic cytokine with a wide range of biological activities. Both lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several tumour cells are known to

produce IL-6(Akira et al., 1993). A potent inducer of terminal macrophage differentiation (Nicola et al., 1993), IL-6 is also known to stimulate the production of acute phase reactant proteins (such as C reactive protein, fibrinogen, α_1 antitrypsin, and serum amyloid A) (Gauldie et al., 1987;Castell et al., 1988).

The detection of acute phase reactants, rheumatoid factors, and increased platelet counts in RA may be due to uncontrolled overexpression of IL-6 (Yoshizaki et al., 1998). Significant amounts of IL-6 have been detected in the sera and synovial fluids of RA joints (Hirano et al., 1988;Houssiau et al., 1988) and IL-6 may promote infiltration of immunocompetent cells into the RA joint as IL-6 is known to upregulate the expressoin of intercellular adhesion molecule (ICAM)-1 (Yamamoto et al., 2000). These findings indicate that IL-6 may play an important role in the pathogenesis of RA.

1.7.3 TNFα

The other abundant cytokine present in the rheumatoid joint, TNFα, is synthesized mostly in monocytes and macrophages, but synthesis has also been detected in activated helper T-,B-, and NK- cells. This is another pro-inflammatory cytokine that exerts its effect by inducing fever, tissue injury, bone resorption and production of acute phase reactant proteins. When stress is induced on the body, TNFα is the most rapidly released cytokine and it has the ability to upregulate the production of other pro-inflammatory cytokines such as IL-1, IL-6, and IL-8 (Tracey et al., 1987; Butler et al., 1995). The many other biological effects of TNFα include the activation of monocytes, upregulation of MMP (matrix metallo-proteinase)-1 (Dayer et al., 1985), inhibition of collagen

synthesis by fibroblasts (Osborn, 1990), and upregulating the expression of adhesion molecules on the surface of endothelial cells (Osborn, 1990).

It is the pro-inflammatory properties associated with TNFα that has made it a major target for therapeutic intervention in RA. Early evidence of TNFα being involved in RA came from the expression of pro-inflammatory cytokines in *in* vitrosynovial cell cultures. Anti-TNFα antibody was able to neutralize TNFα which resulted in the decrease in levels of other pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and GM-CSF (Brennan et al., 1989). *In vivo*, a similar effect has been observed. Anti-TNFα therapy in humans has been successful in reducing the levels of pro-inflammatory cytokines and serum MMP's in patitents with RA (Maini et al., 1999).

1.7.4 IL-15

IL-15 is a 15kDa cytokine expressed at the mRNA level in many human tissues and in a wide variety of cells that include fibroblasts, monocytes, and osteoclasts (Waldmann and Tagaya, 1999). IL-15 has high affinity for the IL-15 receptor complex IL-15R, which is composed of IL-2R β and γ chains, and the IL-15Rα chain. The latter chain can be alternatively spliced to yield 3 different active forms, all of which are capable of binding to IL-15 with high affinity (Giri et al., 1995). IL-15Rα expression has been detected in many tissues, as well as activated T cells. This was evident when it was found that IL-15 can sustain induced TNFα production in macrophages that results from cell contact between T cells and macrophages (Vey et al., 1996;McInnes et al., 1997). IL-15 is expressed in several inflammatory disorders, including rheumatoid arthritis, psoriasis and pulmonary inflammatory diseases. IL-15 promotes activation of T cells,

neutrophils and macrophages, and is critical to dendritic cell function in several model systems (McInnes et al., 2004).

1.8 IL-10 Family of Cytokines

IL-10 is a cytokine involved in the regulation of immunity. Human IL-10 shares around 80% homology with murine IL-10. Human IL-10 is a homodimer that consists of 160 amino acids with a molecular weight of 37kDa. There are several viral IL-10 homologues that include Epstein-Barr virus (BCRF1) (Hsu et al, 1990), cytomegalovirus (Kotenko et al, 2000), herpes virus type 2 (Rode et al, 1994), and Orf virus (Fleming et al, 1997). IL-10 production has been identified in a variety of cells that include T helper 2 cells (Fiorentino et al., 1989), monocytes (De Waal Malefyt et al., 1991a), macrophages (Spits and De Waal Malefyt, 1992), B cells (Pistoia, 1997), eosinophils (Nakajima et al., 1996), and mast cells (Lin and Befus, 1997).

IL-10 plays an important role in a variety of human diseases, especially diseases of the autoimmune and inflammatory nature. IL-10 is thought to be involved in this category of diseases as it has the ability to activate B cells (Itoh et all, 1995; Perez et al, 1995). IL-10 is of relevance in SLE as this is a disease characterized by high antibody levels, and a decrease in the immune response, both of which are phenomena caused by IL-10. Indeed, it has been documented that levels of IL-10 are elevated in SLE patients when compared to normal, healthy individuals (Park et al, 1998) while anti-IL-10 mAb *in vitro* treatment of SLE patient PBMC significantly decreased autoantibody production (Llorente et al, 1995). Inhibitory functions of IL-10 are also known, as IL-10 inhibits

antigen presentation by dendritic cells and macrophages (Fiorentino et al., 1991, Macatonia et al., 1993, Enk et al., 1993, Peguet-Navarro et al, 1994)

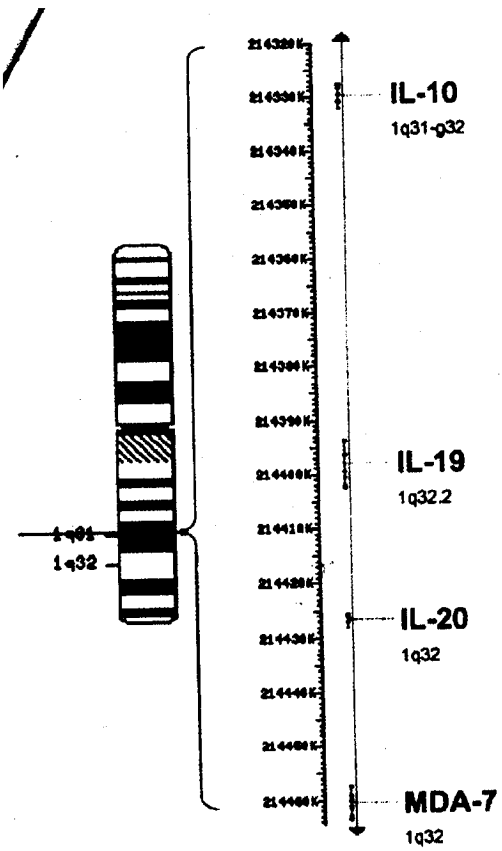
IL-10 may also have a significance in RA as IL-10 has been detected in the serum, synovial fluid, or synovial explants of RA patients (Jenkins et al, 1994; Cush et al, 1995; Al Janadi et al, 1996). The exact role of IL-10 in RA is not completely known; however, IL-10 has been shown to block the production of TNFα, IL-1, and IL-8 by synovial macrophages and synoviocytes (Chomarat et al., 1995; Hart et al., 1995).

Recently, several novel cytokines that demonstrate primary sequence homology and structural similarity to IL-10 have been identified and have been placed into the IL-10 family. These five newly discovered cytokines can be seen in Table 1, and these cytokines of the IL-10 family are similar to IL-10 in that they are α-helical proteins, share similar cysteine locations, and have amino acid sequences that are 30% identical. In the human genome, the genes for IL-10, IL-19, IL-20, and IL-24 are located in clusters on chromosome 1q31-32, while IL-22 and IL-26 are located on chromosome 12q15 (for more detail, see Fig.3).

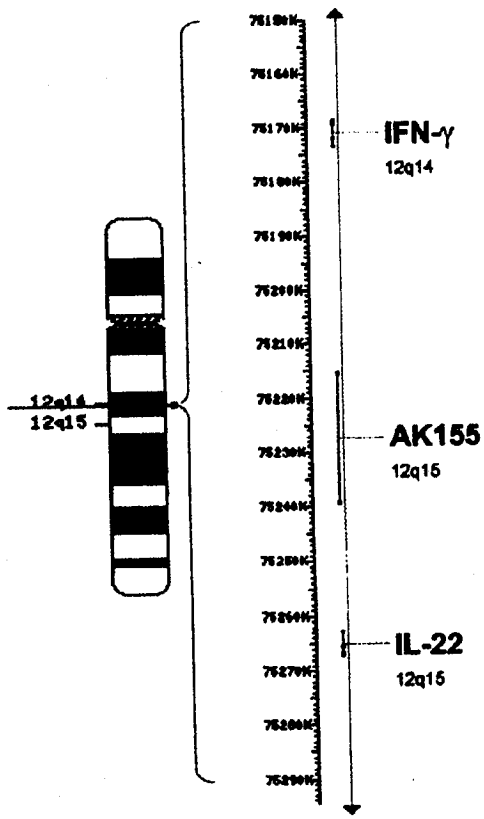
Table 2: Cytokines classified into the IL-10 cytokine family

Cytokine	Reference
IL-19	Gallagher et al, 2000
IL-20	Blumberg et al, 2001
IL-22	Dumoutier et al, 2000a,b,c
IL-24 (mda-7)	Jiang et al, 1995
IL-26	Knappe et al, 2000

Fig.2 Chromosomal location of IL-10 and IL-10 related cytokines. From Asadullah et al, 2003.



Chromosome 1



Chromosome 12

1.8.1 IL-19

IL-19 was originally identified by analyzing DNA sequence databases for possible IL-10 homologs, and eventually as a gene that is transcribed in monocytes after lipopolysaccharide (LPS) stimulation (Gallagher et al, 2000). IL-19 shares 21% amino acid identity with IL-10 and is a secreted protein with a molecular weight of 17.9 kDa. Similarly to IL-10, the gene encoding IL-19 consists of five exons and four introns within its coding region. However, unlike IL-10, the gene for IL-19 can be transcribed as two separate mRNA sequences that differ in their 5' sequences (Gallagher et al., 2000). This suggests the possibility of an intron located at the 5'terminal end of the gene.

1.8.1.1 Cellular Sources of IL-19

Synthesis of IL-19 mRNA has been detected in a variety of cell types including B cells, monocytes, and a population of keratinocytes in psoriatic patients (Wolk et al., 2002; Rømer et al, 2003). Expression in monocytes is detectable after 4 hours of treatment with LPS, which is slightly delayed compared to IL-10 mRNA expression which can be detected within 2 hours after treatment with LPS. The expression level observed is relatively low; however, priming monocytes with IL-4 or IL-13 can increase the IL-19 mRNA levels. Expression of IL-19 in B cells has been detected, but its expression tends to be very low and irregular (Wolk et al, 2002).

1.8.1.2 Biological Properties of IL-19

The biological properties of IL-19 are just starting to be unraveled. IL-19 has been shown to stimulate IL-6, TNFα, and ROS production in monocytes (Liao et al,

2002). This finding, along with knowledge that IL-19 is inducible 2 hours later than IL-10 in LPS-stimulated monocytes may suggest that IL-19 is a pro-inflammatory cytokine. Elevated IL-6, TNFα, and ROS are characteristic of inflammatory conditions, and the 2 hour difference between IL-10 and IL-19 induction may indicate that IL-19 is involved in limiting the anti-inflammatory actions of IL-10. Both of these effects are pro-inflammatory in nature.

1.8.2 IL-20

IL-20 was originally identified as a mRNA expressed in keratinocytes, and in a database search for helical cytokines (Blumberg et al, 2001). IL-20 has 28% sequence similarity to IL-10 while it shares similar three-dimensional structure to IL-19. The gene for IL-20 consists of 5 exons with 4 introns and is located on chromosome 1q32 with tight links to IL-10, IL-19 and MDA-7.

1.8.2.1 Cellular Sources of IL-20

To date, IL-20 expression has been detected in keratinocytes, and in the basal and suprabasal keratinocytes of psoriatic patients (Rømer et al, 2003). Low levels of mRNA transcripts for IL-20 have also been detected in tissue samples from skin and trachea (Blumberg et al., 2001). IL-20 has not been detected in resting or activated PBMC and is thought to be involved in skin development.

1.8.2.2 Biological Functions of IL-20

It seems that the main biological role for IL-20 is in proper development of skin. Receptors for IL-20 have been found in skin and are dramatically upregulated in psoriatic skin. Transgenic mice that overexpress IL-20 have neonatal lethality with skin abnormalities, similar to that obsevered in psoriatic skin (Blumberg et al, 2001). Some other manifestations in these transgenic mice included lack of adipose tissue, apoptotic thymic lymphocytes, and swollen extremities. These abnormalities indicate that IL-20 plays an important role in skin development. Future studies may prove if IL-20 is important in the pathogenesis of psoriasis, or other inflammatory conditions.

1.8.3 IL-22

IL-22 was initially identified as a gene specifically induced by IL-9 in mouse T cells and was alternatively named IL-TIF (IL-10-related-T-cell-derived-inducible-factor) (Dumoutier et al, 2000c). It has recently been identified in humans (Xie et al, 2000) and the cDNA for this gene encodes a protein of 179 amino acid residues which is shares 25% identity to that of IL-10. The gene for human IL-22 is located on chromosome 12q15 and contains 5 introns and 6 exons.

1.8.3.1 Cellular Sources of IL-22

After the initial discovery that IL-22 was detectable in T cells following IL-9 treatment (Dumoutier et al., 2000c), it was found that mitogen-stimulated T cells are able to transcribe mRNA for IL-22. ConA was identified to cause an increase in IL-22mRNA synthesis, along with anti-CD3 stimulation (Dumoutier et al., 2000a; Xie et al., 2000).

Furthermore, the Th1 subset of T cells is responsible for IL-22 synthesis rather than the Th2 subset of T cells (Pittman et al., 2001). mRNA for IL-22 has also been detected in various tissues, such as the brain and thymus (Dumoutier et al, 2000a).

1.8.3.2 Biological Properties of IL-22

Little is known about the biological functions of IL-22. Cell lines that are responsive to IL-22 include TK-10 renal carcinoma, SW480 colon adenocarcinoma, HepG2 and HepG3 hepatoma, and HT29 intestinal epithelial cell lines (Dumoutier et al, 2000a; Xie et al., 2000; Kotenko et al, 2001a; Kotenko et al., 2001b; Dumoutier et al., 2001). There are several genes that are responsive to IL-22 and these include acute phase reactant proteins, more specifically, serum amyloid A, and α1-chymotrypsin. IL-22 was found to upregulate synthesis of these genes in both an *in vivo* and *in vitro* environment (Dumoutier et al, 2000a). Another gene responsive to IL-22 is the gene that encodes for haptoglobin in HepG2 hepatoma cells as it is also upregulated by IL-22 (Dumoutier et al, 2000a).

IL-22 seems to have some properties that are opposite to those of IL-10. IL-10 is known to inhibit the synthesis of TNFα, IL-1 and IL-6 in monocytes following LPS stimulation, but IL-22 did not inhibit the expression of either of these cytokines in LPS-treated monocytes (Xie et al., 2000). IL-22 also seems to have no effect on IFN-γ production in Th1 T cells, while it does inhibit IL-4 production from Th2 cells. Many of the above mentioned properties seem to implicate IL-22 as a cytokine involved in inflammatory processes, and as further research is conducted on this cytokine its role in inflammation may correctly be established.

1.8.4 IL-24(mda-7)

Melanoma differentiated associate gene-7 (MDA-7) was identified in 1995, and its homology to IL-10 along with its cytokine like properties were not discovered until later. MDA-7 was originally identified as a protein with elevated expression levels in terminally-differentiated human melanoma cells (Jiang et al., 1995). Human IL-24 is located on chromosome 1q32 and shares 23% identity to human IL-10. The gene that composes IL-24 consists of seven exons and and six introns, is approximately 2kb in length, and encodes a protein with a molecular weight of 23.8 kDa (Jiang et al, 1995; Sauane et al, 2003).

1.8.4.1 Cellular Sources of IL-24

IL-24 expression was initially observed as elevated in terminally differentiated human melanoma cells. However, expression of IL-24 in these cells is lost as the tumor progresses, suggesting an alternative biological role for IL-24. Human PBMC's treated with PHA or LPS, and microbial infection have been shown to transcribe mRNA for IL-24 and secrete functional protein (Caudell et al., 2002). More specifically, similar to expression of IL-19, expression of IL-24 from LPS treated monocytes follows a time dependant response. IL-24 has been shown to be transcribed from LPS-treated monocytes after 2, 6, and 18 hours of treatment (Wolk et al., 2002). Delayed expression of IL-24 is also observed in T cells that have been activated by treatment with anti-CD3 monoclonal antibody or conA (Huang et al, 2001; Wolk et al., 2002). A 24 hour IFN-β treatment can induce IL-24 mRNA expression in the following cell lines: prostate carcinoma (DU-145), breast epithelial cells (HBL-100), breast carcinoma (MDA-MB-

157, MDA-MB-231), cerebellum astrocytes, glioblastoma multiforme (GBM-18), osteosarcoma cells (Saos-2), cervical carcinoma, and nasopharyngeal carcinoma cells (Huang et al., 2001).

