T cells in Rheumatoid Arthritis

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ABSTRACT

Identification of the role of T cells and their interaction with other cell types remains a major challenge to our understanding of the pathogenesis of rheumatoid arthritis. In this study we have investigated the regulation of the response of T cells infiltrating the rheumatoid joint to IL-6. Furthermore we have investigated the level of T cell activation in the early stages of rheumatoid arthritis.

Interleukin-6 is an important regulator of T cell differentiation and survival. It exerts its biological function by either directly binding to the complete IL-6 receptor consisting of CD126 CD130 or via transsignaling, when sIL6R-IL6 complexes bind to CD130. This study addresses the expression and regulation of these receptor components on the T cells infiltrating the rheumatoid joint. While compared to blood T cells, CD126 expression was found at low levels on synovial fluid and tissue T cells, expression of CD130 on synovial tissue T cells was comparable to that of blood T cells, with lower levels in synovial fluid T cells, both at protein and mRNA level. When exposed to sIL6R-IL6 complexes, tissue derived T cells responded with a higher level of STAT3 phosphorylation compared to cells incubated with IL-6, suggestive of transsignaling. High CD130 expression was demonstrable in T cells in the perivascular cuff area. Among a range of cytokines tested, IL-6 reduced CD126 and CD130 expression while IL-10, which is expressed at high levels in the perivascular infiltrate, induced expression of CD130. Taken together these data suggest that the inflammatory microenvironment maintains responsiveness to IL-6 transsignalling by cytokine driven CD130 expression on CD4 positive T cells.

To address the question whether the role of T cells changes during the course of progression of RA, we analysed the expression of T cells activation markers on synovial fluid and peripheral blood T cells from patients at the very early stage of disease (within 3 months of disease onset) compared to patients with established or self resolving arthritis. Expression of

CD69, CD71 and HLA-DR was upregulated on synovial fluid T cells compared to peripheral blood but there were no differences between the different groups of patients. Furthermore, we quantified the proportion of T cells expressing the invariant TCR Vα24Jα18 in synovial fluid and blood of the same groups of patients. We found a lower frequency of iNKT cells in the synovial fluid of very early arthritis patients compared to other patients. While this is a preliminary result, it suggests that there may be a role for these cells in the regulation of disease susceptibility.

A mi padre, en su memoria

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ABREVIATIONS

ABIA	Antibody induced arthritis
ACPA	Anti-citrullinated protein/peptide antibodies
AHR	Airway hyper-responsiveness
AICID	Activation Induced Cell Death
APC	Antigen Presenting Cells
ARA	American Rheumatism Association
ARC	American College of Rheumatology
BALB	Bagg Albino
Bcl-2	B cell Lymphoma 2
BLyS	B Lymphocyte Stimulator
BSA	Bovine serum albumine
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CIA	Collagen Induced arthritis
CLC	Cardiotrophin Like Cytokine
CNTC	Ciliary Neurotrophic Factor
CNTF	Cilliary Neurotrophic Factor
CRP	C-reactive protein
CT-1	Cardiotrophin 1
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CX3CL1	Chemokine CXC3C Ligand 1
DAPI	4'-6-Diamidino-2-phenylindole
DBA	Dilute Brown Non-Agouti
DC	Dendritic cell
DMARD	Disease Modifying Anti-Rheumatic Drugs
DN	Double Negative
DNA	DeoxyriboNucleic Acid
DP	Double positive
EDTA	Ethylene-diamine-tetra-acetic acid
ELK-1	Ets LiKe gene1
ERK	Extracellular Signal-Regulated Kinases
FACS	Fluorescent activator cell sorter
FasL	Fas Ligand
Fc	Fragment C
FCS	Foetal calf serum
FS	Forward Scatter
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GP	Glucoprotein
GPS	Glutamine-penicillin-streptomycin
GSL	Glycosphingolipid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen

HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular adhesion molecule
IFN	Interferon
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
IL-10R	Interleukin 10 Receptor
IL-1R	Interleukin 1 Receptor
IL-25R	Interleukin 25 Receptor
IL-6R	Interleukin 6 Receptor
iNKT	Invariant natural killer T cell
JAK	Janus kinase
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysacharide
LSM	Laser Scanning Microscopy
MACS	Magnetic Activated Cell Sorting
MAP	Mitogen activator protein
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
NF	Nuclear factor
NFkB	Nuclear Factor Kappa B
NK	Natural Killer
NOD	Non-Obese Diabetic
NP	Neuroprotein
OA	Osteoarthritis
OSM	Oncostatin M
OVA	Ovoalbumine
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PIAS	Protein inhibitor of activated STAT
PMN	Polymorphonuclear
PUA	Persistent Unclassified arthritis
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RBC	Red Blood Cells
RF	Rheumatoid Factor
rIL-10	Recombinant Interleukin 10
rIL-6	Recombinant Interleukin 6
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Real Time Polymerase Chain Reaction
SD	Standard Deviation
SDF	Stromal Cell-Derived Factor
SEM	Standard Error of the Mean

SF	Synovial fluid
SFMC	Synovial Fluid Mononuclear Cells
sIL6R	Soluble IL6R
SJL	Swiss Jim Lambert
SLA	Self-Limiting Arthritis
SLE	Systemic Lupus Erythematosus
SLRA	Self Limiting Rheumatoid arthritis
SLUA	Self-Limiting Undefined arthritis
SOCS	Suppressor of cytokine signaling proteins
SS	Side Scatter
ST	Synovial tissue
STAT	Signal Transducer and Activators of Transcription
STMC	Synovial Tissue Mononuclear Cells
SUMO	Small Ubiquitin-like Modifier
Tc	Cytotoxic T cell
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll like receptors
TNF	Tumor Necrosis Factor
Treg	Regulatory T cells
Tyk	Tyrosine Kynase
VCAM	Vascular Cell Adhesion Molecule
VEA	Very early arthritis
VEGF	Vascular endothelial growth factor
WT	Wild Type
α-GalCer	α-galactosylceramide

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Chapter 1.

INTRODUCTION

1.1 The immune system

The immune system is a complex defense system that has evolved to defend the body against a wide variety of pathogenic microorganisms and cancerous cells. It comprises the organs of the primary and secondary lymphatic system, specialized cells and soluble factors. All of these components of the immune system act in a coordinated fashion to achieve its function: to defend the body.

It is able to discriminate between foreign and self in order to only attack and destroy invading pathogens or altered self. If this discrimination fails and the immune system directs its effector function to the host's own cells and tissues, autoimmune diseases develop.

We can divide this complex system into innate and adaptive responses. These are, however, not independent of each other and interact at multiple levels to ensure rapid and effective removal of pathogens.

An innate immune response is triggered by recognition of patterns characteristic for pathogens by a limited number of receptors and soluble factors. This phenomenon is known as pattern recognition. Examples are Toll-like receptors (TLR) recognizing bacterial and viral structures such as lipopolysaccharide and proteoglycans. The innate immune system acts through four mechanisms: anatomical and physiological barriers, phagocytosis and inflammation. Physical barriers prevent the entry of microbes into the body; a very good example of a physical barrier is the skin. Other physical barriers include body secretions such as tears which can wash away invading pathogens, normal flora which competes for nutrients with invading microorganisms in mucous membranes and structures such as cilia which propel microorganisms that have been entrapped by mucus in the respiratory tract.

Physiological barriers that prevent pathogen invasion as part of the innate immune system include temperature and pH not suitable for microorganism survival. Soluble components of the innate immune system comprise factors such as lysozyme, interferons and complement. The complement system includes a set of proteins that once activated, attacks bacterial cell walls and facilitates pathogen clearance. Activated complement factors also alert cells of the innate and adaptive immune response to a site of infection. Interferons are secreted by virally infected cells and trigger antiviral effects in neighboring cells to limit the spread of infection. Lyzosome is capable of breaking the peptidoglycan layer of the bacterial cell wall. It is present in body secretions such as mucus or tears.

Inflammation is a complex sequence of events that follows a challenge of the body by irritation, injury, or infection. It is characterized by the following symptoms: Redness, Heat, Swelling, Pain and loss of function of the inflamed tissue. Inflammation will be described in more detail in section 1.4.

The adaptive immune system is a more evolutionarily advanced branch of the immune system that recognizes a large range of different antigens specifically. It is characterized by antigenic specificity, diversity, memory and self/nonself recognition. Antigen specificity allows the immune system to distinguish between different antigenic challenges and react appropriately; it also allows the immune system to discriminate between self and non-self antigens. Once the adaptive immune system recognizes a foreign antigen and mounts a response it will retain memory of this particular antigen, often conferring the host life-long immunity to it. The adaptive immune system requires the collaboration between lymphocytes and antigen presenting cells to function. Lymphocytes can be subdivided into B and T cells. B cells express a surface bound immunoglobulin that can bind to native foreign antigen. When a mature B cells encounters the antigen, recognized by the surface immunoglobulins with a

high affinity it divides rapidly and the daughter cells will differentiate into plasma or B memory cells. A proportion of the activated B cells will undergo isotype subclass switching and hypermutation of the variable region genes to generate high affinity antibodies with a range of effector functions. Eventually, Plasma cells will secrete antibodies that bind to the triggering antigen; causing its clearance by phagocytic cells, complement activation, neutralization, and agglutination of pathogens. Memory B cells stay in the body conferring long-term immunity to this particular antigen. In case of a subsequent encounter with the same antigen, memory B cells are much more efficient at proliferating and secreting high affinity IgG antibodies to defend the host. T cells are characterized by expression of the T cell receptor on their surface. This allows them to recognize antigens presented by an APC. Under a strictly controlled set of circumstances the T cells becomes activated and reacts by secreting cytokines that will activate or regulate other cells of the immune system or by differentiating into cytotoxic T Lymphocytes (CTLs) that will kill self altered cells (1)

1.2. T cells

Precursors of T lymphocytes are generated in the bone marrow, from where they migrate to the thymus, where they undergo further maturation steps. During maturation in the thymus T cells begin to express T Cell Receptors (TCR) on their surface. This receptor allows them to bind to antigen. The TCR is unique to each T cell clone and unlike B cells it does not recognize antigen on its own. The TCR recognizes antigen bound to a MHC molecule on the surface of an Antigen Presenting Cell (APC). Following stimulation a mature T cell will proliferate and differentiate, giving rise to memory T cells and effector T cells.

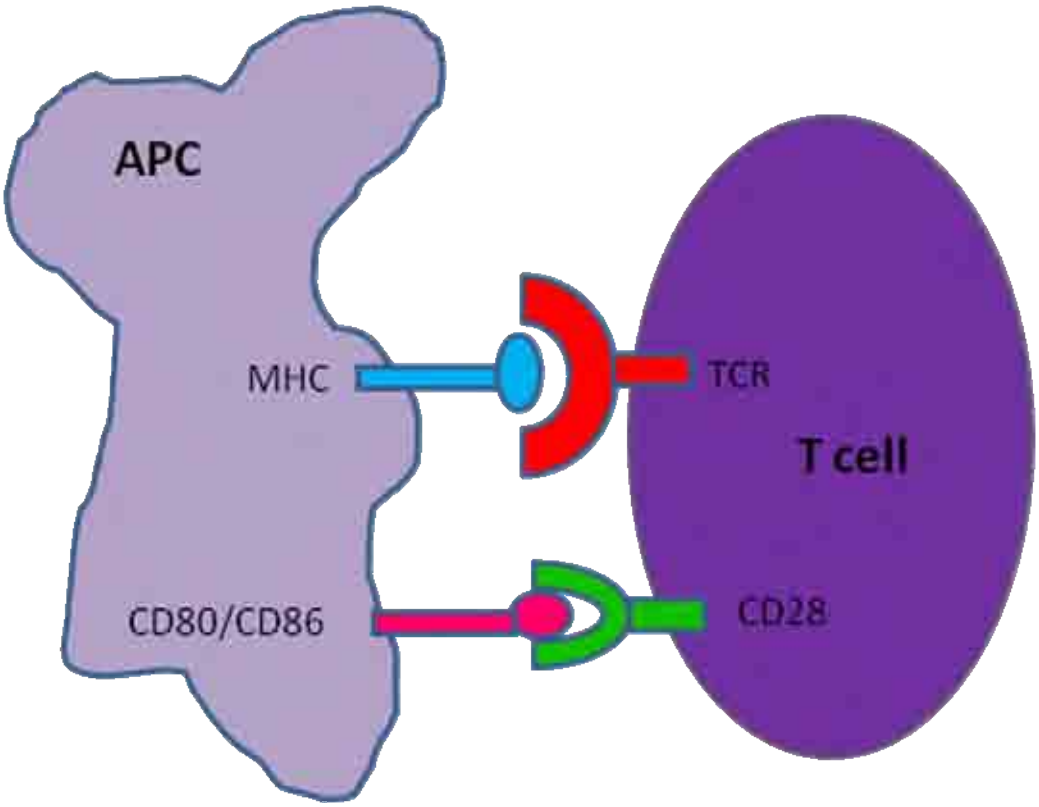


Figure 1. Antigen presentation by an APC to a T cell via TCR/MHC molecule interaction. The CD80/86 CD28 interaction shown here is an example of one of a large number of costimulatory/adhesion molecule interaction.