1.8.4.2 Biological Properties of IL-24

The observation that IL-24 can be assembled by cells of the immune system and by tumor cells has lead to the speculation that IL-24 may be a dual functioning molecule. The expression of IL-24 protein in human melanoma cells tends to decrease with progression of the melanoma (Ekmekcioglu et al., 2001), and this suggests some sort of anti-tumorgenic role for this cytokine. However, this observation has only occurred in melanoma cell lines, and it is thought that the anti-tumorgenic properties may be due to immune function. This observation prevents IL-24 from fitting in the classical definition of a tumor suppressor. Currently, the only property known for IL-24 in the immune system is that IL-10 can downregulate IL-24 mRNA synthesis in monocytes (Caudell et al., 2002). Although it has been detected in cells of the immune system, there have been no studies indicating any possible role for this cytokine in immunity.

1.8.5 IL-26

The last member of the IL-10 family is the cytokine IL-26, and little is known about this molecule. IL-26, originally designated AK155, was initially discovered as a protein expressed by herpesvirus saimiri (HVS)-transformed T cells (Knappe et al., 2000). The gene for this protein is located on chromosome 12q15, and is near to the IL-

22 and IFN-γ genes (Goris et al., 2001), and encodes a protein at approximate size of 18 kDa.

1.8.5.1 Cellular Sources of IL-26

IL-26 mRNA can be detected at low levels in T cells, and at higher levels upon infection with HVS. IL-26 has also been detected at low levels in human T-cell leukemia virus (HTLV)-transformed cell lines, and in PBMC from normal healthy donor patients. Its expression is generally limited to T cells but human herpes virus 8 (HHV-8) transformed B cells can transcribe mRNA for IL-26 at low levels. There is little known about the sources of IL-26, and also little known about any biological functions of this cytokine. Its apparent upregulation after HVS infection may suggest that It is possible that IL-26 plays a role in regulation of the immune response to HVS.

1.9 Class II cytokine receptors

Cytokines exert their effects by binding to specifc cell surface receptors which leads to the activation of the cell through ctyokine induced signalling pathways. Cytokines of the IL-10 family are known to bind to class II ctyokine receptors (Kotenko, 2002). These class II receptors are distinguished based on conserved amino acid sequences within the extracellular domains of the receptors and structurally consist of two subunits. It is accepted that one subunit is critical in binding cytokine ligands, while the other subunit is crucial in causing activation in intracellular signalling once the cytokine has bound.

The designation of nomenclature for these class II ctyokine receptors is based on naming after discovery of the first receptor by *in silico* screening (Lutfalla et al., 1993). The first class II cytokine receptor was designated cytokine receptor family class II member 4 (CRF2-4). Other cytokine receptors were named following identification of CRF2-4 and are known as CRF2-8, CRF2-9, CRF2-10, CRF2-11, CRF2-12 and are shown Fig.4 and table 2. A unique characteristic about receptors for the IL-10 family is that each ligand can be shared among cytokines. For example, from Table 2 it is evident that IL-19, and IL-20 utilize the same receptor complex for binding.

1.9.1 Class II Cytokine Receptor Structure

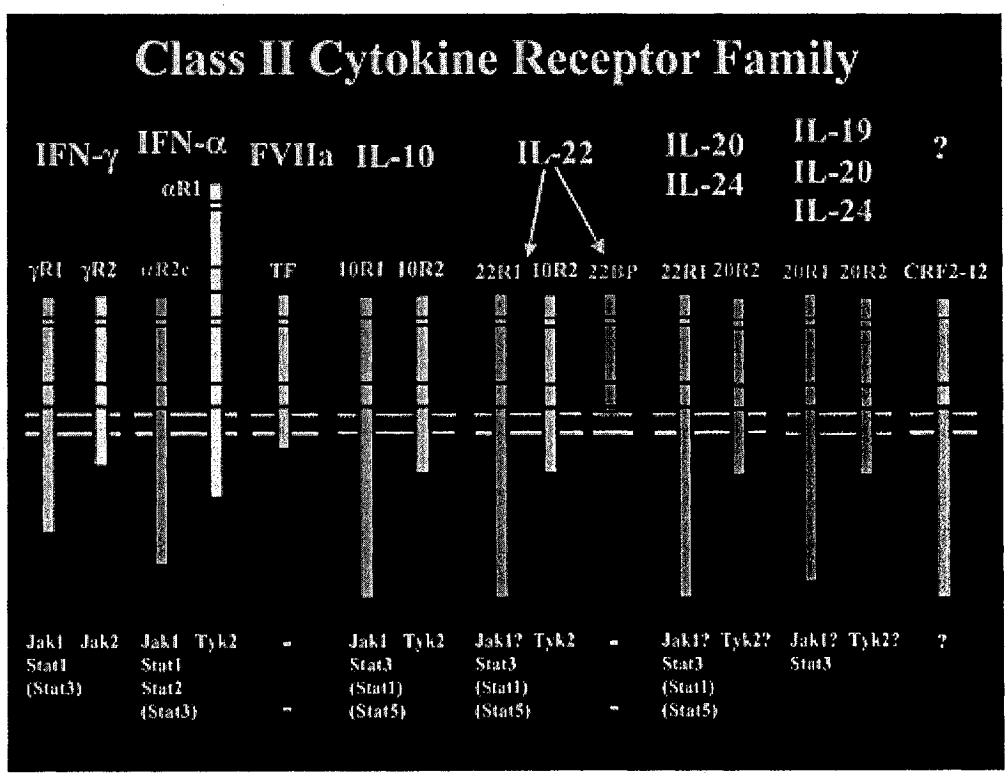
Each receptor chain belonging to class II receptors is given the designation R1 or R2. The extracellular domain of the receptor is composed of tandem fibronectin type III, with most family members having two tandem domains. Each receptor chain is independent of the other, while ligand binding can induce oligomerization of each subunit resulting in Jak/Stat signalling activation. The R1 subunit has high affinity for ligand, and is associated with Jak1 tyrosine kinase. Binding of ligand induces phosphorylation of Tyr residues and allows the recruitment of various Stats to the receptor complex (Kotenko, 2002). This property allocates specificity of signalling to the R1 subunit. This is not the case for the R2 subunits, as this subunit does not bind ligand on its own, does not recruit stats, but is associated with Jak2. It seems the purpose of the R2 subunit is not to bind ligand nor activate sigalling pathways, but rather act as an initiator of signal transduction events.

Activation of stat/jak signalling pathways in this pathway results in homo or heterodimer formation of stats and their translocation to the nucleus. In the nucleus these dimers, along with other factors, can regulate and specify the transcription of cytokine regulatable genes.

Table 3: IL-10 Homologues and their known Receptors.

Cytokine	Receptor	Alternative Nomenclature
IL-10	IL-10R1 IL-10R2	IL-10R, IL-10Rα/CRF2-4,CRFB-4, IL-10Rβ
IL-19	IL-20R1 IL-20R2	CRF2-8, IL-20Rα CRF2-11,IL-20Rβ
IL-20	IL-20R1, IL-22R1 IL-20R2	CRF2-8, IL-20Rα CRF2-11,IL-20Rβ
IL-22	IL-22R1, IL-10R2 IL-22BP	CRF2-9, IL-22R CRF2-10, IL-22RA2
IL-24	IL-20R1, IL-22R1 IL-20R2	
IL-26	IL-20R1	IL-10R2

Figure 3: Class II cytokine receptor complexes (Kotenko, 2002)


Class II Cytokine Receptor Family
IFN-γ
IFN-α
FVIIa
IL-10
IL-22
IL-20
IL-24
IL-19
IL-20
IL-24
?
αR1
γR1 γR2 αR2c TF 10R1 10R2 22R1 10R2 22BP 22R1 20R2 20R1 20R2 CRF2-12
Jak1 Jak2
Stat1
(Stat3)
Jak1 Tyk2
Stat1
Stat2
(Stat3)
-
Jak1 Tyk2
Stat3
(Stat1)
(Stat5)
Jak1? Tyk2
Stat3
(Stat1)
(Stat5)
-
Jak1? Tyk2?
Stat3
(Stat1)
(Stat5)
Jak1? Tyk2?
Stat3
?

1.9.2 IL-20 Receptor Complex

IL-19, IL-20, and IL-24 all bind to the IL-20 receptor complex, which is designated IL-20R1 (CRF2-8) and IL-20R2(CRF2-9); IL-24 can also signal through a complex consisting of IL-22R and IL-20R2.(Blumberg et al., 2001; Dumoutier et al., 2001; Wang et al., 2002; Parrish-Novak et al., 2002). As mentioned above, these three cytokines have diverse biological functions ranging from immune function to skin development, and the observed sharing of receptors is indicative of a unique property of class II cytokine receptors.

IL-20R1 is the most widely expressed of these subunits and both IL-20R1 and IL-20R2 mRNA have been detected in the skin, testis, along with lung and ovary tissue (Blumberg et al., 2001). It seems that the expression of IL-20 R2 is dependent on the expression of IL-20R1 as IL-20R2 is only expressed in tissues that show IL-20R1 expression (Parrish-Novak et al., 2002). It has been shown through luciferase reporter assays that both subunits of the IL-20R complex are needed for proper activation. In the same experiment STAT3 was identified as the signal transduction pathway activated by IL-19, IL-20, and IL-24 binding to the IL-20R complex.

Whereas expression of, and ligand binding to the IL-20R complex seemed to be similar, functional differences of each cytokine binding has been reported. Growth inhibition assays have shown that IL-19 and IL-24 inhibit proliferation of NIH:OVCAR-3 cells, while IL-20 had no effect (Parrish-Novak et al., 2002). This difference is not explained by the receptor specificity, and the differential mechanisms will have to be eluicidated.

1.9.3 IL-22 Receptor Complex

A functional IL-22 receptor consists of IL-22R1 and IL-10R2, indicating that the second subunit of the IL-10 receptor is shared amongst IL-22 and IL-10. IL-22R1 has been detected in various normal tissues including the kidney and liver, with highest expression observed in the pancreas and intestine (Kotenko et al., 2001a; Aggarwal et al., 2001) IL-22 has equal affinity to each of these receptor subunits (Xie et al., 2000) and binding to the receptor complex involves the activation of stats 1, 3, and 5 (Kotenko et al., 2001, Dumoutier et al.,2000; Dumoutier et al., 2001; Kotenko et al., 2001).

Unique to the IL-22 receptor is that a soluble receptor from this family has been identified and is known as IL-22BP (CRF2-10). mRNA for IL-22BP has been detected in mononuclear cells of of inflammatory infiltration sites and plasma cells (Xu et al., 2001). IL-22BP binds IL-22 protein at a higher affinity than the functional membrane bound receptor, and the interaction of IL-22:IL-22BP prevents the binding of IL-22 to the functional membrane receptor. IL-22BP is specific for IL-22 as it can not neutralize the activity of IL-10, or bind to IL-19, IL-20, and IL-24 (Dumoutier et al., 2001c; Kotenko et al., 2001b; Dumoutier et al., 2001a;), suggesting that IL-22BP is a naturally occurring IL-22 antagonist.

CHAPTER 2

2.0 Hypothesis

The synovial fibroblast plays a key role in the development and maintenance of inflammation in RA through the formation of pannus and the secretion of cytokines. SF's are capable of synthesizing cytokines and other genes that are critical in disease development . An improved understanding of gene expression in SF will provide insight into the maintenance of inflammation associated with the diseased RA joint.

2.1 Objectives

1. To study novel cytokine gene expression in RA and OA synovial fibroblasts, and compare this expression to that of dermal fibroblasts;
2. To verify any novel cytokine gene expression by RT-PCR and quantitate expression by real time RT-PCR;
3. To identify cytokine receptors for the novel cytokines.

2.2 Significance of Research

Studies of gene expression in synovial fibroblasts have determined that the synovial fibroblast is a key source of cytokines present in an inflamed joint. Any further elaboration on cytokine expression capabilities may provide new knowledge into mechanisms involved in RA inflammation, and possibly create new alternatives for therapy. Cytokine production in synovial fibroblasts has not been previously studied in great detail, and new research would greatly contribute to the knowledge of cytokine

expression abilities of fibroblasts in general, and synovial fibroblasts in arthritis, in particular.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Synovial Tissue

3.1.1 Synovial Cell Culture

Synovial fibroblast cell cultures were initiated from tissue removed during joint surgery or synovial biopsy of patients with RA or OA. This study was approved by the Research Ethics Board of The Ottawa Hospital. Tissue samples from 12 female (6 RA, 6 OA) and 1 male patient (OA) were studied. The patients average age was 66.4 years and disease duration was 10 years. Synovial fibroblast cultures were established as previously described (Firestein, 1996; Sakurada et al., 1996; Yoshida et al., 1999). Briefly, synovial tissue obtained at surgery was minced into small pieces and treated with 1 mg/mL collagenase for 10-20 min at 37°C. Cells were then washed and cultures were maintained in RPMI-1640 (BioWhittaker) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine. The cell culture medium was changed every 7 days and non-adherent cells were removed; the remaining adherent cells were used for the experiments described. Cells used in this study were from either the 4^{th} or 5^{th} passage in culture.

3.1.2 Foreskin Fibroblast Cell Culture

The dermal (foreskin) fibroblast cell line (Hs68) was obtained from ATCC. Dermal fibroblast samples were maintained in the same media as synovial fibroblasts. Purity of fibroblasts maintained in long term culture were assessed by flow cytometry specific for

the fibroblast marker Thy-1. Cells were also assessed for CD14 and CD3 and were negative (data not shown).

3.1.3 PHA activation of PBMC

Total PBMC (3×10^6) isolated from fresh human blood were maintained in the same media as fibroblasts and were activated with 10 μg/mL PHA for 72 h. Cellular RNA was obtained immediately after incubation at 72 hours.

3.1.4 Monocyte Cell Culture

PBMC were isolated from normal human blood by centrifugation through Histopaque 1077 (Sigma) and were enriched for monocytes by adherence to plastic Briefly, PBMC (2×10^6 cells/mL) were maintained in serum-free DMEM for 1 hour at 37°C, 5% CO_2 and non-adherent cells were removed by pipetting. The medium was replaced with fresh complete culture media and adherent monocytes were incubated in either the presence or absence of 100 ng/mL LPS for 4 h.

3.1.5 EBV B cell Culture

An EBV B cell line was obtained and cultured in complete media (in RPMI-1640 (BioWhittaker) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine) for 4-6 days. Cells were pelleted after 6 days and RNA was isolated.

3.1.6 HeLa Cell Culture

HeLa cell line HeLa 229 were obtained from ATCC and cultured similarily to that of dermal fibroblasts. Cells were resuspended every 7 days, and total RNA was isolated.

3.2 Molecular Biology Techniques

3.2.1 Total Cellular RNA Preparation

Total cellular RNA was prepared from all cell types using TRIzol reagent (Life Technologies). Briefly, 1x 10^6 cells were isolated from culture and prepared for RNA isolation. Cells were pelleted and disrupted using 1mL of TRIzol reagent. Following treatment with chloroform the upper clear aqueous layer was collected and RNA was precipitated with isopropanol. The RNA pellet was washed twice with 75% ethanol, and dissolved in a small amount of diethyl pyrocarbonate (DEPC)-treated water.

3.2.2 Quantitation of Cellular RNA

RNA obtained from RNA preparation was quantitated by spectrophotometry in a Beckman Coulter Du 640B spectrophotometer (Beckman Coulter, USA). The absorbance at 260nm (A_{260}) was used to determine concentration of RNA using the following formula for single stranded RNA (ssRNA):

$$(A_{260}/0.25) \times \text{dilution factor}$$

A stock solution of RNA was made for each sample and stored at a concentration ~0.5 μg/μL in a -20°C freezer.