1.2.1. T cell development, positive and negative selection

T cell precursors travel from the bone marrow to the thymus where they will undergo a series of changes that will lead to development of mature T cells. The thymus is divided into subcapsular region, thymic cortex, corticomedullary junction and medulla. As thymocytes mature into T cells they travel across the different regions of the thymus. Thymocytes enter the thymus through endothelial venules and migrate to the subcapsular region where they will undergo rearrangement of their TCR genes. In the cortex thymocytes interact with cortical epithelial cells, at this point thymocytes are double-positive expressing CD4 and CD8

molecules as well as and αβTCR. If the thymocyte is able to recognize an MHC class I or class II molecule expressed by cortical epithelial cells it will receive a maturation and survival signal. Thymocytes that recognize MHC class I molecules lose the expression of CD4 and maintains CD8 expression, while thymocytes recognizing MHC class II molecules loose CD8 expression becoming CD4 single positive thymocytes. Thymocytes unable to recognize MHC molecules die by apoptosis and thymocytes that recognize self peptide antigens presented by MCH class I or II molecules with high affinity also receive a pro-apoptotic signal and die to avoid the development of self-reacting T cells in a process known as central tolerance. The cells that survive the selection process migrate to the medulla where the remaining single positive CD4 or CD8 naïve T cells leave the thymus directly joining the bloodstream or via the lymphatic system (2)

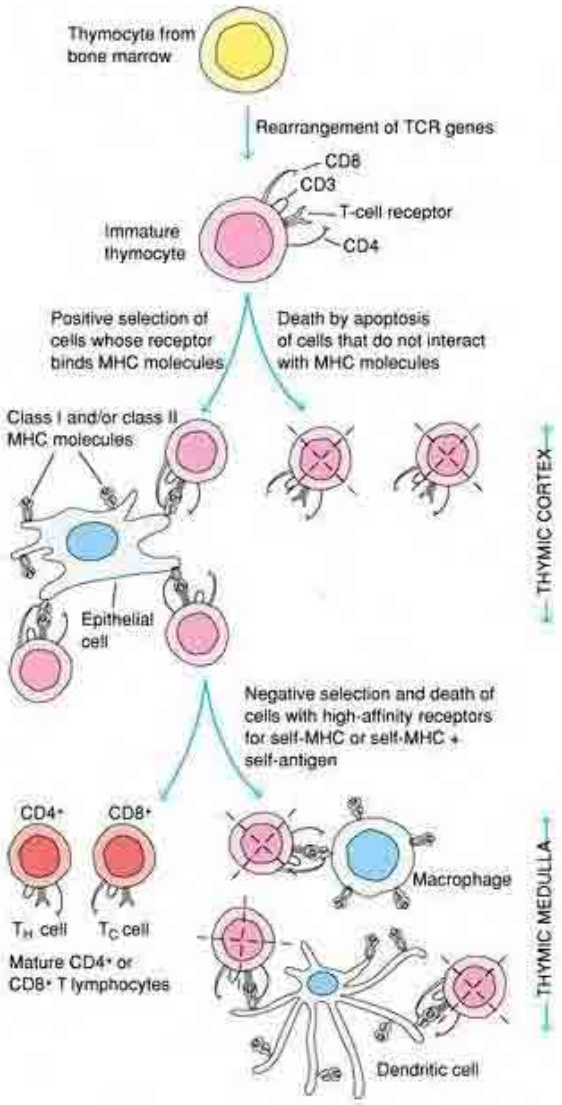


Figure 2. Positive and negative selection process in the thymus. Modified from: http://www.cartage.org.lb/en/themes/sciences/LifeScience/GeneralBiology/Immunology/ImmunityInfection/CellActivation/TCellMaturation/TCellMaturation.htm

From a functional point of view T cells can be divided into T helper cells (Th) and cytotoxic T cells (Tc). In general, Th cells express CD4 molecule on their surface and bind to antigen presented by MHC class II molecules expressed by APC such as B cells or dendritic cells. On the other hand, Tc express CD8 on their surface and only recognize antigen in the context of MHC Class I which is expressed by virtually all nucleated cells (2).

When a naïve T cell encounters antigen, a primary response is initiated. Once the TCR encounters antigen, CD28 binds to CD80 and CD86 expressed by the APC and this triggers a co-stimulatory signal which supports proliferation and survival. There are other co-stimulatory molecules but CD28 interaction with CD80 and CD86 is critical for T cells activation; without CD28 TCR engagement results in anergy. T cells divide repeatedly, giving rise to an expanded antigen specific T cell clone. In the case of Tc cells, following antigen recognition on the surface of a dendritic cell, the CD8 + T cells will evolved into CTL which have the ability to kill virally infected cells or self altered cells. When Th cells encounter antigen, they also proliferate and produce cytokines, but they can also be further polarized to form several functional subpopulations. Among these, Th1 cells predominantly secrete IL-2, IFN-γ, and TNF-β. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10. Th1 cells are responsible for cell-mediated functions such as activation of macrophages, whereas Th2 cells stimulate B cells to switch to IgE production. Th17 cells are a newly identified subtype of T cells. They are defined by their ability to produce IL-17(3), additionally Th17 cells can produce IL-21, TNFα, Lymphotoxin-β, IL-26 and IL-22. Th17 cells are also characterized by the expression of chemokine receptor CCR6 and its ligand CCL20 (4,5). These cells play a role fighting extra-cellular pathogens such as bacteria or fungi (6). The differentiation of T cells into Th17 cells is driven by IL-6 and TGF-β, further contributing factors are IL-1 and TNFα reviewed in

(7). Here, STAT3 is a key transcription factor as STAT3 deficiency results in the inability of T cells to differentiate into Th17 cells (8).

1.2.2 T regs

Regulatory T cells or Tregs are a relatively new T cell subtype with a specific role in regulating the immune response to protect the host from autoimmune disease. Tregs represent about 5-10% of the total CD4+ population in the bloodstream. They have been characterized by the expression of CD4, high levels of CD25 and expression of the transcription factor FoxP3 reviewed by (9). Depending on their origin, two different types of Tregs have been identified: natural Tregs and adaptive Tregs. Natural Tregs are generated in the thymus via TRC-MHC interaction whereas induced or adaptive Tregs are generated in secondary lymphoid organs and tissues (reviewed in (10). Adaptive Treg development was reported to require CTLA4 upregulation while natural Treg development may be CTLA4 independent (11). Treg produce TGF-β, IL-10, IL-12 and IL-35 (12). They are associated with maintenance of self-tolerance; this was established following experiments in which Tregs were depleted from mice. Treg depletion resulted in the development of widespread autoimmunity in these animals, affecting several organ systems, including the joints (13). Several additional studies have demonstrated the role of Tregs in preventing autoimmunity. NOD mice with partial Treg depletion have severe diabetes, which can be prevented by adoptive transfer of Tregs (14). In humans suffering from type I diabetes the number of Tregs seems to be comparable to healthy individuals, however, they appear to be dysfunctional; a similar situation is observed in other autoimmune diseases such as rheumatoid arthritis, myasthenia gravis or multiple sclerosis (15,16).

1.2.3 iNKT cells

iNKT cells are a subpopulation of the T cell lineage that expresses a semi-invariant TCR and markers originally associated with NK cells, such as CD161. The semi-invariant TCR consists of a variable TCR Vβ11 chain in combination with an invariant TCR Vα24Jα18 (reviewed in (17)). In humans, iNKT cells represent about 0.2% of the total T cell population in peripheral blood (18). 40 to 60% of all iNKT cells are CD4 positive, most of the remaining are double negative (DN) (CD4 negative, CD8 negative) and a very small proportion are CD4 negative CD8 positive (19,20).

iNKT cells recognize glycolipid antigens presented in the context of CD1d molecule by an antigen presenting cell, such as dendritic cells, B cells and macrophages (21-23). Several iNKT cell ligands have been described to date. The first iNKT cell ligand described is a component of the extract of marine sponge *Agelas mauritianus*, known as α-galactosylceramide (α-GalCer) (24). Other iNKT cell ligands include bacterial antigens such as a-glucuronylceramide, found in *Sphingomonas* and a glycosphingolipid self Ligand isoglobotrihexosylceramide (iGb3) (reviewed in (17)).

Within minutes after activation, iNKT cells are able to secrete large amounts of cytokines (25). The iNKT subsets secrete different cytokines, CD4 positive iNKT cells can secrete Th1 and Th2 cytokines, in contrast DN iNKT cells only secrete Th1-type cytokines (26-28). The ability of iNKT cells to secrete different combinations of cytokines confers on them the ability to polarize T cell responses towards Th-1 or Th-2 like responses. Therefore a critical immunomodulatory role has been proposed for iNKT cells (29).

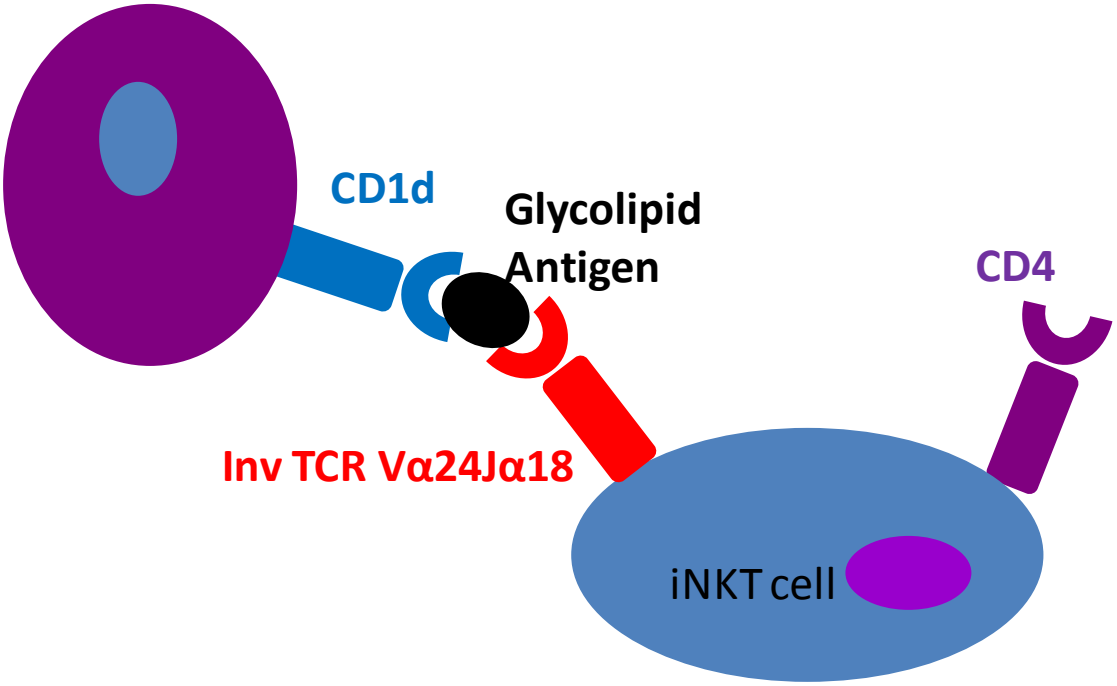


Figure 3. iNKT cells recognizing a glycolipid antigen presented through a CD1d molecule.

1.2.3.1 iNKT cell development

iNKT cells develop in the thymus. Two models were originally proposed to explain iNKT cells development. The first one proposed the concept that iNKT cells come from a T cell precursor originally destined to become an iNKT cell. The second model suggests that iNKT cells derive from a common T cell precursor but segregate from T cell development into iNKT cell development during positive selection where they recognize CD1d molecules by TCRs generated randomly (reviewed in (30)). Recent evidence favors the second model with iNKT cells developing from a common T cell precursor and segregating at the DP thymocyte stage, which is also the time when they express TCRαβ chain (reviewed in (31)). As Vα14-Jα18 rearrangement occurs in a random manner and at a very low frequency, only one thymocyte in a million can potentially become an iNKT cell (32).. Once iNKT cells develop in the thymus they will undergo a maturation process requiring the expression of

costimulatory molecules such as CD28, cytokine receptors, signal transduction molecules and transcription factors. Following this process, some iNKT cells leave the thymus as NK1.1 (CD161) negative cells and will acquire the expression of this molecule in the periphery, becoming mature iNKT cells. A proportion of iNKT cells leave the thymus as NK1.1 negative cells, but will never express NK1.1.This subpopulation has been proposed to have a different biological activity. Another subpopulation of NK1.1 negative NKT cells is thought to never leave the thymus. They acquire NK1.1 expression and are thought to become permanent residents of the thymus. The mechanism underlying thymic export and retention of iNKT cells at the different stages of maturation is not well understood to date (reviewed in (30)).

1.2.3.2 iNKT cell activation and function

iNKT cells, once activated, play a role in many immunological processes including infection, transplant-rejection and autoimmunity .

Their activation occurs by the direct recognition of iNKT cells of an exogenous antigen or via the recognition of self antigens such as iGb3. The function of iNKT cells is not very well understood. It appears to vary depending on the situation as they have the ability to secrete different sets of cytokines in different scenarios.

A cascade of events following iNKT cell activation by α-Gal-Cer has been proposed: α-Gal-Cer is presented by resting DCs to iNKT cells through CD1d and iTCR interactions. This causes iNKT cells to secrete cytokines and chemokines as well as to upregulate CD40L surface expression. CD40L interacts with DCs through CD40. Interaction between iNKT cells and DCs is thought to cause DCs to secrete IL-12 which in turn enhances iNKT cell activation and cytokine production. Activated DCs then upregulate MHC class I and II molecules and present increasing amounts of antigen to CD4 and CD8 T cells. iNKT cells produce high

levels of IFN-γ and cause NK cell cytolysis. They also stimulate B cells to increase antibody production. It is not clear why iNKT cells produce Th1 or Th2 cytokines in different situations. Following massive cytokine production by iNKT cells, these cells have been suggested to undergo apoptosis. New iNKT cells are generated in the bone marrow but the process may take up to three to four days during which very few iNKT cells will be detectable in the circulation (reviewed in (17)).

iNKT cell are known to produce IL-4, IFNγ, IL-2, IL-5, IL-6, IL-13, IL-17, IL-21, TNF-α, TGF-β and GM-CSF. In addition to these cytokines, iNKT cells also secrete a range of chemokines (33). Several transcription factors are known to regulate cytokine and chemokine production by iNKT cells such as NFkβ (34) or STAT6 (35). iNKT cells can also have cytotoxic activity. They express granzyme B, perforin and FasL. iNKT cells have been demonstrated to kill antigen-pulsed APC in vitro (36). iNKT cells secrete cytokines that are activation signals for various cells including: CD4 and CD8 T cells, macrophages, NK cells and B cells. They also have an influence on the type of cells recruited; they can recruit neutrophils through IFN-γ production. IL-2 production by iNKT cells can modulate Treg function but in turn Tregs may act as a negative feedback mechanism for iNKT cell function by suppressing iNKT cell proliferation, cytokine production and reducing iNKT cells cytotoxic activities (reviewed by (37).

In response to bacterial infections, iNKT cells can be activated via two different mechanisms, by direct presentation and recognition of bacterial antigen or triggering self activation by iGb3. Some bacteria, such as *Sphingomonas* contain a compound, known as glycosphingolipids (GSLs) in their cell wall (38). It has similarities to LPS. In *Shingomonas* infection, the bacterium is taken up by DCs and bacterial antigen is presented to iNKT cells via CD1d, these results in iNKT cell activation and cytokine production. On the other hand

many bacteria lack specific antigens that can be recognized by iNKT cells. These bacteria, for example, Salmonella have the ability to activate iNKT cells via an alternative pathway. LPS from gram negative bacteria is recognized by TLR4 on APC and this leads to iGb3 and IL-12 production by APC. iGb3 is then presented to iNKT cells and they recognized it as a self antigen which triggers iNKT cell activation and subsequent cytokine production (reviewed in (17)).

1.2.4 T cell activation markers.

T cell stimulation leads to the upregulation of several cell surface molecules including CD25, CD69, CD71 and HLA-DR. Functionally, CD25, (39) acts as the IL-2R alpha chain. Its expression is not specific to activated T cells but it is also expressed by Tregs and on activated B cells (40). CD69 is a very early activation marker, it belongs to the natural killer cell gene complex family and it is expressed on T cells within 30 minutes of TCR stimulation, it is involved in lymphocyte proliferation and it acts as a signal transducer for NK cells, lymphocytes and platelets (41-43). CD71, the receptor for transferrin, an iron-transport protein, mediates iron uptake, is normally expressed on resting lymphocytes at a very low level and is upregulated upon activation (44). Its expression is very closely associated with the S-phase of the cell cycle. HLA-DR is a human class II major histocompatibility complex antigen. HLA-DR can be detected in elevated levels at the later stages of T cell activation (45).

1.3 Cytokines

Cytokines are small proteins of about 25KDa. A large part of the information exchanged between cells of the immune system is transmitted through this group of soluble proteins. They affect the behavior of several cells in an autocrine and paracrine manner. There are two

structural families of cytokines: the hematopoietin family and the TNF family. The hematopoietin family includes several growth factors and interleukins, an example is IL-6. The TNF family of cytokines comprises 19 members including: TNFα, receptor activator of nuclear factor κB ligand (RANKL) and Fas ligand (FasL) (reviewed in (46). Cytokines from both families play a role in both innate and adaptive immune responses. Cytokines can be both pro and anti-inflammatory, some favor the inflammatory process to fight an invading pathogen and some act in an anti-inflammatory fashion to promote the termination of inflammation and help restore tissue homeostasis (2).

1.3.1 The IL-6 family of cytokines.

The IL-6 family of cytokines currently has ten members: IL-6, IL-11, LIF, OSM, ciliary neurotrophic factor (CNTF), cardiothrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and neuroprotein (NP). The various members of this family of cytokines are produced by a variety of cell types; T cells, monocytes, fibroblasts, osteoblasts, epithelial cells and some cancer cells. They are implicated in different processes such as inflammation, embryonic development, bone homeostasis and hematopoiesis (reviewed in (47)).

1.3.2 IL-6.

IL-6 is a single chain protein with a molecular mass of about 28kd. The gene encoding IL-6 has been mapped to chromosome 7, it has been cloned and sequenced and it appears to contain four introns and five exons and shows a high degree of conservation. The IL-6 encoding gene has three initiation of transcription sites and three TATA-like sequences (reviewed in (48)).

IL-6 is a cytokine secreted by monocytes, lymphocytes, synoviocytes, fibroblasts and endothelial cells (49), it has mainly a pro-inflammatory effect with some anti-inflammatory functions.

1.3.2.1 IL-6, functions

IL-6 upregulates adhesion molecules and induces the production of other pro-inflammatory cytokines (50). IL-6 also upregulates genes that affect important cellular functions such as apoptosis, differentiation, proliferation and survival (51). It has been reported to induce differentiation of activated B cell into antibody-producing cells (reviewed in (52). IL-6 promotes the differentiation of cytotoxic T cells, macrophages and megakaryocytes. It induces secretion of several molecules by hepatocytes such as C-reactive protein (CRP) or hepcidin (reviewed in (48)). CRP is an acute phase protein that is produced during inflammation and it binds to microbes during infection to enhance phagocytosis (reviewed in (53)). Hepcidin, is a hepatic hormone that regulates the intestinal absorption of iron (Nemeth et al. 1271-76). Furthermore, IL-6 contributes to angiogenesis by promoting the production of vascular endothelial growth factor. It also is a growth factor for several cell types including mesangial kidney cells, B cells and some tumor cells (reviewed in (54)).

1.3.2.2 IL-6 signalling.

The IL-6 receptor complex mediates the biological activities of IL-6. It is a heterotetramer combining two IL-6R α chain (CD126) and CD130 proteins respectively. CD130 is a signaling molecule also used by receptors for other cytokines; IL-27, IL-11, LIF, OSM, CNTF CT-1, CLC and NP (reviewed in (51)).

IL-6 classically binds CD126 directly; this causes homodimerization and phosphorylation of CD130 which in turn activates JAK 1 and 2. JAK phosphorylates tyrosine residues of STAT 3 and STAT 1 molecules, these form homo and heterodimers and translocate to the nuclear compartment where they will bind to specific target sites on the DNA of genes such as bcl-2 and bcl-xL. IL-6 binding to its receptor also stimulates the Ras-MAP Kinase cascade, and this activates transcription factors such as ELK-1 (51,55).

In addition to the direct signaling through the IL-6R/CD130 complex, IL-6 may signal through a process known as transsignaling. This occurs when the soluble form of IL-6 Receptor (sIL-6R) forms a complex with IL-6. This sIL6R-IL6 complex binds to a dimer of CD130 (56) triggering the activation of ras/Map-kinase/ERK and Jak-STAT pathways as in direct signaling through CD126 (57). The sIL-6R can be formed by alternative splicing of mRNA or shedding of membrane bound CD126 (56).

IL-6 has a limited capacity to signal directly through CD126 binding, as CD126 is expressed in only a few cell types such as leukocytes and hepatocytes. In contrast CD130 is expressed ubiquitously, this allows IL-6 to influence a wider array of cells via transsignaling (58).

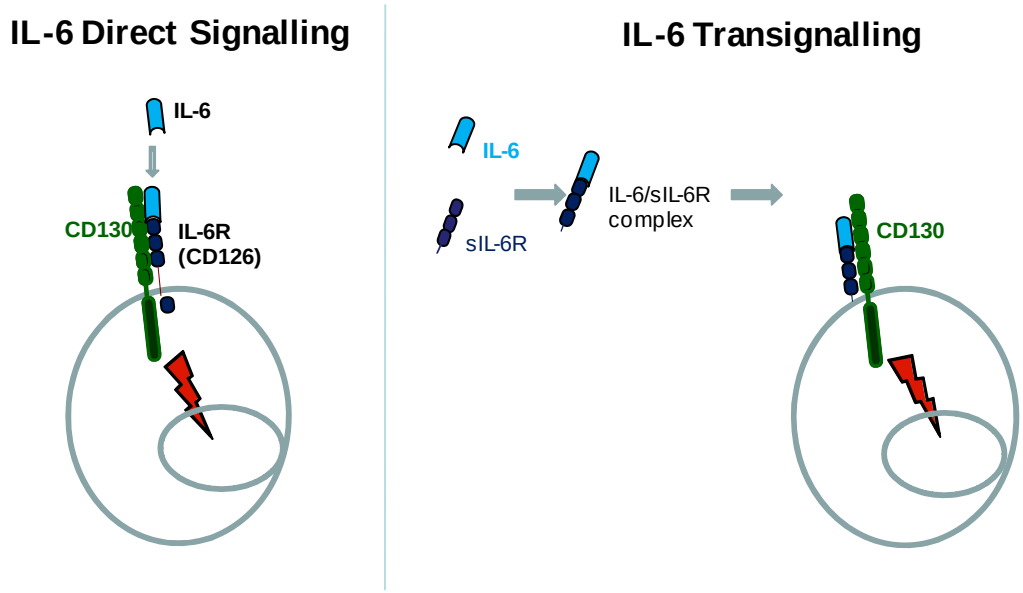


Figure 4. IL-6 signalling. On the left hand side IL-6 direct signalling. On the right hand side IL-6 signals via transsignaling.

The importance of IL-6 transsignaling is highlighted in inflammation where stromal cells exhibit a CD126 low CD130 high phenotype and their responses to IL-6 are therefore via transsignaling. In recent studies it has been shown that leukocyte expression of IL-6R make it possible that the IL-6 signals to leukocytes both via classic signaling and transsignaling (reviewed in (59)). It has been shown that T cell apoptosis can be inhibited in the aqueous

humour of uveitis patients by IL-6 transsignaling (60), indicating that T cells can respond to IL-6 not only via direct signaling but also via transsignaling.

In order to achieve IL-6 transsignaling sIL-6R must be available, there are several instances in which sIL-6R is released from various cell types. Human neutrophils shed IL-6R during apoptosis that can be induced by several causes such as cytokine deprivation or DNA damage (61). Naïve and memory CD4 T cells also produce sIL-6R following TCR activation, activated CD4 T cells shed sIL-6R in a mechanism mediated by ADAM-17 protease (62). In addition the levels of sIL-6R are elevated in serum of healthy individuals after severe exercise, the elevation is found on the second day of severe physical activity and the increase in sIL-6R correlated with an increase in CRP (63)

1.3.2.3. Regulation of IL-6R expression

IL-6R expression can be regulated by internalization or downregulation of mRNA levels coding for the IL-6R. IL-6 causes IL-6R downregulation by internalization, the IL-6-sIL-6R complex is internalized by endocytosis and subsequently most IL-6R is degraded in the lysosomal compartment. This mechanisms acts as negative feedback for IL-6 signal (55,64). In 1992, Falus et al studied the regulation of CD126 and CD130 on human cell lines of myeloid and lymphoid origin. They showed a downregulation of CD126 and CD130, at both protein and mRNA level, in U266 and BMNH cell lines treated with TPA. They also showed an inhibition of CD126 expression triggered by TPA in U937 cells but no effect on CD130. In their experiments they also demonstrated an upregulation of CD130 only in U266 cells. their data suggest that CD126 and CD130 regulation can be independent (65). In rat hepatocytes CD126 mRNA is upregulated by dexamethasone but this has no effect on CD130 mRNA. In addition IL-1 and IL-6 both separately and together cause the downregulation of CD126

mRNA (66). The downregulation of CD126 mRNA has been reported in human monocytes following LPS exposure (67).

1.3.2.4 IL-6 and signaling regulation

There are two families of molecules that negatively regulate the response to IL-6; suppressor of cytokine signaling proteins (SOCS) and protein inhibitor of activated STAT (PIAS). There are 7 SOCS molecules named SOCS1 to 7. Activation of IL-6 signalling through JAK and STAT3 initiates a pathway that limits the extent and duration of the IL-6 response by directly initiating SOCS1 and 3 *de novo* production (47). The expression of SOCS proteins is also upregulated by LIF, IL-11 and OMS (68,69). SOCS1 acts on JAK2 and TYK2, preventing phosphorylation of CD130, STATS and JAKs themselves (70). PIAS are another family of proteins that negatively regulate IL-6 signaling. PIAS are constrictively expressed on many cell types; PIAS1 and PIAS3 has been reported to bind to STAT1 and 3 respectively, preventing STAT1 and 3 mediated gene transcription (reviewed in (51)). The majority of proteins that interact with PIAS are subject to modification by small ubiquitin-related modifier (SUMO). PIAS work by promoting SUMOylation of their interaction partners, which results in an alteration in function, interaction or cellular localization. For example PIAS 4 stimulates SUMOylation of P53, increasing its transcriptional activity (reviewed in (71)

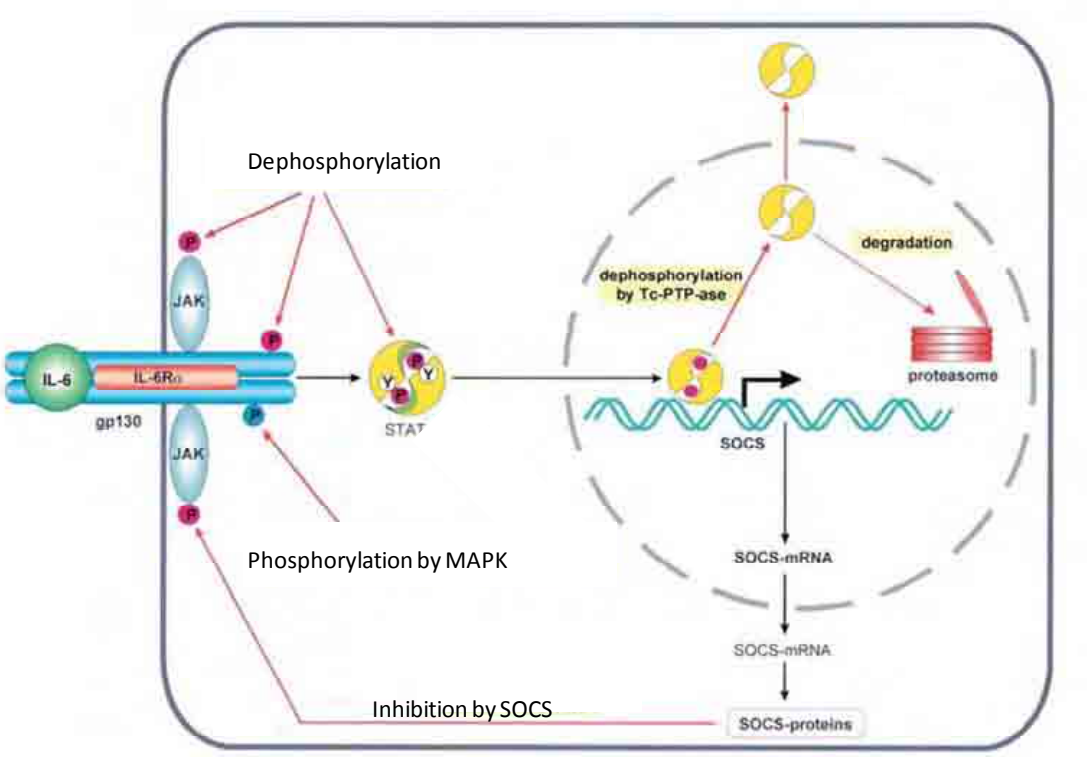


Figure 5. IL-6 signalling. Downstream events following IL-6 signaling and regulation. Adapted from (51)