3.2.3 Agarose Gel Electrophoresis of RNA

To determine purity of isolated RNA after quantitation, each RNA sample was subjected to agarose gel electrophoresis using the Hoefer HE 33 Mini Submarine or Hoefer HE99X Max Submarine Units (Amersham Biosciences) as directed by the manufacturer's instructions. Electrophoresis grade agarose (GibcoBRL) was added to a final concentration of 1.0% (w/v) in 1x TAE buffer that was diluted 1:10 with 10X TAE buffer (48.4 g Tris-base, 11.4 ml glacial acetic acid,20.0 ml 0.5 M Na_2EDTA, pH 8.0 to 1000 mL with deionized water), and 0.02% ethidium bromide. Prior to loading, 2 μL of RNA preparations were mixed with 2 μL of 6x DNA loading dye (MBI Fermentas), and 6 μL of TE buffer(10 mM Tris, 1mM EDTA, pH 8.0). Electrophoresis was carried out under a constant voltage of 100V from anywhere between 40-60 minutes. After electrophoresis the gel was visualized under UV light using a MultiImage Light Cabinet (Alpha Innotech Corp.; San Leandro, CA, USA). The size of each band was determine by comparison to the migration of a 1kb DNA ladder (MBI Fermentas)

3.2.4 Reverse transcription of RNA

To produce complementary (c) cDNA from isolated RNA, reverse transcription using the Superscript II first strand synthesis system (Invitrogen) plus oligo dT was carried out. This system uses the MMLV reverse transcriptase enzyme and is very efficient at producing cDNA. Briefly 1μg RNA was used, and the reaction mixture contained 25 ug/ml oligo dT(12-18), 0.01 M DTT, 0.5 M dNTP (dATP, dCTP, dGTP, dTTP), and 200 units of Superscript II enzyme. After preparation of cDNA, the final mixture was maintained at -20°C for extended periods of time.

3.2.5 Microarray Gene Expression Analysis

The gene expression profile each cell studied was assessed using low- density microarrays [GEArray Q series Human Common Cytokine Gene Array (HS-003), GEArray Q series Human Interleukins and Receptors Gene Array (HS-014), and GEArray Q series Extracellular Matrix & Adhesion Molecules Gene Array (HS-010) from Superarray Inc.]. Total cellular RNA was used to generate cDNA according to manufacturer's instructions using 2 μg of RNA and MMLV reverse transcriptase (Invitrogen) in the presence of biotinylated-dUTP, using primers supplied by the manufacturer (Superarray). After hybridization to the membrane and extensive washing, bound cDNA was detected by chemiluminescence using X-ray film (Kodak, Biomax). The intensity of each sport on the array was calculated using Scanalyze Software (Eisen labs, http://rana.lbl.gov/EisenSoftware.htm) and was compared to internal controls (actin, GAPDH).

3.2.6 Polymerase Chain Reaction (PCR) Amplification

Total cellular RNA (1 μg) was also used as a template for RT-PCR. cDNA was first synthesized using the Superscript II first strand synthesis system (Invitrogen) plus oligo dT. Specific primers for both IL-19 (Forward: 5'-ACATGCACCATATAGAAGAG-3'; Reverse: 5'-GCTGAGGACATTACTTCAT-3') and IL-22 (Forward: 5'-TCAGAACAGGTTCTCCTTCC-3'; Reverse: 5'-GACATGTGCTTAGCCTGTTG-3') were designed based on the known sequence for each gene. Conditions for IL-19 PCR was as follows: denaturation at 94°C for 4 min; 40

amplification cycles of 94°C 1 min, 54°C 1 min, 72°C 1 min, with a final 10 min elongation step at 72°C. Conditions for IL-22 PCR were identical except that that the annealing temperature was 53°C and 10% DMSO was present in the reaction mixture. The resulting PCR products were cloned into the TA vector (Invitrogen) and sequenced at the University of Ottawa Biotechnology Research Institute.

3.2.7 Agarose Gel Electrophoresis of DNA

To determine the accuracy and specificity of each PCR reaction, all PCR products were subjected to agarose gel electrophoresis using the Hoefer HE 33 Mini Submarine or Hoefer HE99X Max Submarine Units (Amersham Biosciences) as directed by the manufacturer's instructions. Electrophoresis grade agarose (GibcoBRL) was added to a final concentration of 1.2% (w/v) in 1x TAE buffer that was diluted 1:10 with 10X TAE buffer (48.4 g Tris-base, 11.4 ml glacial acetic acid,20.0 ml 0.5 M Na_2EDTA, pH 8.0 to 1000 mL with deionized water), and 0.02% ethidium bromide. Prior to loading, 20 μL of PCR product was mixed with 2 μL of 6x DNA loading dye (MBI Fermentas). Electrophoresis was carried out under a constant voltage of 100V from anywhere between 40-60 minutes. After electrophoresis the gel was visualized under UV light using a MultiImage Light Cabinet (Alpha Innotech Corp.; San Leandro, CA, USA). The size of each PCR fragment was determined by comparison to migration distance of a 1 kilobase (kb) DNA ladder (MBI Fermentas).

3.2.8 Isolation and purification of DNA Fragments

DNA fragments obtained from PCR reactions were separated by 1.2% (w/v) agarose gel electrophoresis. The desired PCR fragment was isolated and purified from agarose gel using the Prep-A-Gene® DNA Purification system (BIORAD) according to manufacturers recommendations.

3.2.9 Cloning and Ligation of DNA Fragments

Once PCR generated DNA fragments were isolated from agarose gel, they were cloned into a TA-based vector following manufacturers instructions (Invitrogen).

3.3 Quantitative Real Time PCR Reactions

3.3.1 PCR reaction conditions

cDNA generated from total RNA (as described above) was also subjected to real time PCR as follows. Reaction mix (in a 20 μL reaction volume) contained 1× buffer (Amersham), 0.8 mM of each dNTP. 2.5 mM $MgCl_2$, 0.4 μM of each primer (primers for IL-19 were the same as used for RT-PCR while the sequence of the IL-22 primers were Forward: 5'-TTCCCCAGTCACCAGTTGC-3'; Reverse: 5'-ATAGGGCTGCTGGAAGTTG-3'), 0.5 mg/mL BSA, 1/40,000 SYBR Green I DNA dye (Molecular Probes), 2 U *Taq* DNA polymerase (Amersham) and 2 μL of template. Template was either 2 μL of total cDNA or 2 μL of TOPO plasmid containing the IL-19 sequence at indicated concentrations. The following LightCycler (Roche) protocol was used for IL-19 target and reference sequences: denaturation at 95°C for 30 sec, 40 cycles of 95°C for 0 sec, 54°C for 8 sec, 72°C for 20 sec, and fluorescence acquisition at 79°C

for 2 sec; melting curve program (61°C-98°C, 0.1°C/sec slope, continuous fluorescence measurement) and a cooling step to 40°C for 30sec. The protocol for IL-22 target and reference sequence was as follows: 95°C for 30 sec, 40 cycles of 95°C for 0 sec, 58°C for 8 sec, 72°C for 20 sec, and fluorescence acquisition at 79°C for 2 sec; melting curve program (61°C-98°C, 0.1°C/sec slope, continuous fluorescence measurement) and a cooling step to 40°C for 30 sec.

3.3.2 Generation of Standard Curve

Quantification of product was performed using a standard curve method. A standard curve was generated by 4 to 6 10-fold serial dilutions of each vector, starting at 0.29 ng/μL for IL-19, 0.23 ng/μL for IL-22, and 7.5 ng/μL for GAPDH. The concentrations of IL-19, IL-22, and GAPDH in each cDNA sample were estimated from their crossing points, compared to the standard curve. The final results for each sample were then normalized against GAPDH.

3.4 Cytokine Induced T cell proliferation assays

3.4.1 T cell samples

Total PBMC ($3x10^7$) were isolated from fresh human blood from non-arthritic patients using Histopaque gradient (Sigma) according to the manufacturer's instructions. The lymphocyte band (at the interface) was carefully removed and washed in RPMI 1640 medium (Sigma) supplemented with 10%, v/v, fetal calf serum, 1%, w/v, sodium

piruvate, 0.002 M L-glutamine, 100 UI/ml penicillin and 100 μg/ml of streptomycin (complete medium). T cells were isolated from total PBMC using flow cytometry with markers specific for CD3, CD4, and CD8 at the Flow Cytometry Facility of the Ontario Genomics Innovation Centre. Cells were isolated based on CD3+CD4+, or CD3+CD8+, and the total T lymphocyte population was 75% CD3+CD4+ and 25% CD3+CD8+. In this study, T lymphocytes from 6 patients were used, and two groups of T cells ($1x10^6$) from each patient were either unstimulated, or stimulated with 10μg/mL PHA for 72 hours.

3.4.2 T cell Proliferation assay

To determine the reactivity of activated/unactivated T cells to various cytokines, a T cell proliferation assay was performed. Activated and non-activated T cells were re-suspended in RPMI-1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma, Oakville, ON), penicillin/streptomycin and L-glutamine (Life Technologies). Activated and unactivated T cells were plated in triplicate in 96-well round-bottom tissue culture plates (2×10^5 per well) in a total volume of 200 μL and were incubated in the presence of either IL-2, IL-10, IL-19, or IL-22 at a concentration range of 0.1-0.5 μg/mL. After 3 days incubation, T cells were pulsed with [^{3}H] thymidine (1 μCi per well) for an additional day and then harvested onto filter papers. Incorporated radioactivity was measured using a Matrix direct beta counter (Packard).

3.4.3 Statistical Analysis

For comparison of normalized quantitative values between different cell types from RT-PCR, a paired *t*-test was applied. $P < 0.01$ was considered significant.

3.5 Protein Biochemistry

3.5.1 Western Blot Analysis

Western blot analysis was performed on a small number of OA and RA synovial fibroblasts to detect functional IL-19 and IL-22 at the protein level. 1 x 10^6 cells were removed from culture and resuspended in RPMI-1640, washed two times with ice cold PBS and centrifuged for 5 minutes. All remaining supernatant was removed and the cells were lysed by adding 30 μL of 2x SDS sample buffer. The samples were stored on ice for 5 minutes and immediately boiled for 5 minutes. Before loading into gel, the samples were spun for 30s at 12,000 RPM. Samples were resolved on a 12% SDS-PAGE for 1 hour at 175V. After one hour of resolving, the SDS gel was placed in Bierrum and Shafer-Nielson (BSN) transfer buffer (pH 9.2, 0.005% Tris base, 0.003% glycine, 20% methanol, 10% SDS) for 15 minutes in preparation for transfer to a nitrocellulose membrane. Protein was transferred to a nitrocellulose membrane using a Trans Blot SD (Biorad), and transfer occurred for 35 minutes at 15V. After protein was transfered to a nitrocellulose membrane, the membrane was incubated with western blocking buffer (1% BSA dissolved in PBS and 0.1% Tween 20) overnight at 4°C. The membrane was then incubated with either rabbit-anti-human IL-19 or IL-22 antibody diluted 1:1000 in western blocking buffer for one hour with rocking. Primary antibody was discarded and the membrane was washed 3 times with PBS/0.1% Tween-20. The membrane was then

incubated with a goat-anti-rabbit secondary antibody diluted 1:1000 for one hour. Secondary was discarded and washed as above. After this final washing the membrane was incubated for 10 minutes in AP buffer (1.2% tris base, 0.1M NaCl, 0.02M $MgCl_2$). For detection, AP detection solution (Invitrogen: consisting of Nitro blue tetrazolium (NBT) and 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) diluted in AP buffer) for five minutes. After visualization of protein bands, 5mL of AP stop buffer(PBS, 0.5M EDTA) was added. The membrane was then air dried and sealed in a plastic bag.

3.5.2 Enzyme Linked Immuno Specific Assay (ELISA)

For detection of secreted IL-19 and IL-22 in synovial cell culture supernatants, ELISA was performed. NUNC immuno flat bottom 96 well plates were incubated with synovial cell culture supernatant, while standards consisted of either recombinant human (rh) IL-19 or IL-22 protein(Peprotech), and were incubated overnight at 4°C. After overnight incubation, the supernatant and protein samples were discarded and 200 μL of blocking buffer (1%BSA in PBS) was added to each well and incubated for 1 hour at room temperature. The blocking buffer was discarded after incubation and the remaining wells were washed 3 times using PBS+0.05% Tween-20. Next, goat anti human IL-19 or IL-22 was diluted in 0.1%BSA-PBS + 0.5% Tween-20, and 100 μL were added to each well and incubated at room temperature for 2 hours with rocking. The primary antibody solution was discarded, and the wells were washed 6 times with PBs-0.05% Tween-20. The secondary antibody, biotin-labelled rabbit-anti-goat was diluted similar to the primary antibody and added to each well. Incubation of secondary antibody was for 2 hours at room temperature with rocking, and after incubation, was

washed 6 times with PBS-0.05% Tween-20. For detection, 100μLof avidin HRP (Pharmingen 554058) diluted 1:5000 in 0.1%BSA-PBS + Tween-20 and incubated for 1 hour with rocking at room temperature. The aviding was discarded after an hour and wells were washed 6 times, soaked for 10 minutes, and washed 6 more times with PBS-0.05% Tween-20. 100 μL of TMB peroxidase substrate was then added to each well for development. Once an appropriate standard curve was developed, the reaction was stopped by adding 100 μL of 1N HCl and the plate was read at 450 nm using a Multiskan Ascent plate reader.

CHAPTER 4

RESULTS

4.1 Statistical Data on Patients in this Study

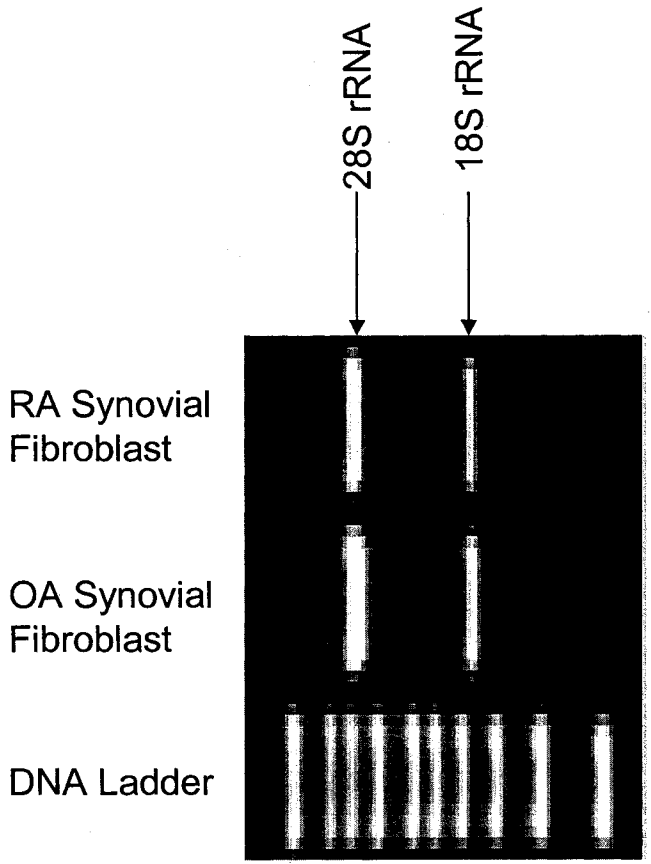
Synovial tissue was obtained from a patient group that consisted of 6 RA, and 7 OA patients who were undergoing joint replacement surgery at the General, Riverside, and Civic campuses at the Ottawa Hospital. The RA patients had an average age of 66±11.3 years and were all female. The OA patients had an average age of 64.3±10.2 years, and included one male patient. Thus the age range of each population of patients is similar. The dermal fibroblast cell line was considered as another fibroblast control.

4.2 Identification of total RNA Isolated from Cells

After total RNA was isolated from each cell line, it was subjected to agarose gel electrophoresis to determine purity of the RNA generated. This was performed on every isolated RNA sample and can be viewed in Figure 4.1. Positions of bands corresponding to the 28S and 18S rRNA are also indicated.

Figure 4.1 Electrophoretic identification of total cellular RNA from representative RA and OA FLS

Total RNA was isolated from 5 x 10^5 OA and RA FLS in long term culture using TRIzol reagent (Life Technologies) according to manufacturers recommendations. RNA samples were loaded into a 1.0%(w/v) agarose gel and viewed under UV light after electrophoresis.