1.3.1.5. IL-6, chronic inflammation and a role for IL-6 in the transition from innate to acquired immunity.

Inflammation is a generally beneficial process that occurs to eliminate a threat to the organism. To prevent damage to the organism, inflammation must be a tightly regulated process. It needs to be initiated rapidly to eliminate the immunological challenge and be downregulated leaving behind as little tissue damage as possible. Controlled termination of inflammation is critical. IL-6 has an important role promoting the acute phase response. In acute inflammation an increase of acute phase proteins, such as CRP, is observed. IL-6 amongst other cytokines is responsible for the increased production of these proteins mainly by the liver (reviewed in (72)). In some cases acute inflammation fails to resolve, this is the case seen in chronic inflammatory conditions such as RA or Crohn's disease (73)**.** IL-6

influences cell recruitment, survival and clearance by apoptosis, all of these functions if dys-regulated by high levels of IL-6 lead to failure to terminate acute immune responses which in turn become chronic and no longer beneficial for the host but rather detrimental (reviewed in (72)). In addition, IL-6 has been shown to rescue mouse resting T cells from apoptosis in vitro, suggesting that IL-6 may be a T cells survival signal in vivo (50) allowing T cell survival for long periods in situations such as chronic inflammation. Furthermore, IL-6 promotes the development of proinflammatory Th17 cells and suppresses Treg development (74). Work in human and murine peritoneal infection has shown that IL-6/IL6R complexes can control the switch from primarily neutrophilic to mononuclear cell dominated infiltrates by regulating expression of chemokines expression. By controlling this pattern of leukocyte recruitment during peritoneal inflammation, sIL-6R released from the initial neutrophil infiltrate contributes to this temporal switch from acute to chronic inflammation.

The role of inhibition of IL-6 in therapy of RA will be discussed in section 1.6.3.

1.3.2 Interleukin 10 (IL-10)

IL-10 was firstly identified by Mosman and colleagues in 1989 (75). It belongs to a family of cytokines that includes: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 (76). All members of this family have two features in common, they bind to receptors with very similar structures and their genes have a similar exon/intron organization (77). Although these cytokines share some characteristics they have very different biological functions that are determined by the secreting cell, the cell receiving the signal and the environment in which the interaction happens (reviewed in (78)).

The gene encoding human IL-10 has been mapped in chromosome 1q21-32 and contains five coding exons separated by four non-coding introns (reviewed in (79)).

IL-10 is produced by a wide variety of cells, the major sources of IL-10 include: Th2, Th1, a subpopulation of Treg known as Tr1, $CD8^+$T cells, monocytes, macrophages, some subsets of DCs, B cells, granulocytes and some non-immune cells such as epithelial cells or tumor cells (80,81). Some of the regulatory effects of Tregs and Bregs are mediated by the secretion of IL-10 and IL-10 produced by DCs can induce Tr1 differentiation (82).

1.3.2.1 The IL-10 Receptor

IL-10 signals through a receptor complex composed of two chains of IL-10R1 and two chains of IL-10R2. IL-10R1 is expressed by all hematopoietic cells while IL-10R2 is expressed almost ubiquitously. IL-10 binds IL-10R1 with high affinity and IL-10R2 enables signal transduction. Following IL-10 binding to the receptor complex Jak1 is phosphorylated and activated and recruited to IL-10R1 while Tyk2 is activated and recruited to IL-10R2, these two proteins phosphorylate the receptor (83). STAT3 is phosphorylated and then forms homodimers. These are released from the receptor and travel to the nuclear compartment where they will bind specific DNA motifs (84). STAT1 and 3 bind to the IL-10 gene promoter sequence and thus can positively regulate IL-10 expression in a positive feedback loop. STAT3 also activates expression of SOCS3 which in turn has a negative regulatory effect on IL-6 but not on IL-10 mediated signals. (reviewed in (79)).

1.3.2.2 IL-10, Biological activity

One of the main biological functions of IL-10 is to reduce the functional activity of APC(85). IL-10 reduces expression of MHC class II and co-stimulatory molecules CD80 and CD86 on DC and macrophages, preventing these cells from presenting antigen to T cells. It also has been suggested to prevent the activation of Th1 and Th2 cells and their secretion of cytokines. IL-10 also prevents maturation of monocytes to DCs. It not only prevents inflammation by

inhibiting pro-inflammatory events but also has a direct anti-inflammatory function by inducing production of IL-1 receptor antagonist.

Even though, traditionally, IL-10 is seen as a potent anti-inflammatory cytokine it also has very important pro-inflammatory functions. It induces recruitment of CD8 T cells and stimulates its proliferation and cytotoxic activities. It also stimulates the proliferation of B cells, enhancing their antibody production (86). Furthermore, it stimulates NK cells to proliferate and produce cytokines. Interestingly, all proinflammatory effects of IL-10 appear to be dose dependent and this may explain why IL-10 caused adverse effect in patients treated with rIL-10 (reviewed in (87).

Even though IL-10 has several proinflammatory functions it is largely known for its anti-inflammatory properties. On the other hand, IL-6 is a well established pro-inflammatory cytokine. Paradoxically, both IL-6 and IL-10 signals activate STAT3. It is not very well understood why cytokines using the same signaling pathway can trigger such opposite effects. Yasukawa and collaborators proposed that SOCS3 prevents STAT3 activation by IL-6 but not IL-10, SOCS3 could therefore be a factor in the different effects triggered by IL-6 and IL-10 (56).

As I have previously mentioned cytokines regulate important physiological processes and excess cytokine activity can be harmful, therefore cytokine signaling must be modulated. There are several ways to in which the immune system modulates cytokine signaling, for example downregulating cytokine receptor expression by endocytosis or allowing a soluble form of the receptor to bind to the cytokine blocking cytokine binding to its cellular bound receptor.

IL-6 and IL-10 are cytokines with very different biological functions but they both activate the JAK-STAT pathway. Both IL-6 and IL-10 lead to the activation of STAT3. Activated

JAKs phosphorylate parts of the cytokine receptor and create binding sites for SH2 domain containing proteins. JAK activation leads to STAT activation. IL-6 activates JAK1 and STAT3, while IL-10 activates tyk2 and STAT3. SOCS proteins interfere with the activation of the JAK-STAT pathway. For example the SH-2 domain of SOCS1 binds to JAK1 while the SH2 domain of SOCS3 binds to Y757 in mice and 759 in humans of the intracellular domain of gp130. Once SOCS3 binds gp130 in proximity to JAK sites it inhibits JAK activity through kinase inhibitory region, which it is thought to act as a pseudosubstrate. There is also literature suggesting that blockade of binding of SHP2 to Y757 leads to subsequente blockade of MAPK and Pi3K activation (reviewed in (88).

One may wonder why the activation of the same transcription factor STAT3 by IL-6 and IL-10 leads to complete different effects. A clear difference between these two cytokines is the fact that SOSC3 prevents the activation of STAT3 by IL-6 but not by IL-10. It has been shown that STAT3 activation by IL-6 is suppressed in wild type mice macrophages in the presence of LPS whereas IL-10 STAT3 activation is not affected, in addition, IL-6 STAT3 activation is not suppressed in macrophages from SOCS3 deficient mice, indicating that SOCS3 is an essential regulator of STAT3 induced by IL-6 but not by IL-10 (89). SOCS3 is thought to have an important role in reducing inflammation in macrophages. In addition it has also been shown that STAT3 phosphorylation is prolonged in SOCS3 deficient mice macrophages following stimulation with IL-6 but not IL-10 (90).

IL-6 and IL-10 have different effects when activating STATS3 and possibly an important factor in these differences is SOCS3, as it clearly suppresses the downstream effect from STAT3 when activated by IL-6 and not IL-10 (reviewed in (88).

1.4 Inflammation

An inflammatory response is a physiological mechanism to defend the body against physical injury or an invading pathogen.

1.4.1 Acute Inflammation

The hallmarks of acute inflammation are calor (heat), dolor (pain), rubor (redness), tumor (swelling) and function laesa (loss of function).

Acute inflammation is characterized by an influx of mainly Polymorphonuclear Neutrophils (PMN) to the site of injury in order to eliminate the inflammatory stimuli. Neutrophil presence at the site of immunological challenge peaks within 6 hours. Neutrophils are generated in the bone marrow; they migrate from the bone marrow into the bloodstream where they circulate until they are needed at the site of inflammation. When the immune system is challenged by an invading pathogen, acute inflammation involves release of mediators of acute inflammation, such as histamine produced by tissue resident mast cells. Furthermore, cytokines and chemokines are released by locally activated fibroblasts. This causes endothelial cells to bind and present chemokines and to express E-selectins and P-selectins, these molecules bind to mucins expressed at higher levels on neutrophils as a consequence of acute inflammatory mediators such as IL-1 or TNF-α. The interaction between selectins and mucins results in attachment of neutrophils to vascular endothelium, neutrophils then roll in the direction of the blood flow. Other inflammatory molecules then act upon neutrophils causing them to undergo conformational changes that will result in neutrophil transmigration through the vascular endothelium. At this point, neutrophils will accumulate at the site of injury due to the presence of several chemoattractants. Neutrophils express Fc receptors and complement receptors which allow them to bind to complement or antibody coated pathogens facilitating their phagocytosis. Neutrophils also release reactive oxygen and nitrogen intermediates which contribute to pathogen killing and also to the tissue

damage resulting from inflammation. Neutrophils secrete pro-inflammatory molecules such as MIP-1α and MIP-1β which are chemoattractants for macrophages. Activated macrophages contribute to phagocytosis of pathogens and release 3 key cytokines: IL-6, IL-1 and TNF-α. These 3 cytokines contribute to the inflammatory process by local and systemic changes, they induce the expression of ICAM-1 and VCAM-1 on the endothelium, circulating monocytes, neutrophils and lymphocytes bind to these molecules via integrin interactions. IL-1 and TNF trigger cytokine realize from macrophages contributing to neutrophil recruitment. IFNγ and TNFα activate macrophages and prime neutrophils (1).

Local inflammation may be supported by systemic changes known as an acute phase response; these changes are caused by the effect of IL-1, TNF-α and IL-6. The acute phase response involves elevated body temperature; include the production of several hormones such as ACTH or hydrocortisone leading to an increase of white cell production by the bone marrow and production of acute phase proteins by the liver. Among these is CRP, a hallmark of inflammation produced by hepatocytes upon IL-6 stimulation (91).

1.4.2 Chronic Inflammation

The hallmark of chronic inflammation is the accumulation of activated macrophages and lymphocytes at the site of infection or injury. Activated macrophages secrete TNFα, while IFNγ is produced by Th1, NK cells and cytotoxic T cells. IFNα acts on macrophages and increases their production of TNFα. Both cytokines cause the upregulation of ICAM1 and E-selectin expression, thus facilitating the recruitment of more immune cells to the site of inflammation. The recruited cells will produce cytokines and inflammatory mediators and contribute to the process. In chronic inflammation, fibrosis can occur as a result of excessive collagen production by fibroblasts. In some chronic inflammatory diseases, such as in sarcoidosis, granulomas can form. These are organized structures mainly consisting of

activated and differentiated macrophages often surrounded by T -cells. Characteristically, fusion of macrophages to multinucleated giant cells can be observed in these structures.

The persistent release of release reactive oxygen and nitrogen intermediates contributes to the extensive tissue damage seen in chronic inflammation (2).

Chronic inflammation is seen in several autoimmune diseases such as RA and in situations a pathogen cannot be cleared by the immune system. An example for a chronic infection that is often not cleared, but kept under control by formation granulomas is tuberculosis (1).

1.4.3 Resolution of Inflammation

The resolution of inflammation is a process in which all infiltrating inflammatory cells are removed from the site of inflammation and tissue homeostasis is restored. The resolution phase was in the past thought to be a passive process in which the chemical mediators of inflammation would gradually disappear once the inflammatory challenge was eliminated (reviewed in (92)). Today, the resolution of inflammation is understood as a complex active process in which several biochemical pathways are specifically regulated (reviewed in (93)). An imbalance in the mechanisms involved in the resolution phase of inflammation results in failure to resolve inflammation which in turn may lead to chronic inflammatory conditions such as Rheumatoid arthritis (RA)(92). During acute inflammation, PMN synthesize proinflammatory lipid mediators such as eicosanoids. However, recent work has shown that during inflammation neutrophils can change their phenotype and start producing anti-inflammatory mediators to actively switch off acute inflammation and promote resolution. To date there are three types of molecules known to actively resolve inflammation: lipoxins, resolvins and protectins (93). Lipoxins are derivates of arachidonic acid (94). Proinflammatory cytokines IL-1β and IL-4 promote the expression of lipoxins. The main action of lipoxins is to decrease PMN recruitment to the site of inflammation. Resolvins and

protectins are recently identified anti-inflammatory molecules. They are very potent anti-inflammatory mediators; their mode of action includes decrease of production of pro-inflammatory molecules and down-regulation of recruitment of inflammatory cells to the site of inflammation. Protectins, on the other hand, stop PMN cell infiltration by downregulation of cytokine expression for example by glial cells (reviewed in (92)).

1.5 Rheumatoid arthritis (RA) and classification criteria

RA is a chronic systemic inflammatory disease affecting the joints. The etiology and pathogenesis of RA are still not fully understood. It affects approximately 1% of the population worldwide (95) being five times more frequent in the indigenous population of North America compared to Caucasians (96). It is also more commonly found in females than in males with a female to male ration of 2-3:1. It may affect individuals of all ages but there is a peak onset between 40 and 60 years of age (reviewed in (97))

Genetic and environmental factors have been suggested to play an important role in the origin of RA (98). About 60% of susceptibility is accounted for by genetic factors (reviewed by (99)). An association has been described between RA and a sequence motif present in the hypervariable region of the β-chain of the HLA-DRB1 molecule (100). Several infectious agents such as *Borrelia burgdorferi* or rubella virus can induce a clinical syndrome difficult to distinguish from RA, most likely by molecular mimicry mechanisms (reviewed by (101,102)) .So far no infectious agent has been conclusively associated with RA development (103,104). Since, at least during their reproductive years, RA is more frequent in females, sex hormones have been proposed as another susceptibility factor for RA (105). Many other environmental factors have been linked with an increased risk of developing RA such as smoking (106,107). Overall the current perception is that RA is a multifactorial disease and that in a genetically

susceptible individual environmental factors will trigger joint inflammation and RA development (reviewed in (101).

Typical symptoms seen in RA such as joint pain and swelling are not very specific to this disease, in fact patients affected with other rheumatic diseases such as SLE or psoriatic arthritis may present to the clinic with similar complaints. To be able to diagnose RA and distinguish it from other conditions, several classification criteria have been developed. The first set of criteria to be used to differentially diagnose RA was the ARA criteria created in 1956. New developments led to their revision in 1987. Currently another revision is on its way; the new set of criteria is expected to be announced shortly in 2010. In the long term it is expected research into new genetic and immunological tools will further improve diagnostic criteria and will also allow the segregation of subsets of patients with different prognoses and optimized treatment choices. The ARA revised criteria is shown in table 1 (reviewed in (108)).

Criterion	Definition
1. Morning Stiffness	Morning stiffness in and around the joint lasting at least one hour before maximal improvement.
2. Arthritis in three or more joint areas	At least three joint areas simultaneously having soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician (the 14 possible joint areas are [right or left] PIP, MCP, wrist, elbow, knee, ankle, and MTP joints).
3.Arthritis of hand joints	At least one joint area swollen as above in wrist MCP or PIP joint.
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as in criterion 2) on both sides of the body (bilateral involvement of PIP, MCP or MTP joints is acceptable without absolute symmetry).
5.Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions, observed by a physician.
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum "rheumatoid factor" by any method that has been positive in less than 5% of normal control subjects.
7. Radiographic changes	Changes typical to RA on PA hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized to or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

1.5.1 Immunobiology and origin of Rheumatoid arthritis.

Neither the origin nor pathophysiology of RA are fully understood but many efforts have been made to investigate both. Many cells and molecules such as B cells, T cells, synovial fibroblasts, IL-6, TNF-α among others are present in the inflamed joint, but the overall role of

each of these players and the causative agent of the disease have not yet been reliably identified. Considerable effort has been made to understand T cell involvement in RA. There is no conclusive evidence for T cell clonal expansion to a particular antigen in the rheumatoid joint. However, a HLA-restricted T cell response has been postulated since 80% of RA patients among the Caucasian population have a shared epitope across the HLA-DR1 and HLA-DR4 haplotypes (109).The shared epitope is a 5-aa sequence motif in the third allelic hypervariable region of the HLA-DRβ chain (110), The presence of hypermutated IgG antibodies in 50% of RA patients suggests there could be an important autoantigen in RA triggering T cell activation (reviewed in (111)). T cells can be important mediators of disease pathology by antigen driven activation or triggering antigen-independent responses. RA T cells trigger TNF-α production by monocytes via contact dependent interactions (112). It has been shown as well that T cells may play a role that does not rely on antigen activation. T cells can be activated by a cytokine cocktail (IL-6, IL-2 and TNF-α), independently of antigen (113). Cytokine activated T cells can induce contact independent production of IL-6 by synovial fibroblasts (114). Brennan et al showed that RA synovial T cells are in fact similar to cytokine activated T cells (115). Independently of the mode of activation, it is clear that T cells are an important player in the RA pathology as treatment with Abatacept, a recombinant construct of CTLA-4 and IgFc that blocks T cell activation by interfering with signaling through costimulatory molecules (CD28 and B7) is proven clinically effective trials (116). The role of T cells in RA will be discussed in section 1.5.4.

B cells are also important in RA. They produce antibodies and inflammatory mediators such as cytokines and chemokines. In addition, B cells can act as APC in RA triggering T cell activation which in turn results in macrophage activation and further TNF-α production (117).

Treatment with rituximab, an anti-CD20 antibody, which works by depleting B cells, has been successfully used in clinical trials, highlighting the important role of B cells in RA (118).

Various autoantibodies have been described to date in RA, such as RF and antibodies to immunoglobulin binding protein (reviewed in (119)). More recently antibodies to citrullinated proteins (ACPA) have been described. They are very specific markers of the disease but their particular role in the pathogenesis has not yet been elucidated. Antibodies to citrullinated peptides were shown in the CIA model following Type II collagen immunization and prior to disease symptoms and it was further demonstrated that antibodies to citrullinated peptides enhances tissue damage in the mice (119).

Even though the research of the past years have shown advances in the understanding of RA, the overall disease process, the interactions between inflammatory cells and inflammatory mediators and a the overall cause of RA are not well understood to date.

1.5.2 The synovium, in health and in RA.

Under normal circumstances the synovial membrane that covers the inner surface of the joints is a thin layer of tissue of about 5mm thickness. The individual cells that compose the synovial membrane are known as synoviocytes. Two types of synoviocytes have been identified; macrophage-like or type A and fibroblast-like or type B synoviocytes (120). Type A synoviocytes or synovial macrophages are derived from bone marrow derived monocytes. They express several surface markers typically seen in macrophages including MHC class II molecules, CD68 and CD14 (121-123). In contrast, type B synoviocytes, or synovial fibroblast, most likely originate from mesenchymal progenitors in the synovium, and they express the adhesion molecules VCAM-1 (121,124) and α6β1 integrin (125). Synoviocytes can secrete large amounts of hyaluronic acid. However under inflammatory conditions they secrete matrix-degrading enzymes. The synovium is an organized structure of lining and

sublining that resembles the structures of epithelium and endothelium. The synovial cavity is filled with synovial fluid (SF), a viscous fluid that contains hyaluronic acid and lubricin and prevents cartilage friction and lubricates the joint. (reviewed in (126)).

Under inflammatory conditions such as RA the synovium becomes thicker and invasive; it invades the cartilage and bone contributing to the devastating effects of RA. Three key events occur in the synovium in RA: synovial hyperplasia, angiogenesis and infiltration of the synovium by immune cells. Synovial hyperplasia is characteristic of RA, the lining layer of the synovium becomes thicker with a depth of about 8 cells when it was compared to a layer of one cell depth in healthy controls (127). Synoviocytes become activated in patients with RA and exhibit higher activity of various transcription factor including NF-kβ, which is involved in the expression of adhesion molecules, inflammatory cytokines as well as matrix-degrading enzymes, all important mediators of RA pathophysiology (125). As described above, in RA the synovium becomes thicker with a larger number of cells that require nutrition and oxygenation to be brought by the bloodstream. As the requirement for nutrition grows angiogenesis is triggered in the synovium. Without the formation of new blood vessels the increased number of cells could not survive. Angiogenesis is encouraged by the production of several factors such as VEGF or PDGR (128,129). Experiments in animal models have shown the importance of angiogenesis in the pathophysiology of RA-like disease. Inhibition of angiogenesis prevented the development of CIA in mice (130). Finally, a central event in the development of RA is the infiltration of immune cells. Various cells types are found in the rheumatoid synovium: neutrophils, T cells, B cells, NK cells, DC and mast cells (reviewed by (126). The role of all these cell types has been extensively studied but the actual pathological mechanism and how all these cells and their secreted products interact with each other has yet not been fully clarified.

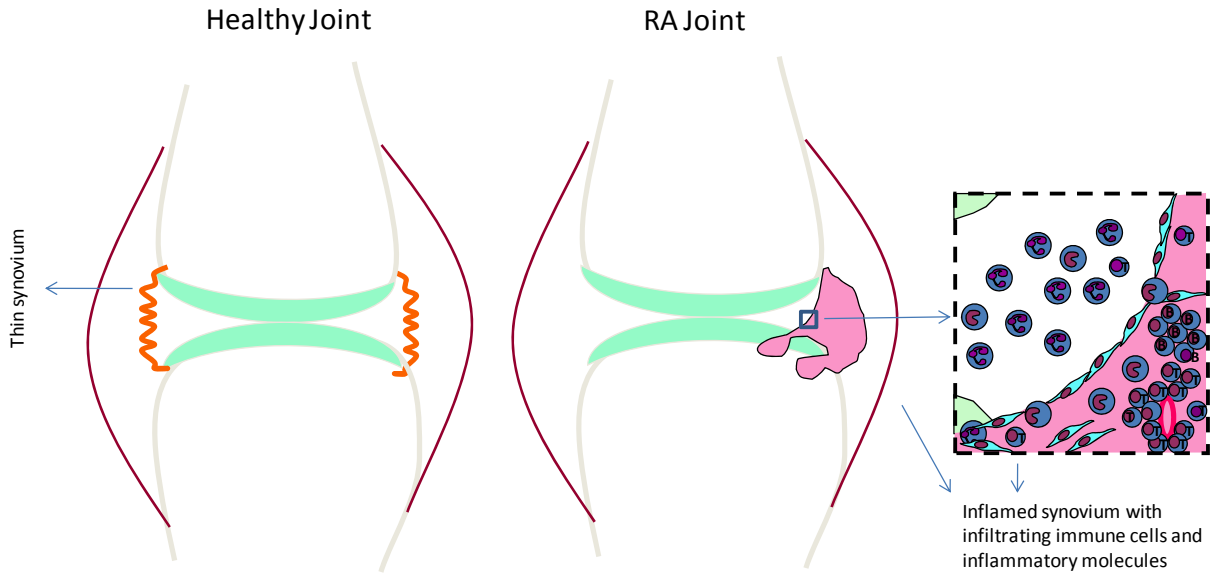


Figure 6. Healthy versus RA joint. The Join in health and in RA. Left hand side shows the schematic representation of a healthy joint. On the right the representation of a RA joint.

1.5.3 RA, a two phase disease

Many people in the course of their lives may present with joint inflammation, many of these patients will resolve the inflammation, some others will develop inflammatory conditions other than RA, such as psoriatic arthritis and some will go on to develop RA. One of the main areas studied in this project is the pathophysiology of very early arthritis patients (VEA), with this term we refer to patients presenting with joint inflammation for a period no longer than 3 months. It is important to identify as soon as possible those VEA patients which will go on to develop RA as treatment is more efficient when administrated at an early stage of the disease (reviewed in (131)).

In a previous study, we have identified a number of that cytokines are only present in the SF at a very early stage, up to three months from symptom onset. These include: IL-2, IL-4, IL-13, IL-17, IL-15, basic fibroblast growth factor and epidermal growth factor. This transient and distinct early cytokine profile found in early RA patients suggests that there may be two

phases in the pathology of RA (132). The source of these cytokines is currently being investigated in a separate project in our group.

1.5.4 T cells in RA

As discussed in 1.5.1 T cells have been proposed to have a central role in the pathology of RA. Although there are other inflammatory cells present in the RA synovium, T cells are found in greater numbers than any other lymphocyte (133). The majority of T cells in the synovium are found forming clusters around perivascular areas and smaller numbers T cells can be found scattered in the RA synovium (134).

During inflammation in response to pathogen invasion or tissue damage large numbers of T cells are recruited to the site of injury (135). Under normal circumstances, when the challenge is removed and the inflammatory response in no longer necessary, the majority of T cells undergo apoptosis (136) caused by cytokine deprivation (137). In RA highly differentiated T cells fail to undergo apoptosis and are kept alive in the synovium by several stromal factors such as IFN-β. The ability of the synovial microenvironment to rescue T cells from apoptosis contributes to T cell accumulation and RA chronicity (138,139).

1.5.4.1 T cell distribution and phenotype in the rheumatoid synovium.

T cells in the synovium of a RA patient display particular phenotypic characteristics, they express activation markers such as CD69 and HLA-DR, but they are hyporesponsive to antigen stimulation (140,141).They express a wide array of other surface molecules including chemokine receptors such as CCR5, CCR6 (reviewed in (142). They also express costimulatory molecules including ICOSL, OX40 and CD28, and receptors for many cytokines such as IL-1R, IL-25R, IL-23, etc which enable them to be targeted by several cytokines (reviewed in (143)).

Several different types of inflammatory infiltrates can be distinguished in the rheumatoid synovium: The synovial infiltrate can be a diffuse infiltrate of T cells, B cells and macrophages and DCs; In a subgroup of patients these can form follicular clusters of B, T and dendritic cells; organized structures resembling germinal centers or in rare occasions lesions resembling granulomatomata have been described (144-146). Germinal centre like infiltrates are of particular importance as they may indicate hypermutation and selection of high affinity B cell clones in the synovial tissue (146). The type of infiltrate seems to be stable and is often observed in different joints of the same patient (147).

1.5.4.2 Potential T cell antigens in the synovium

Autoimmunity involves the recognition of self-antigens by autoreactive T and B cells and the initiation of an anti-self immune response. In the context of RA researchers have been searching for joint specific self antigens that could trigger the disease. Possible candidates identified so far include type II collagen, HCgp-39 (a cartilage protein) and proteoglycans such as aggrecan. These antigens are able to cause murine autoimmune arthritis (148-150). More recently, however greater interest has been given by scientists to citrullinated proteins as potential RA autoantigens (reviewed by (143).