28S rRNA
18S rRNA
RA Synovial Fibroblast
OA Synovial Fibroblast
DNA Ladder

4.3 DNA Microarray Analysis

4.3.1 Cytokine mRNA analysis in Fibroblasts

FLS from OA and RA patients were obtained from synovial tissue removed during routine surgery or biopsy of patients. After *in vitro* expansion, the cytokine/cytokine receptor gene expression pattern was assessed by low density microarray analysis using a common cytokine gene array (Superarray). The genes on this array are grouped into several functional families/groups that include bone morphogenic proteins, colony stimulating factors, fibroblast growth factors, interleukins, and interferons (listed at http://www.superarray.com/gene_array_product/HTML/HS-003.html). An example of the gene expression pattern of FLS from a representative RA patient is shown in Fig. 4.2. This expression pattern was found to be highly consistent and a very similar pattern was observed in all synoviocyte samples regardless of whether they were derived from OA or RA patients. Notably, four cytokine genes, highlighted in Fig. 1, were constitutively expressed by these cells in culture (IL-15, IL-19, IL-22, and TNFα). Of these, IL-19 was found to be the most highly expressed cytokine, based on hybridization intensity. Expression of IL-15 and TNFα by cells of the fibroblast lineage has been previously described (Curfs et al., 1997; Harada et al., 1999) so their expression was not investigated further. However, IL-19 and IL-22 have only been recently discovered and information regarding the source of these cytokines is limited. For comparison, we also assessed the cytokine expression pattern of normal dermal (foreskin) fibroblasts. We observed that these cells constitutively expressed the same pattern of cytokines, including IL-19 and IL-22 (see Fig. 4.3). The quantitative level of expression for five of the genes expressed

by these cell types (after normalizing against the constitutively expressed gene GAPDH) is shown graphically in Fig 4.4. FLS and dermal fibroblasts also express a similar profile of mRNAs encoding various fibroblast growth factors (upper part of array), providing additional evidence that these two cell types are highly related. Fibroblast growth factors are thought to be involved in the development of the skeletal system and their expression was not followed. The only other member of the IL-10 family represented on this gene array was IL-20, which was not expressed in any of the samples studied. The positive and negative controls on this array were GAPDH and PUC18, respectively. The positive control was expressed, while the negative control was not expressed, again validating this methodology for mRNA analysis.

4.3.2 Cytokine Receptor Analysis in FLS

Based upon our novel observation of IL-19 and IL-22 expression by cells of the fibroblast lineage, we next assessed whether fibroblasts also express mRNAs encoding IL-19 and IL-22 receptor molecules. Such expression would allow these cytokines to function in an autocrine fashion. The IL-10 family of cytokines exhibits extensive sharing of receptor subunits. IL-19 is known to signal through the IL-20Rα/IL-20Rβ complex and IL-22 is thought to signal through a complex comprised of IL-10Rβ plus IL-22Rα. A low density microarray analysis examining 72 human interleukins and interleukin receptors (Superarray) indicated that FLS do not express IL-20Rα and thus would not be expected to signal in response to IL-19 (Fig.4.5). This array does not contain IL-20Rβ, so we have no information about the expression of the second receptor subunit. Furthermore, these cells expressed neither the IL-22R or the IL-10Rβ thus they are unlikely to be responsive

to IL-22. Interestingly, these same cells strongly expressed the mRNA encoding IL-22Rα2, also known as IL-22BP, a soluble form of IL-22R that is thought to act as an IL-22 antagonist (Kotenko et al., 2001b; Dumoutier et al., 2001a; Gruenberg et al., 2001. IL-19 and IL-22 hybridization signals were also present on this membrane consistent with results obtained using the common cytokine gene array (see Fig. 4.2 and 4.3). Also of interest was the finding that FLS also express the mRNA encoding IL-23, a recently discovered member of the IL-12 family with a potential role in the induction of Th1 type cytokine production by memory T cells (Oppmann et al., 2000). Quantitative data after normalization against the constitutively expressed gene GAPDH from this experiment is shown graphically in figure 4.6.

4.3.3 ECM and Adhesion Molecule Analysis in FLS and Dermal Fibroblast.

FLS from a representative RA patient, and from a dermal fibroblast cell line were obtained as previously described. After *in vitro* expansion, the extra cellular matrix and adhesion molecule receptor gene expression pattern was assessed by low density microarray analysis using a extra cellular matrix gene array (Superarray). This microarray contains 96 genes encoding cell adhesion and extracellular matrix proteins, both of which are relevant to the pathogenesis of RA. ECM proteins are known to be involved in the pathology of RA and thus the FLS expression capabilities are of interest. The genes on this array are grouped into cell adhesion molecules, ECM proteins, proteases, and protease inhibitors
(Listed at: http://www.superarray.com/genetable.php?pcatn=MM-010). An example of the gene expression pattern of FLS from a representative RA patient is shown in Fig. 4.7.

Of special notice, this array showed the FLS can transcribe mRNA for a variety of matrix MMP's including MMP-2, MMP-26, while Fig. 4.8 displays that dermal fibroblasts can transcribe mRNA for MMP-10, and MMP-14. Common genes expressed by both of these cell lineages was TIMP-1, TIMP2, and TIMP-3. The functions of these proteins will be presented in Chapter 5. A quantitative representation of this gene expression in both FLS and dermal fibroblasts can be seen in Fig. 4.9.

Figure 4.2 Analysis of cytokine mRNA expression in RA synovial fibroblasts

Total RNA from FLS obtained from tissue explants removed during joint replacement surgery of an RA patient and maintained *ex vivo* for 7 days was analyzed by low density microarray using the Q-series human common cytokine array (Superarray Inc.). Positions of spots corresponding to Fibroblast Growth Factors, IL-15, IL-22, IL-19, TNFα and GAPDH are indicated by circles on the left and right side of the array, respectively.

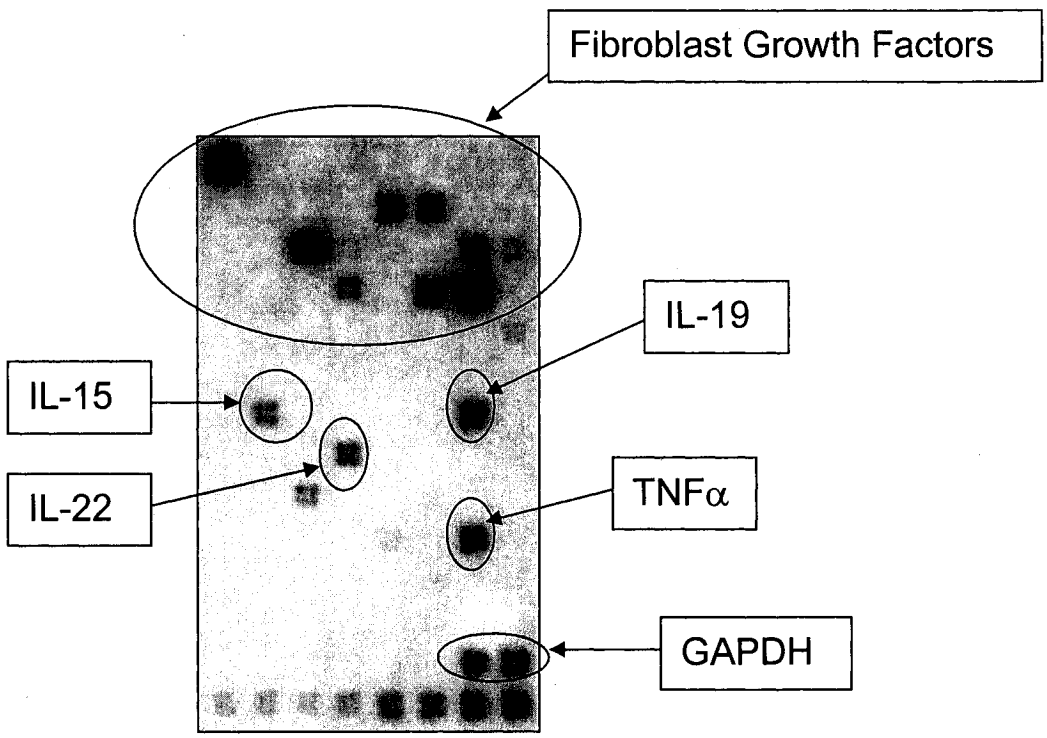

Fibroblast Growth Factors
IL-19
IL-15
IL-22
TNFα
GAPDH

Figure 4.3 Analysis of cytokine mRNA expression in dermal (foreskin) fibroblasts

Total RNA from dermal (foreskin) fibroblasts purchased from ATCC and maintained *ex vivo* for 7 days was analyzed by low density microarray using the Q-series human common cytokine array (Superarray Inc.). Positions of spots corresponding to Fibroblast Growth Factors, IL-15, IL-22, IL-19, TNFα, and GAPDH are indicated by circles on the left and right side of the array, respectively.

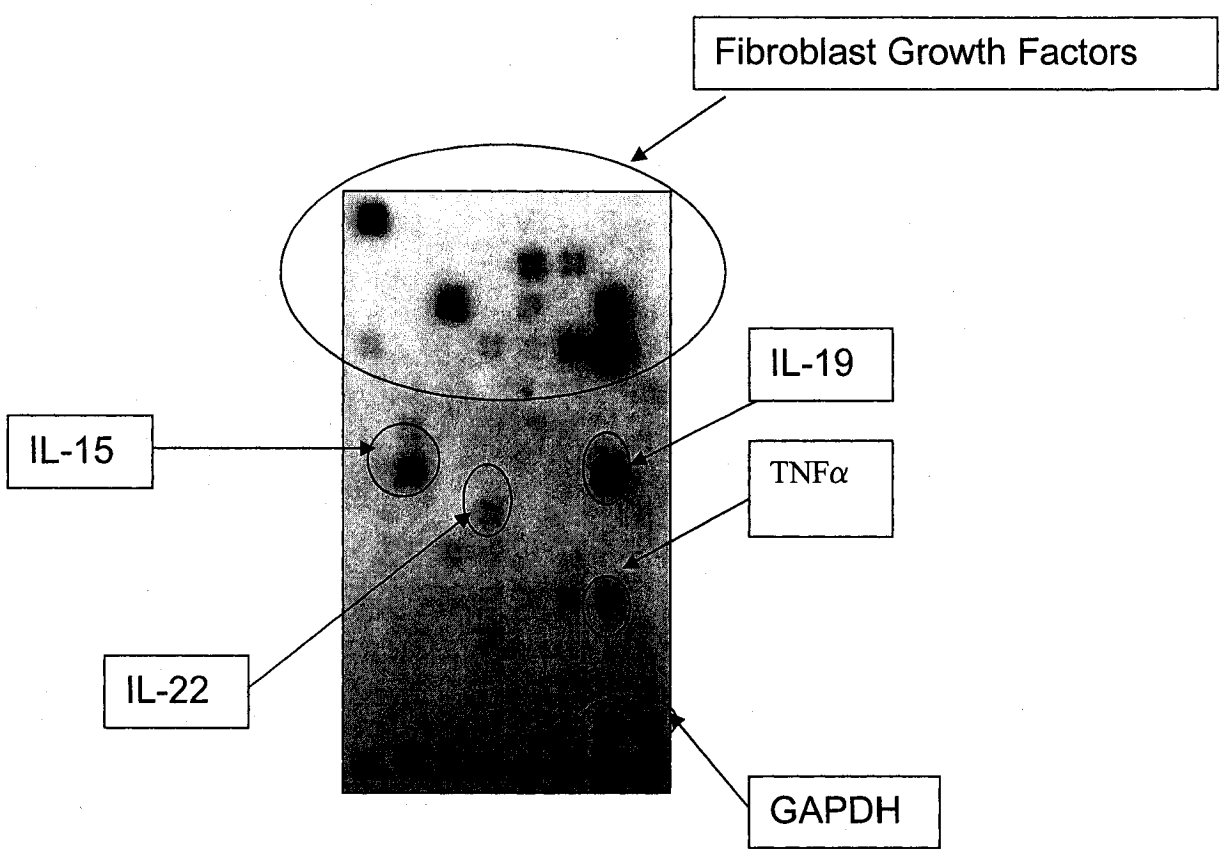

Fibroblast Growth Factors
IL-19
IL-15
TNFα
IL-22
GAPDH

Figure 4.4 Quantitative representation of data shown in figures 4.1 and 4.2.

Signals of spots corresponding to genes that were consistently found to be expressed by FLS and dermal fibroblasts are shown graphically after normalization to GAPDH mRNA. Genes represented include IL-9, IL-15, IL-19, IL-22, and TNFα are shown. This pattern was similar for all samples studied. There were no significant differences observed between each.

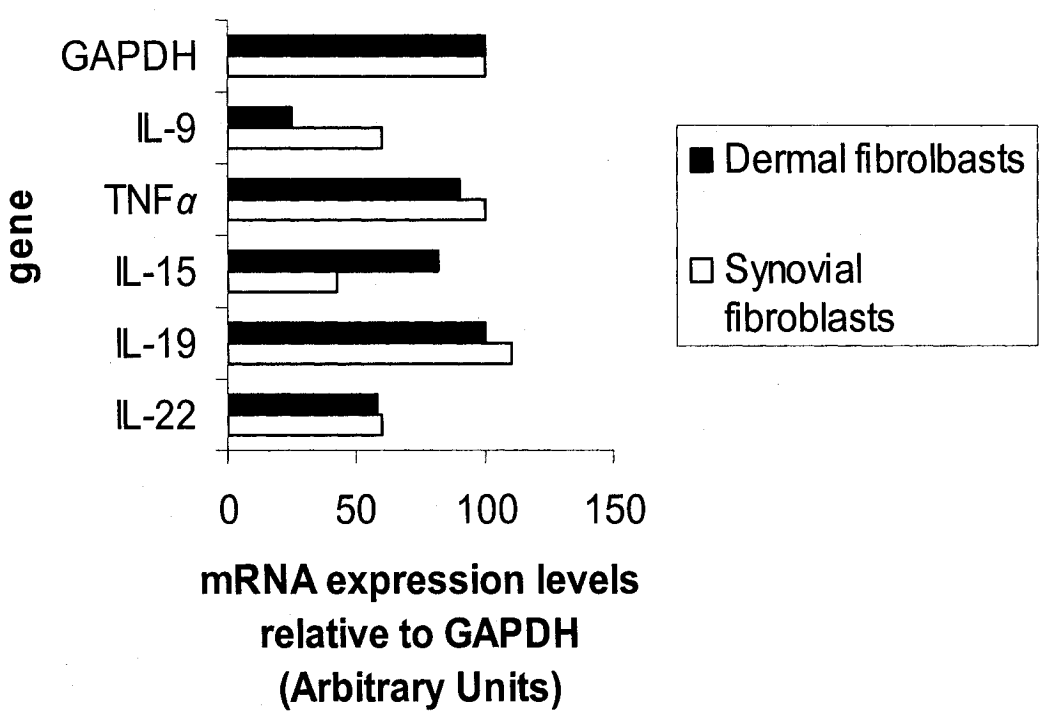

GAPDH
IL-9
TNFα
IL-15
IL-19
IL-22
gene
0
50
100
150
mRNA expression levels relative to GAPDH (Arbitrary Units)
Dermal fibrolbasts
Synovial fibroblasts

Figure 4.5 Analysis of Interleukin and Interleukin Receptor mRNA expression in RA FLS.

Total RNA was obtained from RA FLS and was analyzed by low density microarray analysis using the Q-Series human Interleukin and Receptor Gene Array (Superarray Inc.) Positions of spots corresponding to IL-22BP (IL-22Rα2), IL-20Rα, IL-23A, IL-10Rβ, IL-19, IL-22, and GAPDH are indicated.

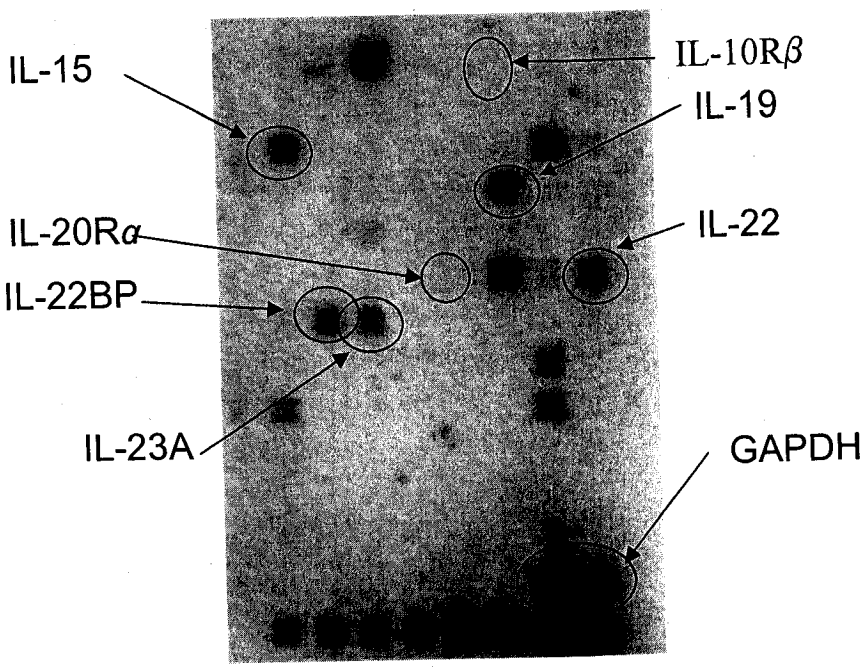

IL-15
IL-10Rβ
IL-19
IL-20Rα
IL-22
IL-22BP
IL-23A
GAPDH

Figure 4.6 Quantitative representation of data shown in figure 4.5.