1.5.4.3 Interactions of T cells with other cells in the rheumatoid joint.

T cells can interact both with infiltrating immune cells as well as with stromal cells resident in the joint. These interactions can occur through direct cell to cell contact or via secreted factors such as cytokines. T cells and NKT cells are activated by interactions with DCs (151). While the existence of antigen specific T cells in the rheumatoid synovium is still a matter of considerable debate, the efficacy of treatment with soluble CTLA-4 Fc constructs that block costimulation suggests that T cell activation is an important process even in established

disease. T cells in the rheumatoid synovium have been compared to T cells activated in vitro by a combination of cytokines (113).

Interaction between T cells and monocytes through CD40-CD40L interaction can lead to osteoclastogenesis. This process is dependent on RANKL and enhanced by several cytokines including IL-15, IL-17, TNF and IL-1 (152). The process of osteoclastogenesis is enhanced by further interaction of T cells with synoviocytes, requiring the presence of TNF and IL-1 (153).

T cells found in RA synovium can acquire expression of chemokine receptor CX3CL1 or the NK receptors KIR2DS2 and NKG2D (reviewed in (142) . Fractalkine-producing synoviocytes can therefore stimulate T cell activation and proliferation through the interaction with its newly expressed receptor (154). Expression of the NK cell related receptors confers the ability to respond to activated protein C to synovial T cells. The generation of hypermutated IgG antibodies is a process that depends upon cell cell contact and cognate interaction between T cells and B cells. Since both anti-citrullinated protein antibodies and a proportion of rheumatoid factor are IgG, such an interaction is likely to take place in patients with rheumatoid arthritis. Whether this interaction takes place in the synovium or in draining or distant lymph nodes remains subject of discussion (reviewed in (143)).

1.5.4.4 Role of the newly identified Th17 T cell subset in RA

Traditionally RA has been considered as a Th-1 like disease in which characterization of T cells from synovial origin from established patients revealed expression of Th-1 cytokines such as IFN-γ and TNF-α (reviewed in (155)). It has been proposed that a defect in Th2 differentiation mechanism leads to the lack of Th2-like cytokines and allows Th1 cytokines to dominate the situation leading to chronic inflammation. It has been proposed that the mechanisms underlying Th2 differentiation defects are IL-4R allelic variations and

consequently attenuated STAT-6 activation and GATA-binding protein 3 induction, both requisites for Th2 differentiation (156).

Recently a newly identified Th subset, Th17 cells, has been proposed to have a critical role in RA. This T cell subtype is characterized by the production of IL-17 and IL-22. IL-17 is a strong effector inflammatory cytokine; its effects include the induction of cytokine secretion such as TNF, IL-1 and IL-6. IL-17 stimulates stromal cells, neutrophils, macrophages, osteoclasts and chondrocytes contributing to bone and cartilage degradation seen in RA (reviewed in (143)).

The role of IL-17 in arthritis has been studied in the context of CIA. In CIA IL-17 produced by CD4 positive T cells unable to produce IFN-γ was pathogenic. IL-23 is a requirement for the survival of Th17 cells. IL-23 deficient mice failed to develop CIA (reviewed in (157)).

In the synovial fluid and tissue of patients with RA high levels of IL-17 are detectable(158,159) other cytokines present in the rheumatic joint; particularly IL-1β, IL-23 and IL-6 are known to support Th differentiation towards Th17.

1.5.4.5 Regulatory T cells in RA

The role of Treg in RA has received considerable attention recently. Their role has been highlighted in several animal models. When DBA/1 mice are depleted of Tregs using anti-CD25 antibodies CIA becomes more several than under normal conditions (160). Mice with a gene mutation in the Foxp3 coding gene, essential for Treg function, develop a multi organ autoimmunity, these mice are known as scurfy mice (reviewed by (9). In patients with Idiopathic juvenile arthritis, numbers of CD4+CD25bright T cells are reduced, supporting the idea that Tregs may be beneficial against autoimmunity (161). In RA PB and SF the number of Tregs seem to be comparable to healthy PB and SF from other conditions (162,163). However, Treg functionality has been demonstrated to be decreased or impaired in RA SF and

PB. Under normal circumstances Tregs suppress TNF-α and IFN-γ production by CD4 T cells, in SF Tregs fail to do so this, which in turn results in maintained effector T cell proliferation (163). Ehrenstein and coworkers showed that Tregs from RA patients were unable to prevent proinflammatory cytokine production by T cells and monocytes, in addition they showed that TNF-α treatment (infliximab) restored the ability of Tregs to suppress T cell and monocyte cytokine production as well as to cause a rise in the number of circulating Tregs which coincided with amelioration of disease symptoms (164). In addition, TNF-α overexpression in the synovium increases the expression of TNF type II receptor which has the effect of lowering Foxp3 expression. Foxp3 is essential for the suppressive effects of Tregs in other cells, the decreased levels of Foxp3 translate in decreased ability to suppresses other immune cells (165). This may suggest that Treg involvement in RA may not be due to abnormal development but is more likely explained by the altered functionality of these cells.

1.5.5 iNKT cells and autoimmune disease

Due to the ability of NKT cells to secrete large quantities of immunomodulatory Th1 and Th2 cytokines, these cells have been implicated in immune responses controlling several diseases such as infection, cancer or autoimmunity (18).

iNKT cells may help to maintain tolerance to self-antigens and therefore contribute to the prevention of autoimmunity (reviewed in (21)).

The levels of iNKT cells in the peripheral blood have been found to be reduced in patients suffering from several autoimmune diseases and in transgenic mice prone to autoimmune disease. In addition, estrogens have been found to induce IFN-γ secretion by iNKT cells, this could contribute to the differential sex distribution of autoimmune diseases (166).

Multiple Sclerosis (MS) is an autoimmune disease in which an immune response is mounted against the myelin sheath covering the nerves. In this autoimmune disease IL-4 secretion by

iNKT cells was reduced and a reduction was also found in the mRNA levels for Vα24 in the peripheral blood of MS patients.

iNKT cells have also been associated with the pathogenesis of Type I diabetes. In the murine Type I diabetes model, non-obese diabetic mice (NOD mice), the numbers of iNKT cells have been reported to be low and their function defective, and disease progression is ameliorated by the adoptive transfer of iNKT cells.

Similarly in the Swiss Jim Lambert (SJL) murine model for autoimmune encephalomyelitis there are reduced numbers of iNKT cell and reduced IL-4 production by iNKT cells.

In patients suffering from Lupus Erythematosus as well as in lupus susceptible mice, iNKT cell levels were reported to be reduced. However the transfer of activated iNKT cells to susceptible young mice induced an autoimmune inflammatory reaction (reviewed in (167)).

Recent studies suggested a link between iNKT cells and animal models of the autoimmune disease bronchial asthma. In BALB/c mice, airway hyperesponsivenes (AHR) can be induced by challenging the mice with Ovalbumine (OVA). CD1d deficient mice and iNKT cells deficient mice failed to develop AHR after OVA sensitization. But when iNKT cells were transferred to iNKT cell deficient, OVA challenged mice, the AHR was restored. In addition, iNKT cells present in the airways of asthma patients have been reported to produce Th2 type cytokines, which seems to contribute to the Th2 phenotype seen in this autoimmune disease (29).

Due to their ability to modulate the immune response iNKT cells have to be carefully regulated to not contribute to the development of autoimmunity.

1.5.6 NKT cells and RA

iNKT cells have been associated with RA in many studies. However the specific role of this cell type in the pathology of RA is not fully understood yet. A protective role for iNKT cells

against the development of RA has been reported by Chiba and colleagues (168). In contrast, other groups suggest that the presence of these cells may be detrimental in RA pathology. The CIA murine model has been used by several groups to elucidate the role of iNKT cells in RA. Administration of OCH, an α-GalCer analogue, was shown to be of therapeutic value in the CIA mouse model, while it did not alter the outcome of the disease in iNKT deficient mice (168). When IL-4 and IL-10 blocking antibodies were administrated in CIA OCH treated mice the effects of OCH treatment were suppressed. It was therefore suggested that OCH may induce a shift from Th1 to a protective Th2 immune response mediated by iNKT cells. The usage of α-GalCer as a therapeutic agent is controversial as conflicting results have been reported. Chiba A. and colleagues reported no benefits from α -GalCer administration in the CIA mouse but Miellet et al 2005 reported attenuated clinical signs of arthritis following prophylactic and therapeutic administration of α-GalCer in the CIA mouse. Involvement of iNKT cells in CIA was confirmed in iNKT cell deficient mice. Upon type-II collagen immunization the iNKT cell deficient mice developed a much less severe arthritis and inhibition of CD1d by monoclonal antibodies, blocking therefore the interaction between CD1d and iNKT cells in the CIA mice also resulted in a suppression of arthritis.

In a further murine model, Antibody Induced Arthritis (ABIA), iNKT cells have not been linked with a protective effect against the disease but rather suggested a harmful role. ABIA is induced in a healthy mouse by transferring Immunoglobulins from the K/BxN mice which spontaneously develops an autoimmune inflammatory condition affecting the joints (reviewed in (169)). iNKT cell deficient mice failed to develop ABIA, and the activation of iNKT cells by administration of α -GalCer resulted in aggravation of the disease. It was also reported that in CD1d deficient mice the levels of Transforming Growth Factor-β (TGF- β) were elevated in the joint (170). The same group reported later on that iNKT cells express FcγRIII and that

FcγRIII engagement by IgG causes iNKT cells to become activated and produce IL-4, IL-10, IL-13 and IFN-γ within the context of ABIA (171). It is possible that iNKT cells activated by IgG through FcγRIII engagement produce pro-inflammatory cytokines that downregulate the production of TGF-β in the joint which in turn contributes to the inflammatory process seen in ABIA (reviewed in (27)).

1.5.7 IL-6 and RA

The first evidence that IL-6 plays a role in RA pathology derives from the observation that IL-6 is present at high concentrations in the SF of RA patients (172). Some of the systemic symptoms observed in an RA patient, such as fever or autoantibody production, can be associated with excess production of IL-6, and the blockage of IL-6 has proven to ameliorate the clinical signs of RA (173,174). An increase in RA susceptibility has been associated with polymorphisms detected in the IL-6 promoter, in particular the -174G>C (175). IL-6 promotes the development of Th17 cells, clear contributors to autoimmunity, via IL-gp130-STAT3 pathway while it suppresses the development of Tregs (74).

IL-6 has also been demonstrated to play a role in the development of autoimmunity and in particular the development of RA in several animal models. Using a model of Antigen Induced Arthritis (AIA) Boe and colleagues illustrated the role of IL-6 disease pathology (176). For this purpose, they induced AIA by injecting the mice with methylated bovine serum albumin, in both wild-type and IL-6 deficient animals. They observed that while wild type mice developed arthritis, IL-6 knockout mice fail to present any of the symptoms typically seen in this model. Furthermore, when IL-6 was injected into IL-6-/- mice, they were able to induce AIA (176). Alonzi and coworkers injected IL-6-/- DBA/1J mice with type II collagen and complete Freund's adjuvant, in wild type mice. Under these circumstances, type II collagen injection leads to Collagen Induced arthritis (CIA). CIA is a murine animal model

of arthritis often used to study the pathogenesis of arthritis and to investigate possible treatment strategies. Susceptible mouse strains, if immunized with type-II collagen, raise a severe B and T cells response, characterized mainly by the presence of Th1-type cytokines. Mice deficient in IL-6 failed to develop this condition (177). These animal models suggest that the contribution of IL-6 is essential for the development of arthritis in mice. However, some controversial results were shown using the K/BxNOD (KBN) murine model where IL-6 was not necessary for the induction of arthritis (178)**.**

Synovial fibroblasts have an active role promoting inflammation in the synovium. They produce a variety of inflammatory mediators (179). IL-6 supports proliferation of synovial fibroblasts, since they do not express CD126 this response is likely to be mediated by transsignaling. Synovial fibroblasts themselves are a major source of IL-6 in the synovium therefore they may provide a positive feedback mechanism.

IL-6 has been pointed out as a factor in cartilage destruction in the joint. Osteoclasts are major mediators of joint erosion and IL-6 has been demonstrated to promote differentiation towards this cell type (180).

The importance of IL-6 transsignaling in RA pathology was highlighted by Nowell M. A *et al*. They demonstrated the presence of high levels of sIL-6R in the SF of RA patients compared to patients with Osteoarthritis. The major source of sIL-6R comes from shedding from cell membranes. To further highlight the importance of transsignaling they showed that IL-6-/- mice that resisted the development of AIA were susceptible to the disease when reconstituted with sIL6-IL6 complexes. Furthermore, they demonstrated that transsignaling is involved in the recruitment of leukocytes via chemokine CCL2. The addition of the sIL-6R antagonist, spg130, to AIA mice model blocked the development of the disease (181).

1.5.8 IL-10 and RA

IL-10 is present in the SF and ST of RA patients. RA was demonstrated by immunohistochemistry in the joint by (reviewed in (182)). IL-10 mRNA was also demonstrated in RA and OA patients (182). Sources of IL-10 include T cells and monocytes (reviewed in (183). It seems like IL-10 plays an anti-inflammatory role as neutralizing endogenous production of IL-10 in synovial membrane cultures results in a dramatic increase of TNF-α and IL-1β, while the addition of rIL-10 to the same cocultures results in decreased production of TNF-α and IL-1β, interestingly the levels of IL-6 are not affected (reviewed in (182)). However, the presence of IL-10 has been associated with exacerbation of the disease. Lard and colleagues conducted a study looking at the relation of IL-10 promoter polymorphism and RA. They demonstrated that there was an association between the -2849 IL-10 promoter polymorphism which causes high IL-10 production was correlated with a high level of joint destruction in RA (184). Another study compared synovial membrane mRNA levels of various cytokines with disease progression, and demonstrated that the cytokine most closely associated with damage was IL-10 (185). With an anti-inflammatory function of IL-10 in mind, it was tested in a clinical trial as a treatment for Rheumatoid arthritis and. Even though IL-10 did not cause major toxicity the patients did not show significant improvement (reviewed in (186)).

1.6 Cytokine targeting therapies of RA

The etiology of RA is not very well understood; therefore it is very difficult to develop a curative treatment. All treatments available to date aim to minimize the signs and symptoms of the disease. An important target is to delay the bone erosion that leads to loss of joint function and eventually to disability. It is now understood that it is crucial to start treatment as early as possible in the disease process, the sooner the treatment begins the better the likely outcome is for the patient. Established treatment regimes for RA include glucocorticoids and

disease modifying antirheumatic drugs (DMARD) such as methotrexate and sulfasalazine (reviewed in (187)).

More recently, a new class of drugs has been developed based on our increasing understanding of the cell types and cytokines involved in the pathophysiology of RA. This group of drugs, termed “biological”, contains monoclonal antibodies specific for cytokines and cell populations and soluble receptors that can prevent cell-cell or cell-cytokine interaction. Combinations of TNFα blocking antibodies or soluble receptors with methotrexate have considerably improved the treatment of severe RA (reviewed in (188)).

1.6.1 B cell depletion

B cells are well known to contribute to RA pathology. Antibody-secreting B cells are responsible for the production of RF in RA, and in turn RF contributes to the disease by triggering the formation of immune complexes that lead to complement activation, leukocytes infiltration and the formation of membrane attack complex (189,190) . B cells also play a role as APC, they process and present antigen to T cells in RA and as a consequence T cells proliferate and act as pro-inflammatory agents. Furthermore, B cells contribute to RA pathology by the secretion of cytokines such as IL-4 (191). A monoclonal antibody has been developed against a B cells transmembrane proteins expressed in mature B cells known as CD20 (192). This drug, Rituximab, causes B cell death by three different mechanisms. It promotes B cell apoptosis, a Rituximab-CD20 complex activates complement leading to the formation of the membrane attack complex which causes B cell lysis (193) and it induces cell mediated cytotoxicity (194). Several clinical trials using rituximab alone or in combination with other drugs have shown that this compound is very beneficial for the treatment of RA as well as not showing any significant toxicity (reviewed in (195)). Although the response to rituximab is very satisfactory, clinical relapses have been observed after treatment, these

relapses seem to be associated with the reappearance of B cells in the bloodstream (reviewed in (196)). B cells may survive rituximab treatment by several mechanisms. For example, levels of BLyS, a B cell anti-apoptotic can be increased upon B cell depletion, supporting the repopulation of the B cell pool. BLyS has also been suggested to counteract the cytotoxic effect of rituximab (197) (reviewed in (195)).

1.6.2 TNF targeting therapies

TNF-α is a critical cytokine with a role in adaptive immune responses and homeostasis. It is generated by several cell types including monocytes, T and B cells (198). The expression of TNF-α is induced by several stimuli including: TLR stimulation, various cytokines, viral agents and neoplasmic transformation. TNF-α triggers the activation of immune cells such as NK cells, Cytotoxic T cells and PMN cells, it also stimulates proinflammatory cytokine production, it has an role in cartilage breakdown as it promotes osteoclastogenesis and inhibits collagen synthesis (199). The contribution of TNF-α to RA pathology has been highlighted by several studies using murine models for RA. TNF-α overexpressing mice spontaneously develop a systemic inflammatory condition affecting the joints that resembles RA (200).

Currently four anti-TNF drugs are in clinical use: infliximab, etanercept, adalimumab and Certolizumab pegol. Infliximab is a monoclonal antibody against TNF-α, it has a humanized constant region and a murine variable region. It binds soluble and membrane-bound TNF-α, neutralizing their effects. Serious adverse reactions have not been reported but some patients seem to make antibodies against this drug lowering its efficacy. Etanercept is a recombinant form of the TNF receptor 2 obtained by recombinant DNA technology. This drug binds both soluble TNF-α and TNF-β blocking their biological action, antibodies against this drug have also been observed in about 10% of the patients (201,202). Adalimumab is a human

monoclonal antibody that specifically targets TNF-α both in soluble and bound form. (202). Certolizumab pegol is a humanized Fab fragment of an anti TNF-α antibody coupled to polyethyleneglycol (203)

1.6.3 IL-10 as a therapeutic agent.

IL-10 has a very important role in the prevention of autoimmunity. This has been demonstrated using IL-10 deficient mice in autoimmune models. In all murine models of autoimmune disease tested, disease is exacerbated in the absence of IL-10. The observations of the anti-inflammatory role of IL-10 formed the scientific basis for its use as a therapeutic agent to treat autoimmune diseases (reviewed in (204)).

Therapeutic use of recombinant IL-10 has been tested in patients with a range of autoimmune disease such as psoriasis, Crohn's disease or RA. rIL-10 treatment may be beneficial when used in small doses if delivered locally to the site of inflammation. When higher doses of rIL-10 were used to treat RA it resulted in an enhanced inflammatory response (79). It was observed by van Roon and colleagues that IL-10 treatment caused the upregulation of Fc gamma receptor on monocytes/macrophages in IL-10 treated RA patients, it seems that Fc gamma receptor upregulation may counteracts the anti-inflammatory action of IL-10 possibly by triggering a proinflammatory response to immune complexes (205).

1.6.4 IL-6 as a therapeutic target for RA

It has been demonstrated that IL-6 plays an important role in the development of RA both by contributing with its proinflammatory actions. Animal models have also shown that the lack of IL-6 results in failure to induce RA-like disease or cause diseases with less severity and milder symptoms. IL-6 actions are caused by both direct signaling through its receptor or via transsignaling. So it is logical to think that interfering somehow with the actions of IL-6 you may be able to ameliorate the symptoms of RA.

A humanized anti-IL-6R monoclonal antibody is known commercially as Tocilizumab (206). Anti-IL-6R is able to block IL-6 signaling mediated by membrane bound IL-6R or via interactions between IL-6/sIL-6R that lead to transsignaling (reviewed in (207)). Administration of anti-IL6R antibody led in phase I clinical trial to reduced levels of CRP and SAA that reach normal levels, suggesting that IL-6 signaling was required for the overproduction of these protein in RA (49,208). Since then different clinical trials have demonstrated the beneficial effects of IL-6 receptor blocking therapy. It has been shown to improve patients' outcomes including a substantial delay in joint erosion. It has also been tested in combination with other anti-rheumatic drugs such as methotrexate also leading to very positive results. No major adverse reactions have been described, however some moderate changes in cholesterol levels and in serum liver enzymes have been described following treatment with this drug (reviewed in (207)). Anti-IL-6R prevents neutrophil adhesion and joint damage caused by neutrophil release of proteolytic enzymes and ROS intermediates, it prevents osteoclastogenesis which in turn prevent bone reabsorption, it also prevents proteoglycan synthesis and pannus formation (reviewed in (209). Anti-IL-6R normalizes Amyloid levels and suppresses acute phase reactions so it is important to monitor patients under this treatment as they can be at risk of infection. Another possible adverse effect is the rise in cholesterol levels but cardiovascular complications have not been reported so far. Importantly IL-6R blocking does not loose its efficacy over time unlike other biological treatments such as anti-TNF (reviewed in (210)

1.7 RA therapies targeting T cells.

The association of RA with specific MHC haplotypes suggests a role for T cells in RA pathophysiology. They are present in high numbers in the synovium and their interaction with other cell types is thought to be driving inflammation. Several approaches to T cell therapy

have been used over the years. Anti-CD4, anti-CD5 and anti-CD52 antibodies have been used to deplete T cells; this therapeutic approach has had a transient effect that ends with the repopulation of autoreactive T cells. Autologous stem cell transplants following ablation of the bone marrow has been used experimentally. It is associated with a considerable risk to the patient and is mostly used as a last resort in patients with treatment resistant SLE and scleroderma. Although an improvement is observed in these patients they often relapse when the synovium is repopulated with naïve T cells (211).

Abatacept is a biological that blocks costimulation following TCR engagement, interferes with T cell activation. This treatment has been successfully used in clinical trials on RA patients (reviewed by (212).

1.8 Project rationale

The role of T cells in the pathogenesis of rheumatoid arthritis is not very well understood and remains subject of controversial discussion. It has been proposed that the role of T cells changes during the progression from early to established stages of rheumatoid arthritis. Antigen specific activation of T cells may occur early, while in established disease mechanisms independent of cognate interaction take over. One of the questions we wanted to address was in this project was whether such a change is reflected in differences in expression of activation markers on infiltrating T cells.

Previous work in our laboratory has shown that there is a change in the profile of cytokines detectable in the synovial fluid of patients. Intriguingly, we detected an increased level of Th2 cytokines IL-4 and IL-13 in the SF of very early RA patients. With iNKT cells being described as a source of IL-4 and IL-13, we were interested to examine whether these cells are specifically accumulated in the very early phases of RA. In addition, the transient but cytokine profile seen in early RA suggest that RA pathophisiology could be different in very

early stages compared to later stages. So we hyphothesised that presence of iNKT cells and T cell activation status varies between very early stages of RA and established RA.

IL-6 plays an important role in the regulation of T cell responses. It affects differentiation into Tregs and Th-17 cells and can also act as a survival factor. We hypothesized that T cells infiltrating the Rheumatoid Joint can respond to IL-6 via direct signaling and transsignaling.

In this study, we investigated how the responsiveness of T cells in different compartments of the rheumatoid joint is regulated. In particular, we were interested in dissecting the expression of the two components of the IL-6 receptor, CD126 and CD130, in the synovial tissue, synovial fluid and peripheral blood of patients with RA. Having established that there is a difference in the expression of CD130 in T cells found in these three compartments, we examined the biological consequences of differential CD130 expression and possible mechanisms that regulate its expression in the synovium.

1.9. Project aims.

- To investigate the levels of iNKT cells in PB and SF of very early RA, very early arthritis and established RA compared to healthy controls.
- To investigate the activation status of T cells from PB and SF from the same groups of patients and controls.
- To investigate the level of expression of CD126 and CD130 on CD4 T cells from PB, SF and ST of RA patients.
- To determine the biological consequence of the differential expression of CD126 and CD130 on T cells from SF and ST of RA patients.
- To determine how CD126 and CD130 expression is regulated.

Chapter 2.

MATERIALS AND METHODS

2.1 Patients

We collected PB and SF samples from two groups of patients: patients with established RA as categorized by the 1987 ACR criteria and patients recruited from the Birmingham Early Arthritis Clinic, this group of patients was seen at the clinic within the first three months of symptoms. Within 18 months of presentation these patients were classified as having RA, resolving arthritis, or chronic arthritis other than RA. Informed consent was taken and ethical approval was obtained from the local ethical committee. Patients were not on biological treatments although some were on disease modifying drugs (DMARD). ST was obtained from RA patients, undergoing joint replacement surgery. Further information regarding the patients' age, gender, disease duration, CCP status, disease activity and drug regime can be found in the attachment.

2.2 Isolation of PBMCs by density gradient centrifugation

Peripheral venous blood was collected from patients and healthy volunteers in tubes containing 10 U/ml of heparin (Multiparin r, Product License Authorization holder CP Pharmaceuticals, Ltd, Ash Road North, Wrexham, UK). The blood was gently mixed to ensure an even heparin distribution. The required volume of blood was mixed with an equal volume of RPMI1640 (Sigma), 2mM Glutamine 100U/ml Penicillin and 100 µg/ml Streptomycin. 2 ml of *Ficoll-Paque*™ PLUS (GE Healthcare Bio-Science, Uppsala, Sweden) were dispensed into 12 ml tubes, then 10 ml of RPMI diluted blood were slowly pipetted in an angle on top of the Ficoll layer. All tubes containing ficoll and diluted blood were centrifuged

at 220 g for 30 minutes at 20ºC with no brake. Subsequently a layer of PBMCs located above the ficoll was extracted extracted carefully using a fine plastic Pasteur pipette; the PBMCs were transferred into a 25ml tube. The tube was filled up with RPMI up to the 25 ml mark and centrifuged again at 1200 rpm at 20C for 8 minutes. After centrifugation the supernatant is discarded, and the cells were washed again. At this stage cells may be counted in a haemocytometer. PBMC's obtained were used for several experiments including the isolation of CD4 T cells.

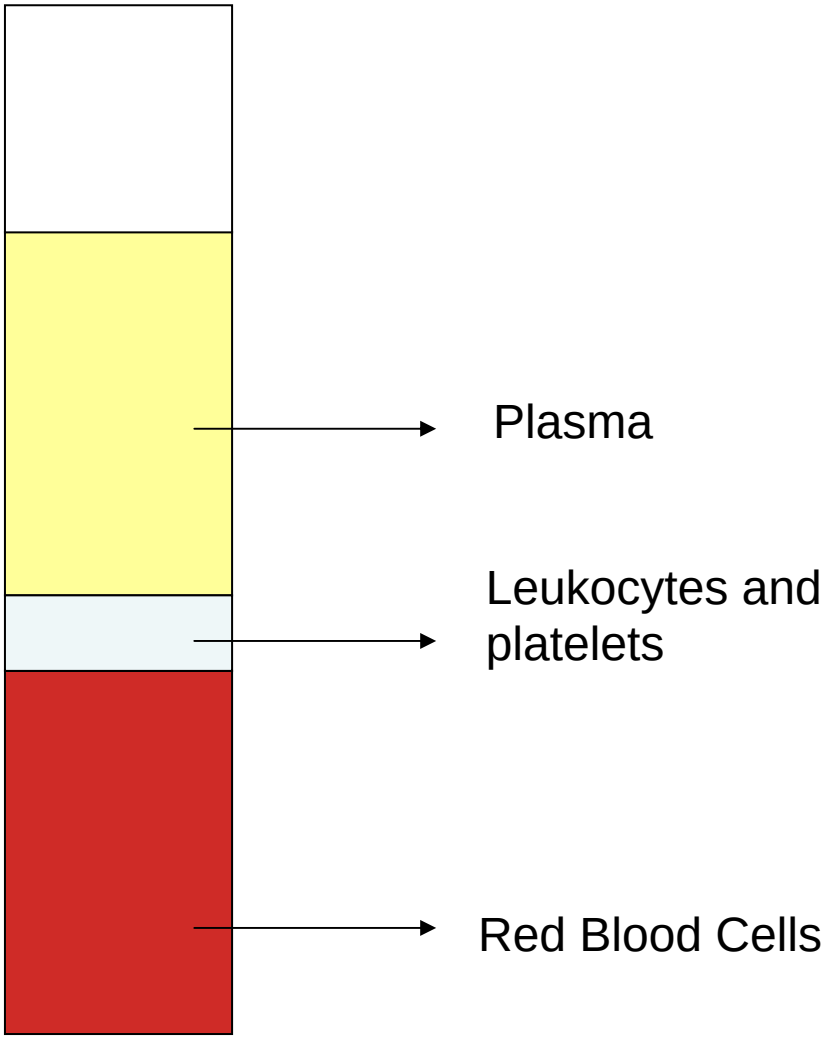


Figure 7 : Schematic representation of a tube following density gradient centrifugation. The figure shows the three layers found following centrifugation, plasma, leukocytes and red blood cells.