Signals of spots corresponding to genes corresponding to interleukins and receptors that were consistently found to be expressed by RA FLS are shown after normalization to GAPDH mRNA. This pattern was similar in all FLS samples tested (n=1).

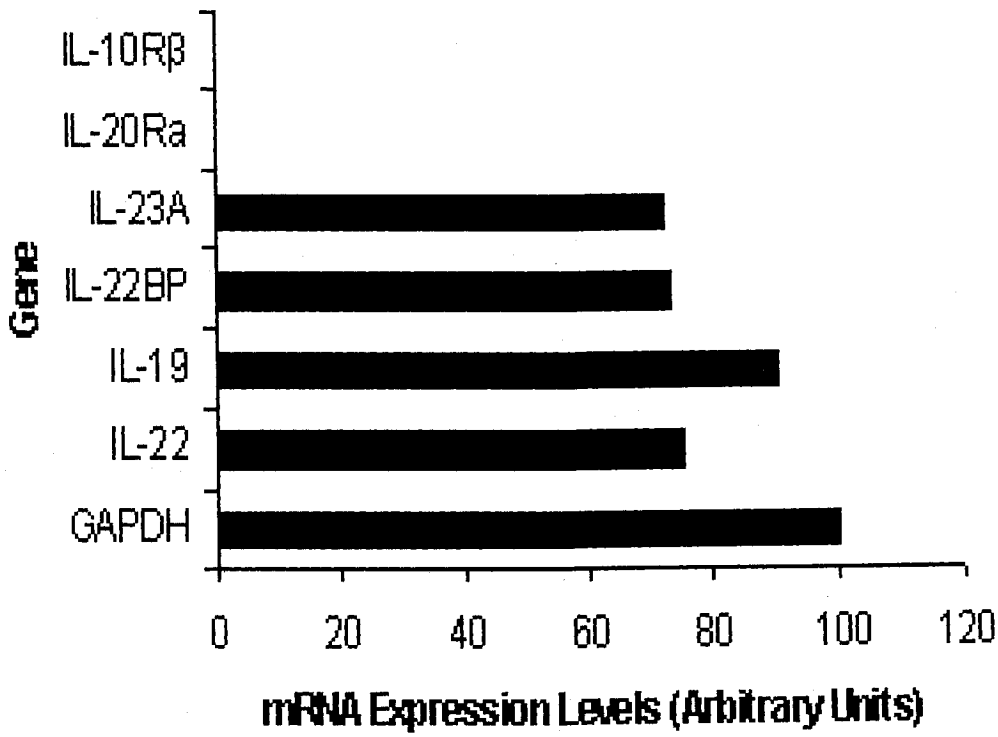

IL-10Rβ
IL-20Ra
IL-23A
IL-22BP
IL-19
IL-22
GAPDH
Gene
0
20
40
60
80
100
120
mRNA Expression Levels (Arbitrary Units)

Figure 4.7 Extra Cellular Matrix and Adhesion Molecule Expression in RA Synovial Fibroblasts.

Total RNA was obtained from RA FLS and was analyzed by low density microarray analysis using the Q-Series human Interleukin and Receptor Gene Array (Superarray Inc.) Positions of spots corresponding to MMP-2,10,14, 26, TIMP1, 2, 3, CAM-1, and fibronectin (FN)-1 are indicated

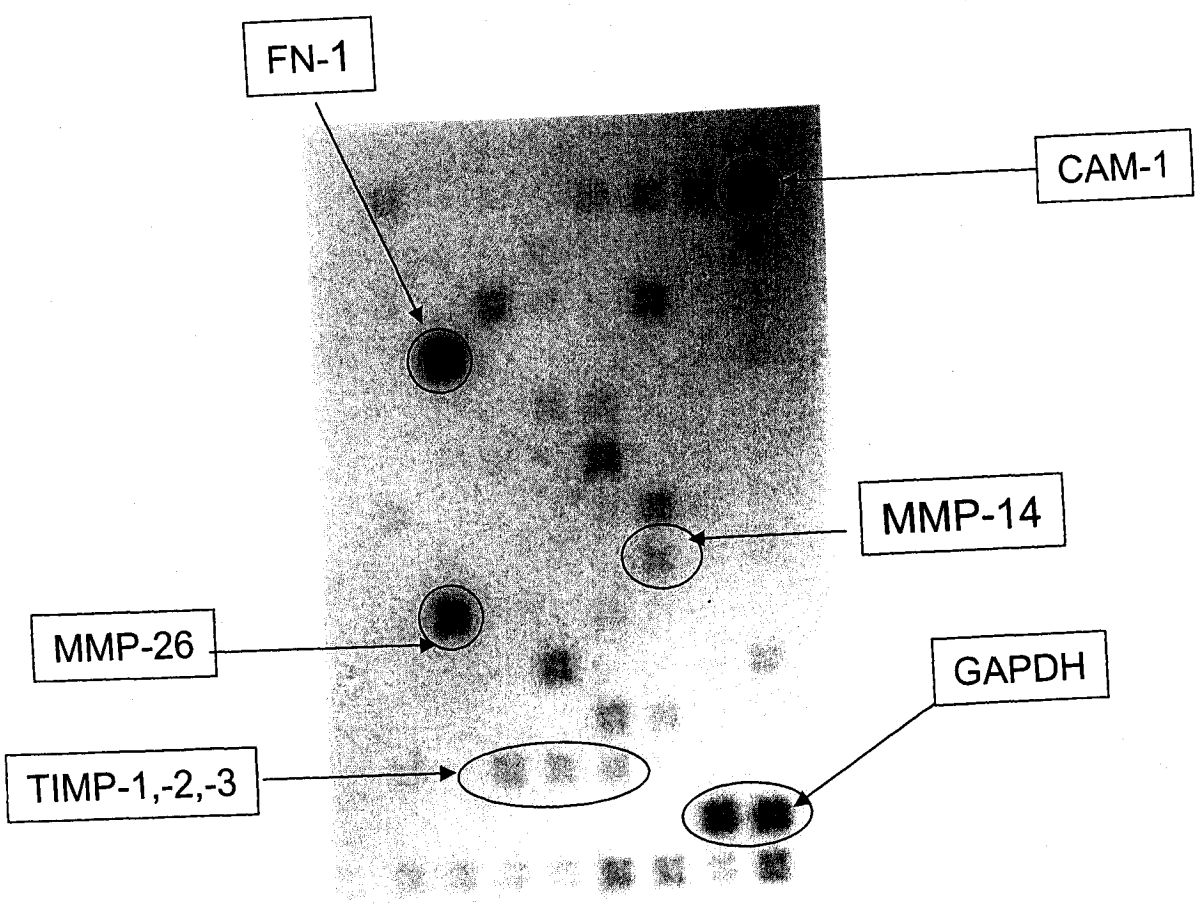

FN-1
CAM-1
MMP-14
MMP-26
GAPDH
TIMP-1,-2,-3

Figure 4.8 Extra Cellular Matrix and Adhesion Molecule Expression in Dermal (Foreskin) Fibroblasts.

Total RNA was obtained from dermal (foreskin) fibroblasts was analyzed by low density microarray analysis using the Q-Series human Interleukin and Receptor Gene Array (Superarray Inc.) Positions of spots corresponding to MMP-2,10,14, 26, TIMP1, 2, 3, CAM-1, and fibronectin (FN)-1 are indicated

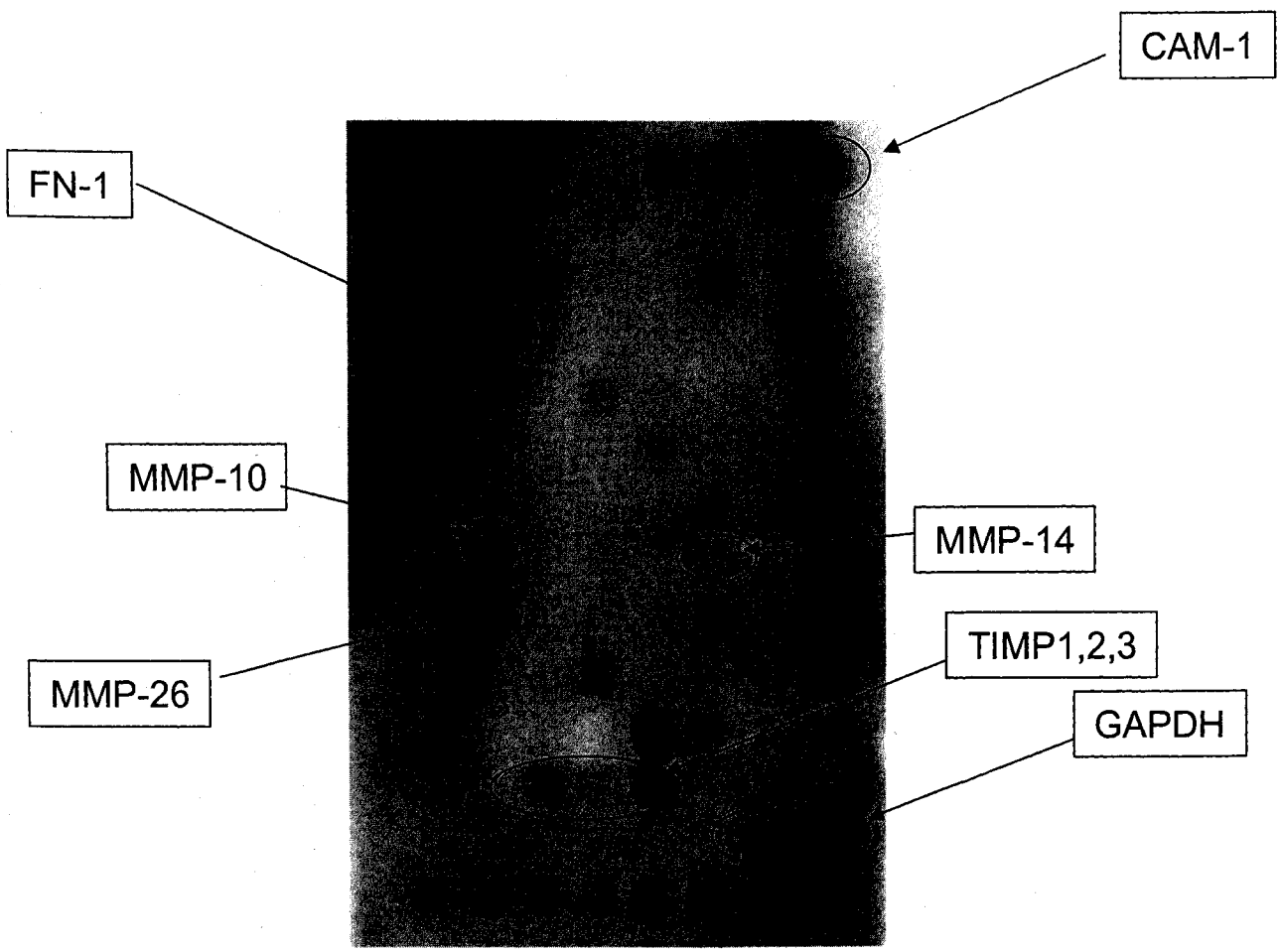
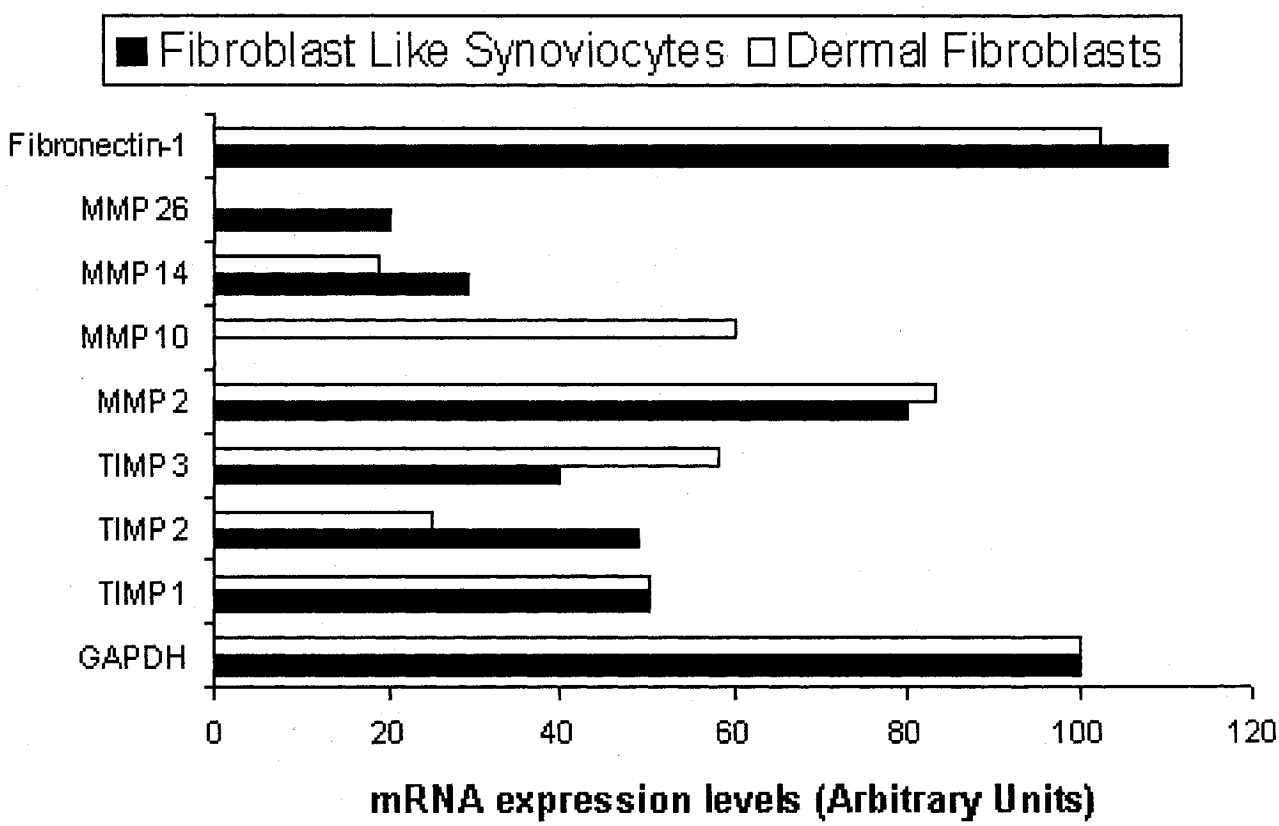

CAM-1
FN-1
MMP-10
MMP-14
TIMP1,2,3
MMP-26
GAPDH

Figure 4.9 Quantitative Representation of Data Presented in Figure 4.7

Signals of spots corresponding to genes that were consistently found to be expressed by FLS and dermal fibroblasts are shown after normalization to GAPDH mRNA. Genes represented include TIMP-1,-2,-3, MMP-2, -10,-14, 26, and FN-1 are indicated. There was no significant differences between each cell type


Fibroblast Like Synoviocytes
Dermal Fibroblasts
Fibronectin-1
MMP 26
MMP 14
MMP 10
MMP 2
TIMP 3
TIMP 2
TIMP 1
GAPDH
0
20
40
60
80
100
120
mRNA expression levels (Arbitrary Units)

4.4 Sequence analysis of IL-19 and IL-22 mRNAs expressed by synovial fibroblasts

Previously, LPS-activated macrophages and mitogen-stimulated T cells were the only cell types known to express significant levels of the IL-10-related cytokines IL-19 and IL-22, respectively. Thus, to confirm that fibroblasts also expressed these cytokines and to exclude the possibility that the signals observed on microarrays were due to spurious cross-hybridization or mis-hybridization, we performed RT-PCR analysis of total cellular RNA from untreated adherent monocytes, LPS-treated monocytes, FLS, PHA-treated T cells, dermal fibroblasts, and Jurkat T cells using IL-19 and IL-22 specific primers. PCR products of the expected size were obtained when IL-19 specific primers were used to amplify cDNA from untreated adherent monocytes, LPS-treated monocytes, RA FLS, OA FLS, and dermal fibroblasts (Fig. 5.0). As a negative control, we found no product when cDNA of Jurkat T cells was used as a template. Similarly, PCR products of the expected size were obtained when IL-22 specific primers were used to amplify cDNA from PHA-stimulated total PBMC, RA FLS, OA FLS and dermal fibroblasts but not Jurkat T cells (Fig. 5.1). IL-19 and IL-22 RT-PCR products obtained from synoviocyte RNA samples were subcloned into a TA-based cloning vector (Invitrogen) and were subjected to nucleotide sequence analysis. A nucleotide BLAST search confirmed that these PCR products corresponded to authentic human IL-19 and IL-22 mRNAs and that no sequence changes indicative of alternative splicing or other variations in expression were observed (data not shown). These findings provide the first clear evidence that FLS are capable of transcribing mRNAs coding for the cytokines IL-19 and IL-22.

Figure 5.0 IL-19 mRNA expression in monocytes, LPS-treated monocytes, and FLS.

Total RNA (1 μg) obtained from untreated adherent monocytes (*lane 2*), monocytes stimulated with LPS (100 ng/mL) for 4 hours (*lane 3*), RA FLS (*lane 4)*, OA FLS (*lane 5*), dermal (foreskin) fibroblasts (*lane 6)*, or Jurkat T cells (*lane 7*) was analyzed for expression of IL-19 by RT-PCR.

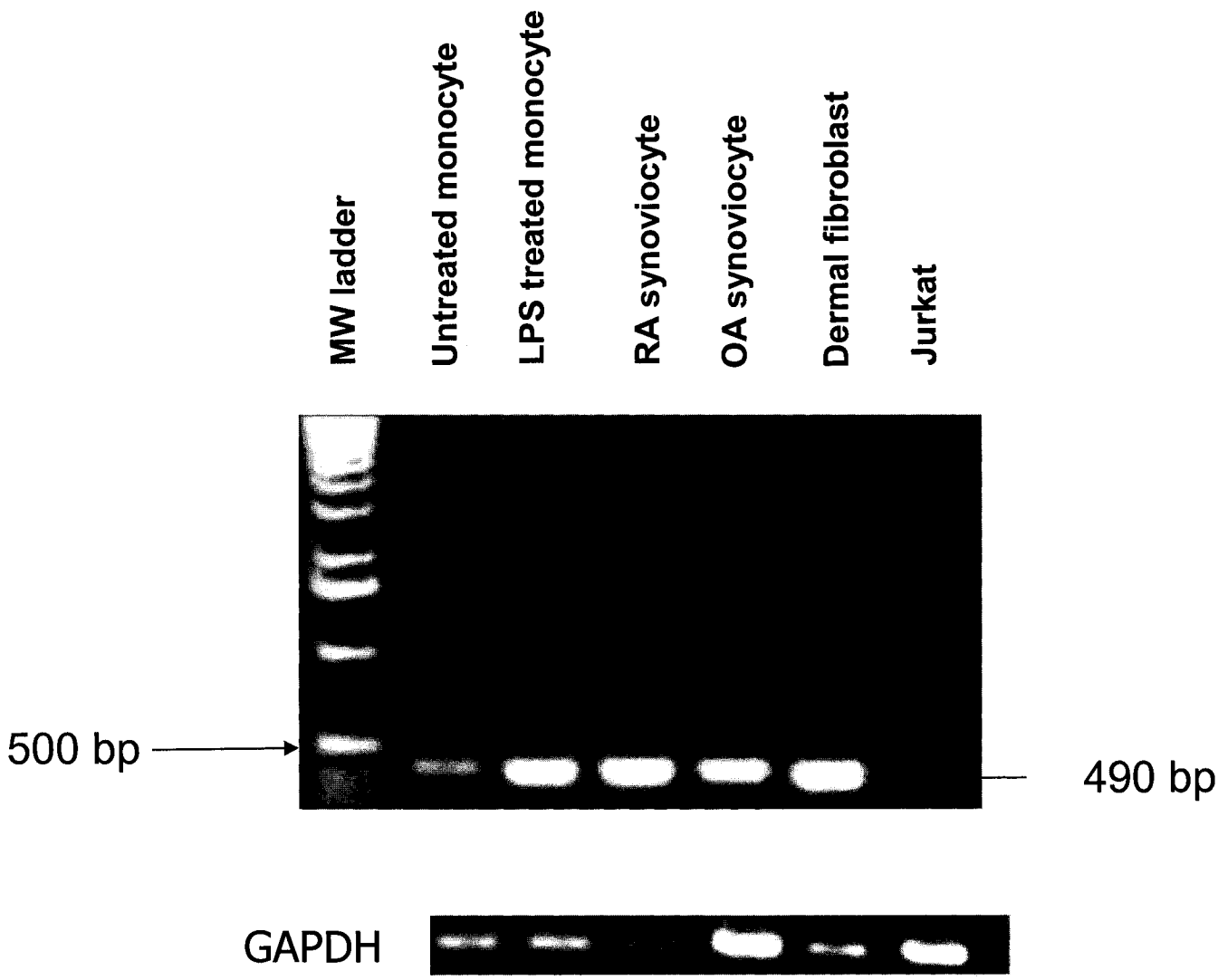
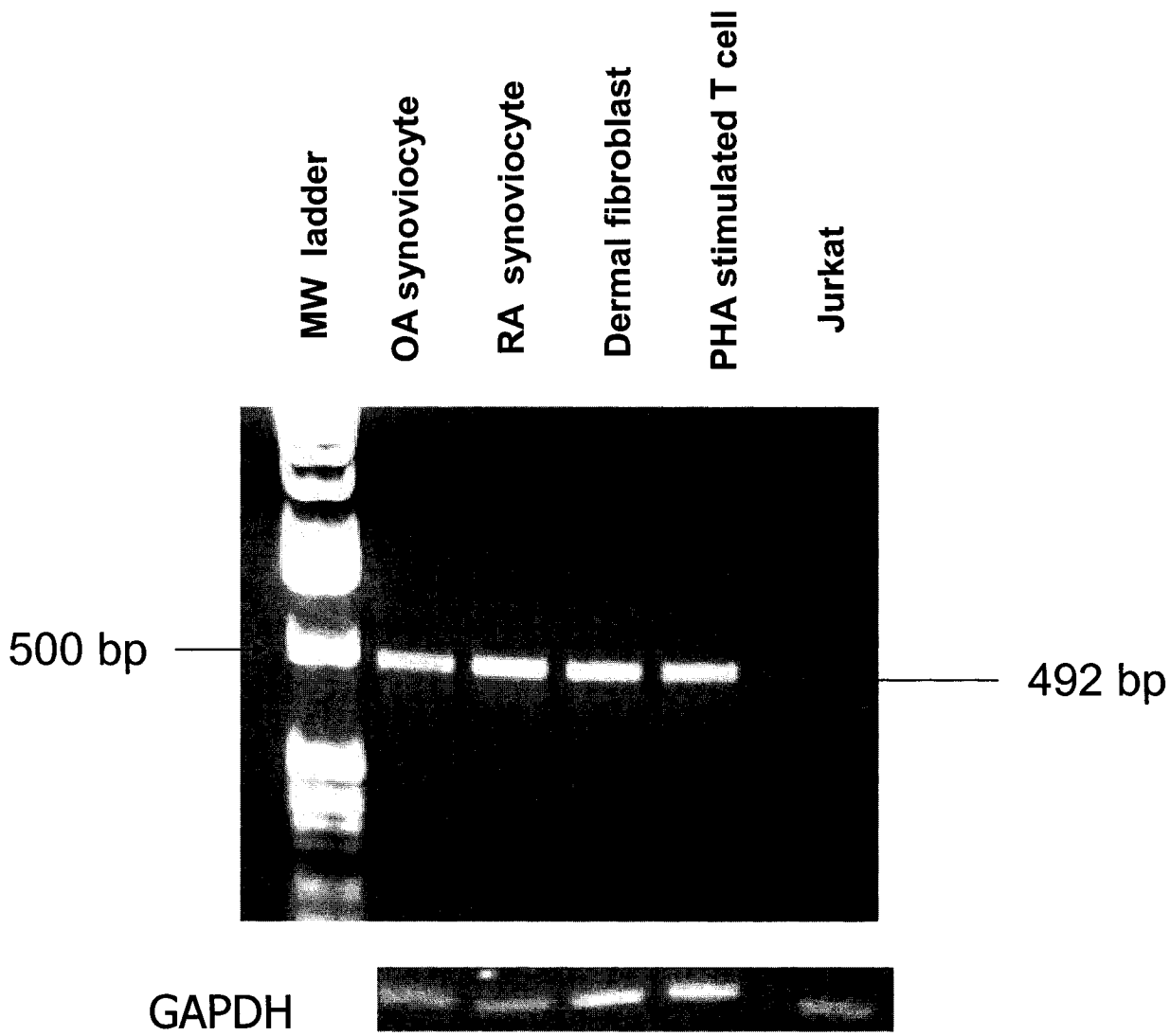

MW ladder
Untreated monocyte
LPS treated monocyte
RA synoviocyte
OA synoviocyte
Dermal fibroblast
Jurkat
500 bp
490 bp
GAPDH

Figure 5.1 IL-22 mRNA expression in, mitogen-stimulated T cells and FLS.

Total RNA (1 μg) obtained from OA FLS (*lane 2*), RA FLS (*lane 3*), dermal (foreskin) fibroblasts (*lane 4)*, PHA-activated T cells (*lane 5*), or Jurkat T cells (*lane 6)* was analyzed for expression of IL-22 expression by RT-PCR. Primers specific for GAPDH were used as an internal control for all samples


MW ladder
OA synoviocyte
RA synoviocyte
Dermal fibroblast
PHA stimulated T cell
Jurkat
500 bp
492 bp
GAPDH

4.5 Quantitative real time RT-PCR analysis of IL-19 and IL-22 mRNA expression

To compare the levels of IL-19 and IL-22 expression in FLS with the previously known sources of IL-19 and IL-22 (LPS-treated monocytes and activated T cells, respectively), we used quantitative real time PCR. To facilitate quantitation, we first established PCR parameters and standard curves using TA plasmid constructs that contained IL-19, IL-22, and GAPDH cDNA inserts. The plasmid templates were used to construct a standard curve over a wide range (4 to 5 logs) of concentrations of each of the plasmids (see Fig. 5.2 and Fig. 5.3). Once standard curves were established, we utilized cDNA generated from total cellular RNA of FLS, LPS-stimulated monocytes, and unstimulated monocytes for quantitation of IL-19 expression, while cDNA from total cellular RNA of FLS, dermal fibroblasts, and activated T cells was used for quantitation of IL-22 expression. All of these templates were used for real time PCR using the GAPDH standard as an internal control for relative RNA amounts. Results from four samples from each of FLS (4 RA, 3 OA), dermal fibroblasts, monocytes, LPS treated monocytes, and PHA stimulated T cells are shown in Fig. 5.4 , 5.5, 5.6, and Fig. 5.7. IL-19 mRNA expression by FLS was similar to LPS-treated monocytes, while expression of IL-22 mRNA by FLS was similar to that of activated T cells. In the limited series tested, it was also noted that there were no significant difference between IL-19 and IL-22 expression levels in RA and OA FLS (data not shown).

Figure 5.2 Quantitative analysis of GAPDH mRNA expression in FLS and LPS-treated monocytes.

cDNA obtained from untreated FLS or monocytes grown in the absence or presence of LPS (100ng/mL) for 4 hours was analyzed by real time PCR using GAPDH-specific primers. Standard curves were established using known concentrations of TA plasmids containing GAPDH inserts at the indicated dilutions. Shown graphically is the incorporation of mean fluorescence for each PCR product at the given PCR cycle.

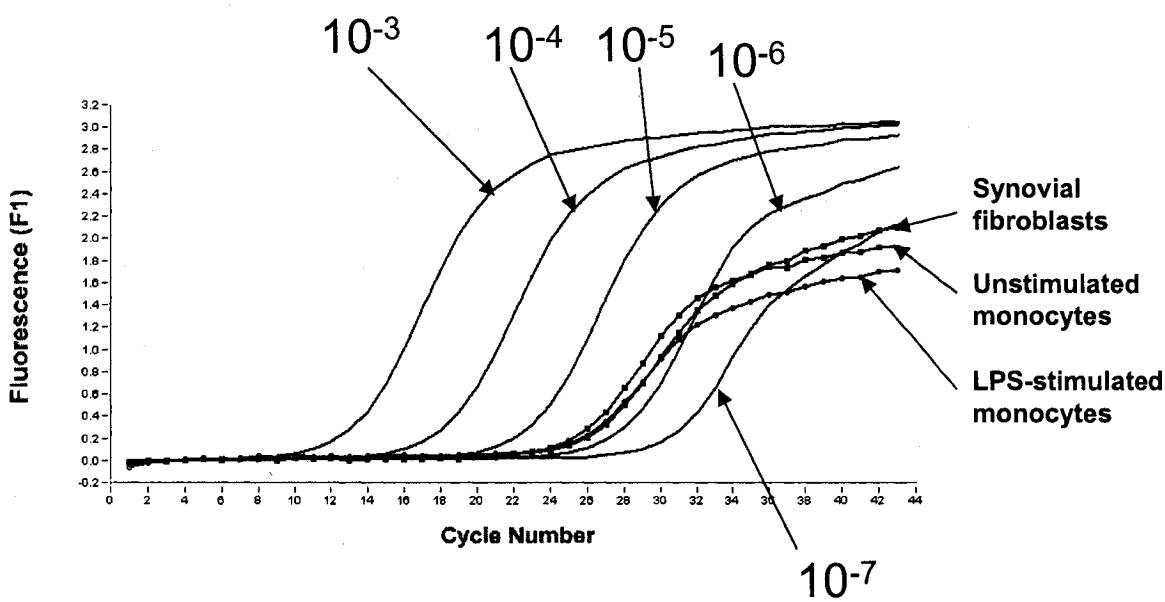

10-3
10-4
10-5
10-6
10-7
Fluorescence (F1)
Cycle Number
Synovial fibroblasts
Unstimulated monocytes
LPS-stimulated monocytes

Figure 5.3 Quantitative analysis of IL-19 mRNA expression in FLS and LPS-treated monocytes.

cDNA obtained from untreated FLS or monocytes grown in the absence or presence of LPS (100ng/mL) for 4 hours was analyzed by real time PCR using IL-19-specific primers. Standard curves were established using known concentrations of TA plasmids containing IL-19 inserts at the indicated dilutions. Shown graphically is the incorporation of mean fluorescence for each PCR product at the given cycle. This pattern was similar in all samples studied using IL-19 primers

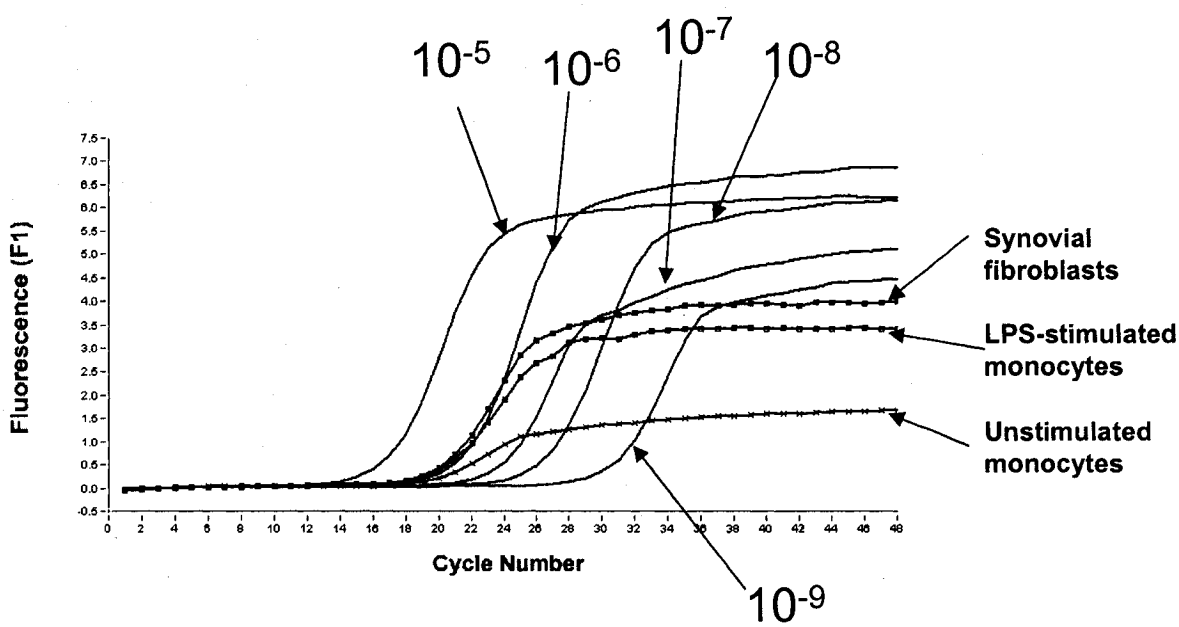

10-5
10-6
10-7
10-8
10-9
Fluorescence (F1)
Cycle Number
Synovial fibroblasts
LPS-stimulated monocytes
Unstimulated monocytes

Figure 5.4 Comparison of IL-19 mRNA expression in FLS and Monocytes

Relative levels of IL-19 mRNA expression in FLS, unstimulated and LPS-stimulated monocytes after normalization against GAPDH (data are plotted as mean after normalization ± STDEV; n=7 for each). Quantitation was established using the ratios of expression between IL-19:GAPDH for each cDNA sample. The numerical value of this ratio is shown graphically.

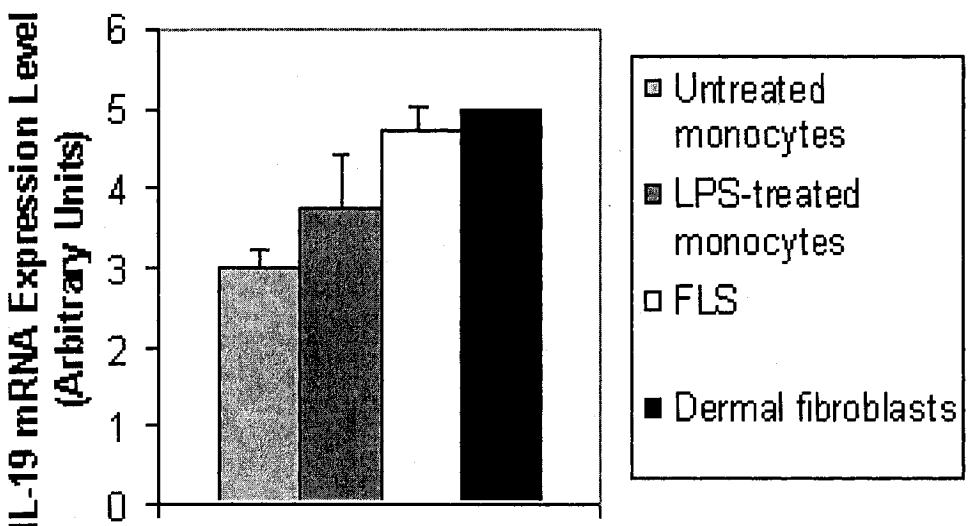

IL-19 mRNA Expression Level (Arbitrary Units)
0
1
2
3
4
5
6
Untreated monocytes
LPS-treated monocytes
FLS
Dermal fibroblasts

Figure 5.5 Quantitative analysis of GAPDH mRNA expression in FLS and mitogen-treated T cells.

cDNA obtained from untreated FLS and dermal fibroblasts, or PBMC stimulated with PHA (10μg/mL) for 72 hours was analyzed by real time PCR using GAPDH-specific primers. Standard curves were established using known concentrations of TA plasmids containing GAPDH inserts at the indicated dilutions. Shown graphically is the incorporation of mean fluorescence for each PCR product at the given cycle.

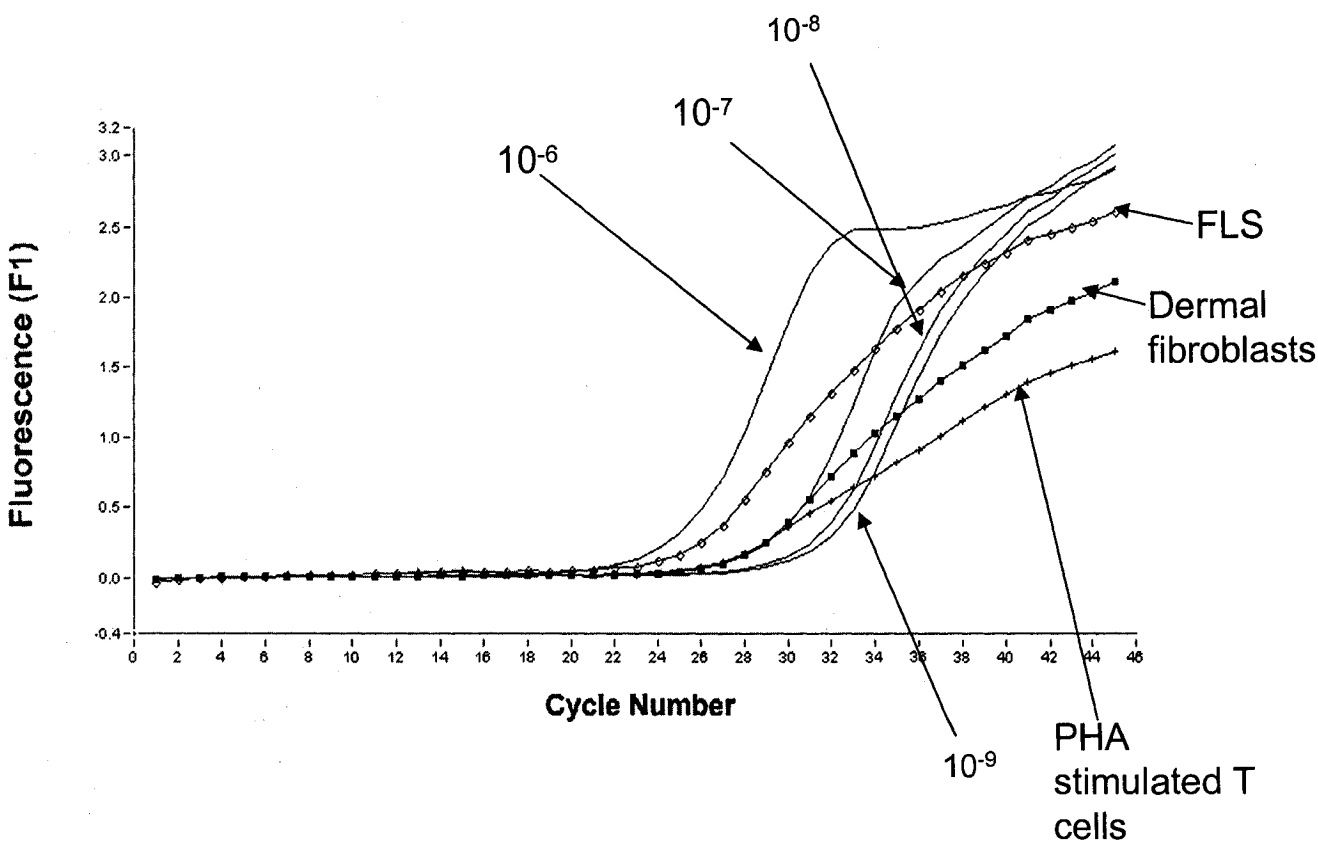

10-8
10-7
10-6
FLS
Dermal fibroblasts
PHA stimulated T cells
10-9
Fluorescence (F1)
Cycle Number
3.2
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46

Figure 5.6 Quantitative analysis of IL-22 mRNA expression in FLS and mitogen-treated T cells.

cDNA obtained from untreated FLS and dermal fibroblasts, or PBMC stimulated with PHA (10μg/mL) for 72 hours was analyzed by real time PCR using IL-22-specific primers. Standard curves were established using known concentrations of TA plasmids containing IL-22 inserts at the indicated dilutions. Shown graphically is the incorporation of mean fluorescence during each PCR cycle. This pattern was similar in all samples studied using IL-22 primers.

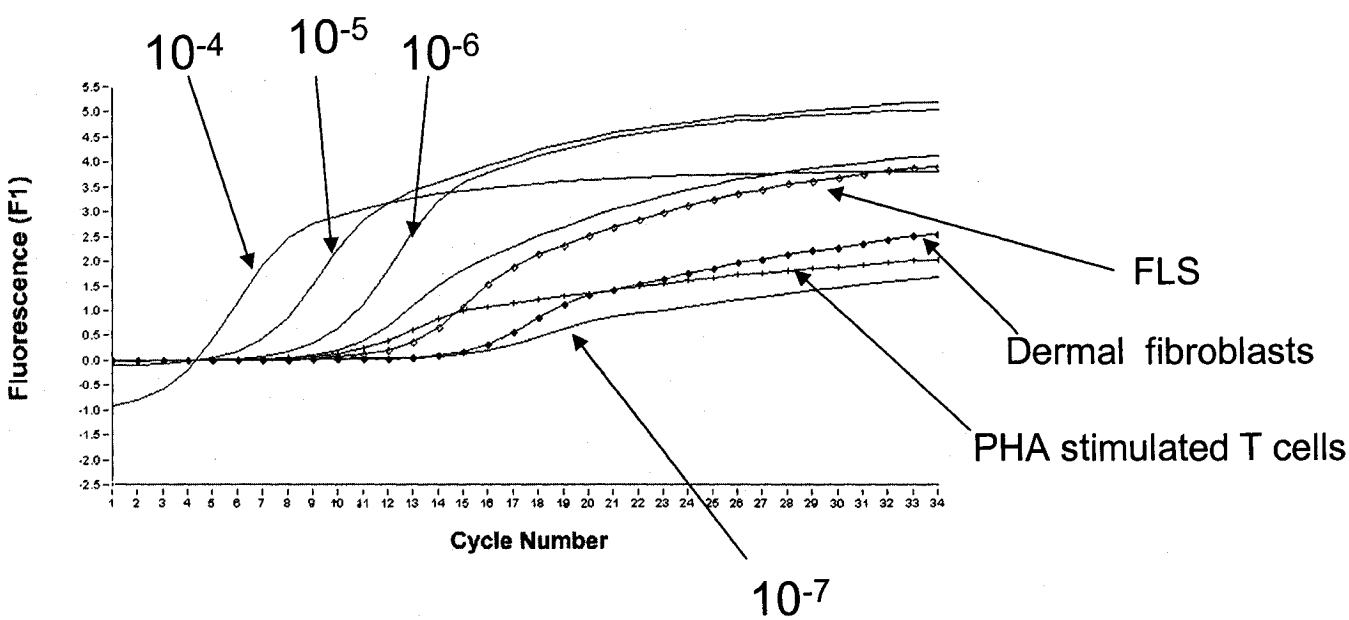
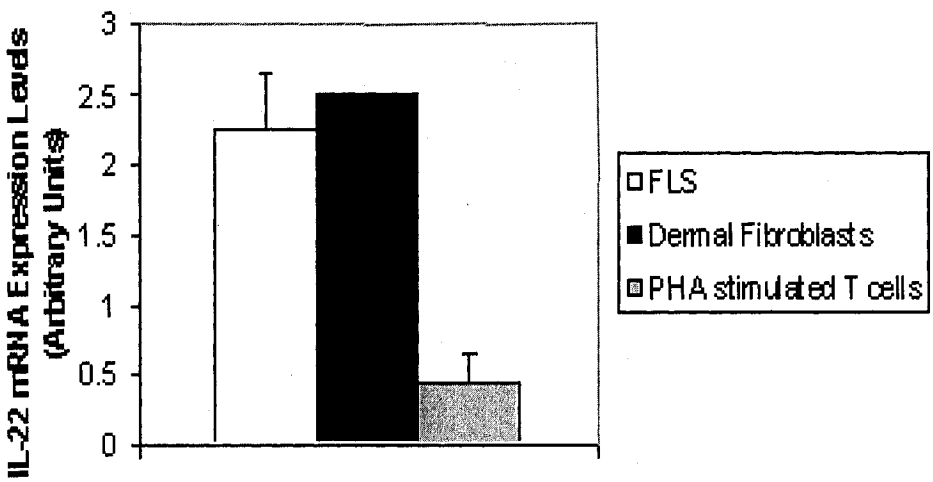

10-4
10-5
10-6
10-7
Fluorescence (F1)
Cycle Number
FLS
Dermal fibroblasts
PHA stimulated T cells

Figure 5.7 Comparison of IL-22 mRNA expression in FLS and PHA stimulated T cells

Relative levels of IL-22 mRNA expression in FLS, dermal fibroblasts, and activated T cells after normalization against GAPDH (data are plotted as mean after normalization ± STDEV; n=7 for each). Quantitation was established using the ratios of expression between IL-22:GAPDH for each cDNA sample. The numerical value of this ratio is shown graphically. There difference in expression between each cell type was not significant.


IL-22 mRNA Expression Levels (Arbitrary Units)
3
2.5
2
1.5
1
0.5
0
FLS
Dermal Fibroblasts
PHA stimulated T cells

4.6 Protein Biochemistry

4.6.1 Western Blot Analysis to Detect IL-19 at Protein Level

To confirm the novel discovery that fibroblasts transcribe mRNA for IL-19, Western blot analysis was used to detect IL-19 protein in the cell lysates of fibroblasts. Much of the secretion method of IL-19 from known sources is not known, and our nucleic acid results suggested that cultured fibroblasts are a constitutive source of mRNA for IL-19. Indeed cytokine production at the protein level has been detected in fibroblast cell lysates (Ritchlin et al., 2001), and confirmation of IL-19 at the protein level is very important step in the identification process. Western blot analysis of cell lysates from FLS, dermal fibroblasts, activated PBMC, and along with varying amounts of IL-19 recombinant protein are shown in Fig. 5.8. From this figure it is evident that the sensitivity of this experiment is 5 ng, as recombinant protein was not detectable at 2.5 ng. Activated PBMC ($3x10^6$) should be an abundant source of this cytokine, but as seen in the figure, no visible band was detectable. Bands for IL-19 in FLS and dermal fibroblasts were also not evident, indicating that the antibody used in this experiment was not sensitive enough to detect functional protein in cell lysates from various sources.

4.6.2 Detection of IL-19 and IL-22 in Fibroblast Cell Culture Supernatants

To detect whether fibroblasts can regularily secrete functional IL-19 protein into their environment, an ELISA was performed. However, various ELISA methods were used to try and detect each cytokine at the protein level, but each trial was unsuccessful. This was likely due to the lack of sensitivity of all current antibodies against IL-19 and IL-22.

Figure 5.8 Western Blot Analysis for IL-19 in Synovial and Dermal Fibroblast Cell Lysates

30 μL of cell lysates from FLS ($1x10^6$ cells), dermal fibroblasts ($1x10^6$ cells), and LPS-stimulated PBMC ($3x10^6$ cells) were run on a 12% SDS gel and transferred to a PVDF membrane and probed with 0.2 ug/mL anti-IL-19 antibody. The anti-IL-19 antibody had a detection limit of 5ng as observed by detection of recombinant human IL-19 protein in lanes 4-8. Protein from cell lysates in lanes 1-3 was not detectable. *Lane 1* Dermal fibroblast cell lysate, *Lane 2* FLS cell lysate, *Lane 3* PHA stimulated monocytes cell lysate, *Lane 4* 2.5ng recombinant human (rh) IL-19, *Lane 5* 5ng rhIL-19, *Lane 6* 50ng rhIL-19, *Lane 7* 100ng rhIL-19.

100 ng rIL-19

50 ng rIL019

10 ng rIL-19

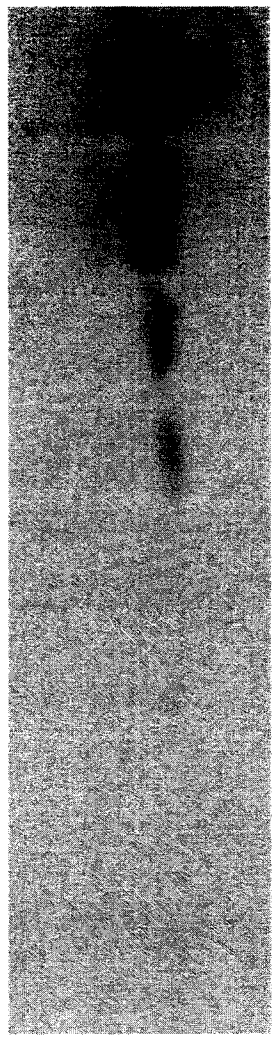
5 ng rIL-19

2.5 ng rIL-19

Activated PBMC

FLS

Dermal Fibroblasts



prevented detection of either protein in the cell supernatants (data not shown). Once commercially available antibody becomes more available, detection of these cytokines may be a possibility.

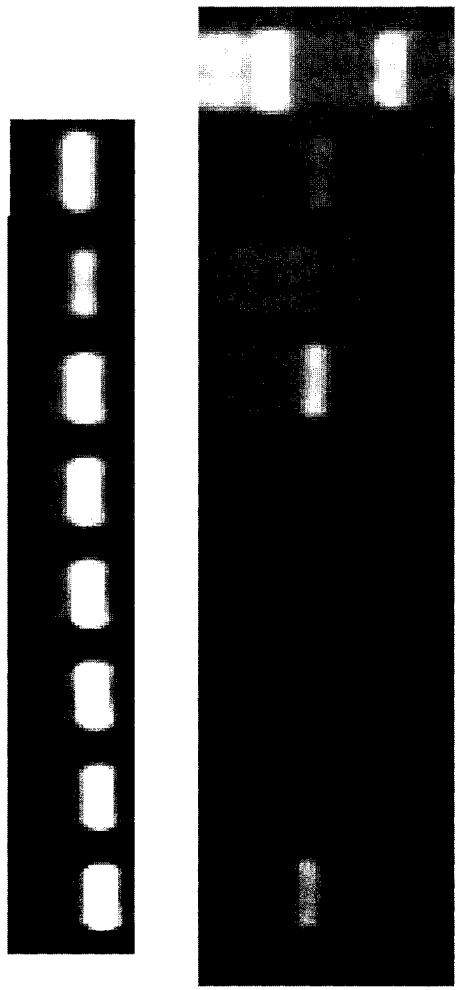
4.7 IL-20R Expression in T cells

Determining another biological role for IL-19 is a very important next step after identifying that fibroblasts are capable of transcribing mRNA for this cytokine. A not so obvious effect, might be the effects of IL-19 and IL-22 on T cells. Blumberg et al., have shown that resting PBMC (isolated directly from fresh blood) do not express either the α or β chain of the IL-20 receptor complex. However, certain receptors such as IL-18Rα chain, are known to be upregulated in the presence of certain cytokines, or after T cell activation (Smeltz et al., 2001). PHA-stimulated and unstimulated T cells were incubated with rhIL-19 and rhIL-22 for 72 hours at varying concentrations. PHA-stimulated T cells responded to IL-19 by increased proliferation rates (data not shown), while untreated T cells showed no response. IL-19 tended to increase the proliferation rates of PHA-stimulated T cells at a dose dependant rate, whereas IL-22 had no effect on either treated or untreated T cells (data not shown). IL-19 had no effect on untreated T cell proliferation and this suggested the possibility the IL-20R receptor complex was up-regulated upon stimulation. To look for expression of each IL-20 receptor subunit, RT-PCR was performed on total cellular RNA isolated from these PHA-stimulated T cells after one day of culture. Primers were designed specifically for IL-20Rα and IL-20Rβ and PCR of mRNA from a variety of cells is shown in Fig. 5.9 and Fig. 6.0. From these figures it is clear that treated T cells, treated B cells, and Jurkat T cells express the IL-

20Rα chain while only Jurkat T cells expressed the IL-20Rβ chain. PCR products were of the expected size and were sequenced at the University of Ottawa Biotechnology Institute. A BLAST search was performed on each sequence obtained and they were identical to human IL-20Rα and IL-20Rβ.

Figure 5.9 IL-20Rα expression in T cells

Total RNA (1 μg) obtained from PHA-stimulated T cells (*lane 2*), untreated T cells (*lane 3*), EBV infected B cells(*lane 4)*, unstimulated B cells (*lane 5*), HeLa cell line (*lane 6)*, synovial fibroblasts (*lane 7*), dermal fibroblasts (*lane 8*), Jurkat T cells (*lane* 9) was analyzed for expression of IL-20α expression by RT-PCR. Primers specific for GAPDH were used as an internal control for all samples



Activated T cells

T cells

EBV B cells

Unstimulated B cells

HeLa Cell line

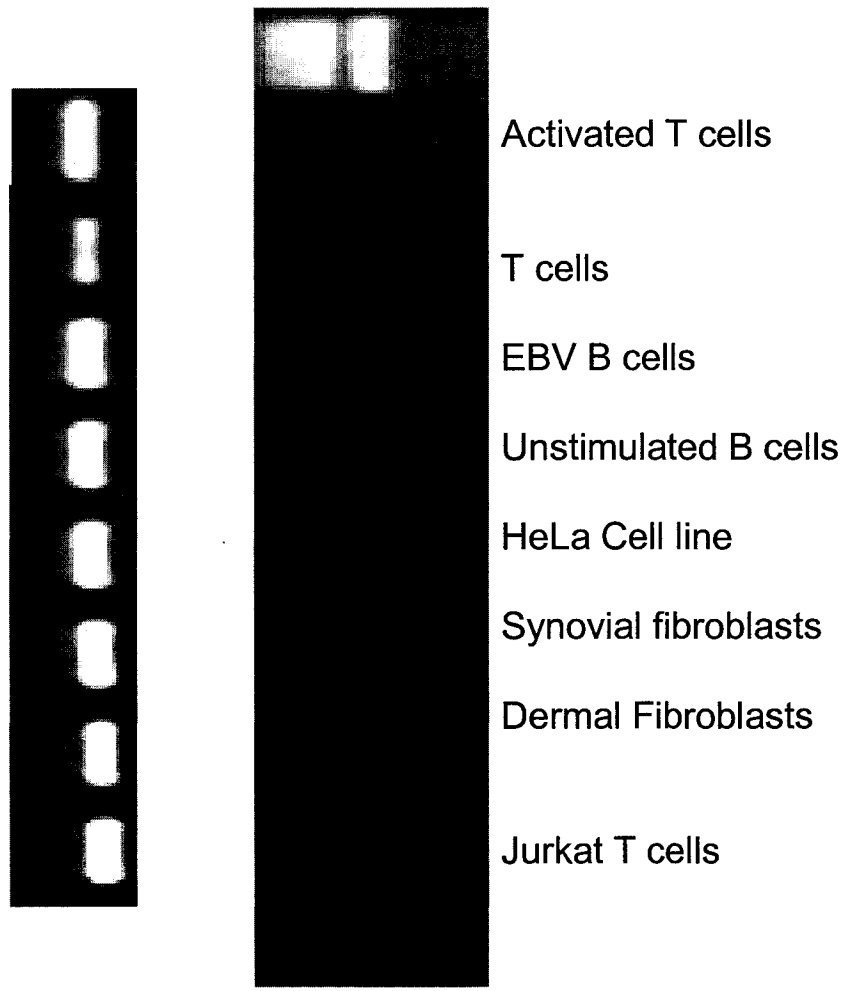
Synovial fibroblasts

Dermal Fibroblasts

Jurkat T cells

Figure 6.0 IL-20Rβ Expression in T cells

Total RNA (1 μg) obtained from PHA-stimulated T cells (*lane 2*), untreated T cells (*lane 3*), EBV infected B cells(*lane 4)*, unstimulated B cells (*lane 5*), HeLa cell line (*lane 6)*, synovial fibroblasts (*lane 7*), dermal fibroblasts (*lane 8*), jurkat T cells (*lane* 9) was analyzed for expression of IL-20β expression by RT-PCR. Primers specific for GAPDH were used as an internal control for all samples.


Activated T cells
T cells
EBV B cells
Unstimulated B cells
HeLa Cell line
Synovial fibroblasts
Dermal Fibroblasts
Jurkat T cells

CHAPTER 5.

DISCUSSION

RA is a debilitating condition that affects the lives of millions of Canadians, and millions more around the world. The pathogenesis of this disease is moderated and maintained by cells of the immune system, and by cells native to the synovial environment. Gene expression in immune cells was once thought to be the major contributor to inflammation associated with RA, and this has been studied in great detail. Functional capabilities of synovial fibroblasts in RA have been extensively studied (Springer et al., 1990., Ohgoda et al., 1998). Synovial fibroblasts have been shown to produce cytokines and MMP's as well as forming invasive pannus. On the basis of such observations, this cell lineage is considered to be central to the pathogenesis of RA.

5.1 Cytokine Gene Expression in Fibroblasts

This study focused on the cytokine mRNA production capabilities of the synovial fibroblast. We have identified that cells of the fibroblast lineage (FLS as well as dermal fibroblasts) can transcribe mRNA for both IL-19 and IL-22 at levels similar to previously known sources. Detection of both of these cytokines at the protein level, was however, not possible due to the lack of a monoclonal antibody suitable for these experiments.

IL-10 is an important anti-inflammatory and immunosuppressive cytokine that has been used to treat various autoimmune conditions including psoriasis, and RA (Asadullah et al., 2003). Over the past couple of years, several novel cytokines that demonstrate primary sequence homology and structural similarity to IL-10 (that include

IL-19, IL-20, IL-22, IL-24, and IL-26) have been identified, forming what is now referred to as the "IL-10-related cytokine family". However, little is known regarding the biology of the newer members of this family. Expression of cytokines by fibroblasts is not a novel concept, indeed fibroblasts are well known to express a variety of cytokines with important effector functions including angiogenesis, chemoattraction, and inflammation (Harada et al., 1999; Curfs et al., 1997). Previously, LPS-activated macrophages and mitogen-stimulated T cells were the only cell types known to express significant levels of IL-19 and IL-22, respectively. The quantitative PCR results in this project show that that FLS constitutively express mRNA for IL-19 at levels similar to LPS-activated macrophages, and IL-22 at levels similar to that of activated T cells.

Our data also suggests that the levels of IL-19 and IL-22 mRNA synthesis are similar in cultured RA and OA FLS as well as in dermal fibroblasts. However, the mRNA used from each sample was from cells in long term culture. It is possible that *in situ* in the inflamed joint, the levels of IL-19 and IL-22 mRNA expression by FLS may reflect the level of inflammation associated with the underlying disease, and this would require further study.

IL-19 was first identified as a novel homologue of IL-10 by *in silico* screening and subsequently as a product of LPS-treated monocytes (Gallagher et al., 2000). IL-19 is also reported to be produced by activated B cells (Wolk et al., 2002). IL-19 has a high affinity for IL-20R receptor complex which leads to JAK/STAT activation, specifically via STAT3 (Parish-Novak et al., 2002). The biological roles of IL-19 are just starting to be unraveled and currently the only known actions of IL-19 are the stimulation of IL-6, TNFα and ROS production in monocytes (Liao et al., 2002). All three of these IL-19-

inducible pro-inflammatory activities are known to play a role in the etiology of RA.

IL-22 was originally identified as a product of IL-9 stimulated T cells (Dumoutier et al., 2000a; Dumoutier et al., 2000b), and is known to be produced in a variety of tissues. Constitutive expression of IL-22 is found in both the brain and thymus, while expression of IL-22 in T cells appears to require stimulation with reagents such as anti-CD-3, or Con A (Dumoutier et al., 2000c; Xie et al., 2000). IL-22 binds to class II cytokine receptors (CRF2-4), but not the IL-10 receptor, and involves activations of STATs 1, 3, and 5(Xie et al., 2000; Blumberg et al., 2001 ; Dumoutier et al., 2001 ; Kotenko et al., 2001). Like IL-19, the biological function of IL-22 is not yet fully understood. However, IL-22 can play a role in inflammation through its ability to activate STAT3 in acinar cells of the pancreas while at the same time inducing mRNA synthesis for pancreatitis associated protein (PAP1) and osteopontin (Conti et al., 2003).

5.2 Cytokine Receptor Expression in Fibroblasts

The strong hybridization signal corresponding to IL-22BP (IL-22RA2) on microarrays for both OA and RA FLS is another novel and potentially interesting discovery. A fully functional IL-22 receptor molecule consists of IL22R1 (CRF2-9), and IL-10R2 (CRF-4) (Xie et al., 2000; Kotenko et al., 2001, Kotenko et al., 1997)). Unlike this membrane bound receptor complex, IL-22BP is a soluble receptor (Kotenko et al., 2001b, Gruenberg et al, 2001). IL-22BP is a naturally occurring antagonist of IL-22 and has a higher affinity for IL-22 than the membrane-associated IL-22 receptor complex (Kotenko et al., 2001). IL-22BP seems to be specific for IL-22 only, as it cannot neutralize the activities of IL-10 or other similar homologues (Kotenko et al., 2001b,

Dumoutier et al., 2001a). It is possible that the expression of IL-22BP in specific tissues may moderate local inflammation. Tissues previously known to express IL-22BP include skin, lung, and lymph node, and certain epithelial cells (Xu et al., 2001).

5.3 ECM and Adhesion Molecule Expression in Fibroblasts

FLS are known to produce MMP's (Stuhlmeier, 2003) which are key players in the morbidity associated with RA, and a study on MMP's as well as other related molecules may identify novel MMP's produced by fibroblasts. The method of detection for MMP's used in this study was a low-density microarray study similar to that of cytokine genes. Indeed, FLS in long term culture were capable of transcribing mRNA for a varitey of MMP's along with other adhesion molecules. The expression pattern observed in FLS was similar to that of the expression in dermal (foreskin) fibroblasts but there were a few exceptions. Both cell lines expressed TIMP-1,-2,-3, along with MMP-2, and MMP-10. However, the only observable difference in both of these cell lines was that FLS expressed MMP-26, while dermal fibroblasts expressed MMP-14.

MMP's have been implicated in angiogenesis, tumor invasion, and tissue remodeling. MMP-26 was originally identified in the uterus and was named endometrial tumor-derived metalloproteinase (Park et al., 2000). MMP-14 expression was found to be limited to dermal fibroblasts and not FLS. This protein is known as an activator of pro-gelatinase A (MMP-2), and is expressed in fibroblasts during wound healing and cancer progression (Mignon et al., 1995). MMP-2 is known as gelatinase (or collagenase type IV) (Nagase et al., 1992). MMP-2 is responsible for degrading type IV collagen, and its expression has been previously observed in fibroblasts (Smolian et al., 2001).

MMP-10, is one of the four members of the collagenase gene family (Muller et al., 1988), and has been shown to promote FLS growth and maturation (Tolboom et al., 2002).

The TIMP gene family encodes a number of proteins that are natural inhibitors of the MMP's. The ECM and adhesion molecule gene array performed in this project indicated that both fibroblast lines studied transcribe mRNA for TIMP-1, TIMP-2, all of which have been previously detected in fibroblasts (Takagi et al., 1998; Moe et al., 2000; Watanabe et al., 2002) thus validating the results of this analysis.

5.4 IL-20R Expression in Activated T and B cells

The finding that activated T and B cells express the IL-20R α chain was an interesting and unexpected discovery. Previously, Blumberg et al. reported that unstimulated PBMC, including T cells, did not express either chain of the IL-20R complex. As previously mentioned, members of the IL-10 cytokine family are structurally similar, therefore sharing of class two cytokine receptors is common for members of this family. A prime example of this phenomenom, is the recent finding that IL-26 signals through a complex of the IL-20R1 receptor and the IL-10R2 receptor (Sheikh et al., 2004). The observation that the IL-20Rβ chain is not expressed in either activated T or B cells may suggest that IL-19 can signal by binding to the IL-20Rα chain, and cause signal transduction through possibly another β chain, perhaps the IL-10Rβ chain. However, this concept is purely speculation, as in some systems, IL-19 requires both the IL-20Rα and IL-20Rβ chain to bind properly and initiate signal transduction in a specific cell line (Pletnev et al., 2003). Interestingly enough, Jurkat T cells expressed both chains indicating that IL-19 binding to these cells is possible.

5.5 Detection of IL-19 and IL-22 at Protein Level

A limitation of my study is that neither IL-19 nor IL-22 was detectable at the protein level in fibroblast cell lysates or in fibroblast culture supernatants. This was at least in part due to lack of reagents to study IL-19 and IL-22 at the protein level. As shown from Figure 5.7, the detection limit of the monoclonal IL-19 antibody was 2.5ng, which is not low enough to detect this cytokine in cell lysates. ELISA was performed to detect secreted protein but the sensitivity of this experiment was not low enough for proper detection. It is probable that if a high quality commerical antibody was available for this study, protein levels of IL-19 and IL-22 would have been detectable in both supernatants and cell lysates. However, that may not be the only reason, as the protein may be degraded upon secretion, or may be altered in form. There are cell lines that are responsive to IL-19, and this would be a good biological assay to detect functional IL-19 in culture supernatants, however, this cell line was not attainable at this current time.

5.6 Future Work

There are many directions to follow with the results of this project. Obviously, the first and most important step is to generate reagents, including monoclonal antibodies for both IL-19 and IL-22 that could be used to study IL-19 and IL-22 at the protein level. Also, RT-PCR on the IL-20R complex suggested that the IL-20Rα receptor chain is upregulated in stimulated T and B cells. It would be worthwhile to study these receptors at the protein level in these cell lines, while also determining the effect of IL-19 binding has on these cell lines. It would also be worth investigatin if IL-19 can signal through the STAT-3 pathway in these cells. This work may identify another potential ligand for IL-

19 signalling as the IL-20Rβ ligand is not expressed on either of these cells. Determining the effects of IL-19 on other cell lines, such as macrophage-like synovioctyes, may also prove worthwhile.

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