2.3 Isolation of CD4 T cells by cell-sorting from PBMCs and STMCs

PBMC's and STMCs are obtained from PB by Ficoll density gradient centrifugation or by mechanical degradation of ST followed by Ficoll gradient centrifugation. PBMCs or STMCs are resuspended in 300µl of an antibody cocktail prepared in PBS 2%BSA. Antibodies used are shown in the following table:

Antibody	Dilution
CD4 Tricolor (Caltag)	(1:50)
CD11b Fitc (ImmunoTools)	(1:20)
CD11c Fitc (ImmunoTools)	(1:20)

Cells are incubated for 30 minutes on ice and then washed twice by adding 1ml PBS 2%BSA and spinning down at 220 g for 4 minutes at 4C. After the washes cells are resuspended in 500µl PBS 2% BSA and then filtered into a FACS tube. An irrelevant control and compensation tubes are set up. The cell suspension is run though the cell sorter (Mo Flow^{tm}). A gate is set around the lymphocytes in the FS/SC window. CD4 positive, CD11b and CD11c negative lymphocytes are collected into a fresh FACS tube containing 500µl of PBS 2%BSA. Cells are centrifuged, the supernatant is discarded and the cells are resuspendend in RPMI BSA 1%, GPS 1%, HEPES 1% at a concentration of 5 million cells per ml. Sorted CD4 T cells will be used to study the expression of CD126/CD130 by flow cytometry after various treatments.

2.4 Isolation of CD45RO CD4 positive T cells T cells by cell-sorting from PBMCs

PBMCs and STMCs are obtained from PB by density gradient centrifugation or by mechanical degradation of ST followed by density gradient centrifugation. PBMCs or STMCs are resuspended in 300µl of an antibody cocktail prepared in PBS 2%BSA. Antibodies used are shown in the following table:

Antibody	Dilution
CD4 Tricolor (Caltag)	(1:50)
CD3Pe (ImmunoTools)	(1:20)
CD45RO Fitc (DAKO)	(1:40)

Cells are incubated for 30 minutes on ice and then washed twice by adding 1ml PBS 2%BSA and centrifugation at 220 g for 4 minutes at 4C. After the washes cells are resuspended in 500µl PBS 2% BSA and then filtered into a FACS tube. An irrelevant control and compensation tubes are set up to control that there is no unspecific staining and to subtract spectral overlap of the individual fluorochromes. The cell suspension is run though the cell sorter (Mo Flow^{TM}). A gate is set around the lymphocytes in the FS/SC window. CD4 positive, CD3 positive and CD45RO positive lymphocytes are collected into a fresh FACS tube containing 500µl of PBS 2% BSA. Cells are centrifuged, the supernatant is discarded and the cells are re-suspended in RPMI BSA 1%, GPS 1%, HEPES 1% at a concentration of 5 million cells per ml. Sorted CD45RO positive CD4 T cells will be used to study the expression of CD126/CD130 by flow cytometry after various treatments and to study the effect of HUVEC transmigration on the expression of CD130.

2.5 Processing of ST samples to obtain STMCs.

ST is brought to the laboratory after surgery. The tissue is placed in a Petri dish with ca 10ml RPMI 1640. ST is cut in ca 1mm^3 pieces using a sterile scalpel. The tissue fragments are transferred into a 50ml tube using a 25ml pipette and the tube is filled with RPMI (2nM EDTA). Cells are then pelleted at 220 g for 8 minutes. After centrifugation the supernatant in discarded and tissue is resuspended in 20ml RPMI. The tissue is then transferred into a plastic bag produced to fit into the stomacher. The bag is heat-sealed to avoid spillage. The tissue-

fragments are mechanically processed in a Stomacher 400 Circulator for 5 minutes at 230 rpm. Processed tissue is filtered into a falcon tube. The filtered solution is ficolled to obtain STMCs. STMCs obtained from ST are used for various experiments.

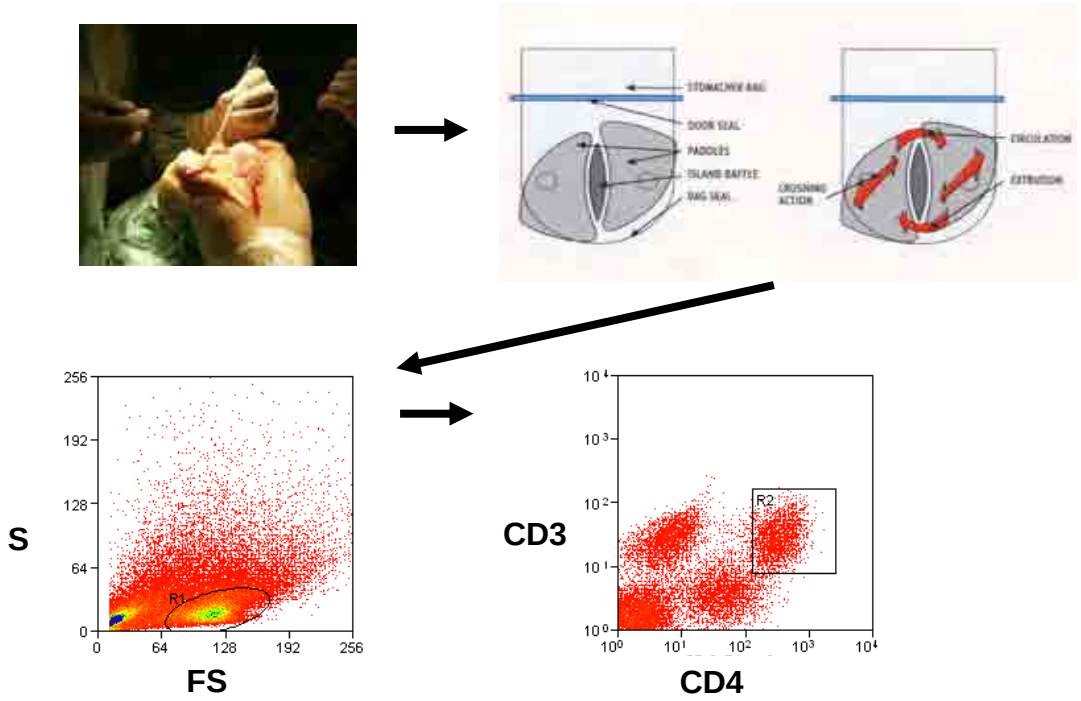


Figure 8. Processing of ST to obtain STMC. Tissue from Joint replacement operations is cut into small pieces and then processed in a Stomacher 400 Circulator to obtain a cell suspension from which we obtain STMCs via density gradient centrifugation.

2.6 Flow cytometry

The flow cytometers used in our experiments were an EPICS XL Flow Cytometer from Beckman Coulter and a Cyan ADP 9 color from DAKO. The expression of cellular surface markers was detected using direct labeled antibodies directed to those particular markers. The labels used included PE, Fitc, PeCy5 and PeCy7. Bound labeled antibodies were detected by excitation using a 488 nm argon laser. Every experiment was calibrated using Flow-setTM Flurospheres standardization reagents (Beckman Coulter and Cyan). Compensation was used

for all colors to make sure each laser only stimulates the particular fluorescent molecule that is supposed to and not others. Isotype species and concentration matched controls antibodies were used to determine the level of background staining.

Flow cytometry data was analyzed using WinMDI software (version 2.8, Scripps Research Institute, La Jolla, CA) and Summit™.

2.6.1. Three-color flow cytometry to quantify iNKT cells and determine T cells activation status.

PB and SF are collected from our patients and consented healthy donors. 10 U/ml of Hyaluronidase are added to the SF. SF is then incubated at 37ºC for 20 minutes to reduce viscosity.

Subsequently 2ml of SF and 2 ml of PB are washed with 1ml of MACS buffer (PBS, 0.5% BSA, 2nM EDTA, pH 7.2). The supernatants are discarded and 50 µl of SF packed cells and PB packed cells are pipetted into tubes for immunostaining. 50 µl of the required antibody mixture at the appropriate dilution are added to the cells. Antibodies used and dilutions are as follows:

- Irrelevant antibodies: IgG1Pe (1:20) (DAKO), IgG1Fitc (1:20) (DAKO) and CD3Pe/Cy5 (1:30) (ImmunoTools)
- Compensation antibodies: CD4Pe (1:50) (BD biosciences), CD4Fitc (1:40) (BD biosciences), and CD3Pe/Cy5 (1:30) (ImmunoTools)
- 4 combinations of anti- iNKTpe (1:15) (BD Pharmingen™) and CD3Pe/Cy5 (1:30) (DAKO) with the following activation markers: CD25Fitc (1:5)(BD), CD69Fitc (1:25) (BD), CD71Fitc (1:200) (ImmunoTools) and HLA-DRFitc (1:100) (BD) . The anti-iNKT antibody, isotype IgG1, k, it is a monoclonal antibody 6B11 which reacts with a

unique determinant in the CDR3 region of the invariant (Vα24-JαQ) TCR chain. Thus 6B11 identifies a subset of all Vα24 positive T cells

After 30 minute incubation on ice 1 ml of RBC Lysis buffer (NH4Cl Tris-HCL dH2O) is added to all tubes and incubated for 7 minutes at RT. Then cells are washed twice by adding 1 ml of MACS buffer and spinning down in the centrifuge at 220 g for 6 minutes at 4º C. Finally the cells are resuspended in 500µl of ice-cold PBS.

2.6.2. Three-color flow cytometry to determine the expression of CD130 and CD126 on freshly isolated CD4 T cells.

CD4 T cells are FACS sorted from PBMC's (as described in section 2.3) or isolated by negative selection using MACS beads (as described in section 2.2). CD4 T cells are resuspended in PBS 2% BSA at a concentration of 5 million cells per ml. 20µl of cell suspension are pipetted into a FCS coated well of a 96 well flexi-plate. The desired treatment is added to the cells; treatments, concentration and incubation condition are described in the following table:

Treatment	**Concentration**	**Incubation conditions**
Medium (RPMI)	Undiluted	0h, 1h, 3h, and 18h at 37ºC
IL-6 (Pepro Tech)	10ng/ml	1h at 37ºC
Anti-IL6R antibody	1ug/ml	1h on ice
SF from RA patients	Undiluted	1h at 37ºC

In seven experiments two treatments are used in combination, first a blocking α-IL6R antibody is added to the cells and after incubation IL-6 or SF are added without washing off the previous treatment. Following a second incubation period, cells are washed and resuspended in 50µl of the required antibody or antibody mixture at its optimum dilution; the

antibodies used vary depending on how $CD4^+$ T cells are isolated. If cells are obtained by negative selection the antibodies used are as follows:

- Irrelevant antibodies: IgG1PE (1:20) (DAKO), IgG1Fitc (1:20) (DAKO) and CD3Pe/Cy5 (1:30) (ImmunoTools)
- Compensation antibodies: CD4PE (1:50), CD4Fitc (1:40) and CD3PE/Cy5 (1:30) (all from ImmunoTools)
- A mixture of CD4Fitc (1:40) and CD3PE/Cy5 (1:30) (both from ImmunoTools) combined with either CD126PE (1:2.5) (Immunotech) or CD130PE (1:5) (BD Pharmingen^{TM}).

In the case where $CD4^+$ T cells were FACS sorted the cells are already stained with CD4-Tricolor so the antibodies used are:

- Irrelevant antibodies: IgG1PE (1:20) (DAKO), IgG1Fitc (1:20) (DAKO) and CD3Pe/Cy5 (1:30) (ImmunoTools)
- Compensation antibodies: CD4PE (1:50), CD4Fitc (1:40) and CD3PE/Cy5 (1:30) (all from ImmunoTools)
- CD126PE (1:2.5) (Immunotech) or CD130PE (1:5) (BD Pharmingen^{TM})

In both cases compensation tubes are set up using PBMCs rather than sorted CD4 positive cells. After incubation for 30 minutes on ice the cells are washed twice by filling up the wells with PBS 2% BSA and centrifuging the plate at 220 g for 4 minutes at 4ºC, then they are transferred to FACS tubes containing 500µl of ice-cold PBS. Irrelevant controls and compensation tubes were included. Cells are read by flow cytometry.

2.6.3 Four color flow cytometry to investigate the expression of CD126 and CD130 on CD45RO positive CD4 T cells from PB, SF and ST.

PBMCs and SFMCs are obtained by density gradient centrifugation. STMCs were obtained processing ST using mechanical disintegration with a Stomacher 400 Circulator as described in section 2.4 and then by ficoll gradient centrifugation. PBMCs, SFMCs and STMCs were stained with 50ul of the antibodies shown in the following table:

Antibody	Company	Dilution
Anti-CD126Pe or anti-CD130Pe	Immunotech and BD Pharmingen™ respectively	1:2.5 and 1: 5 respectively
Anti-CD3PeCy5	Immunotools	1:15
Anti-CD4PeCy7	BD Pharmingen™	1:10
Anti-CD45ROFitc	DAKO	1:40

The cells were incubated with the antibodies for 30 minutes on ice, and then cells were washed with PBS/BSA twice and resuspended in 500ul of PBS/BSA. Cells were analyzed using a DAKO Cyan flow cytometer.

2.7 Cytokine CD4 T cell treatments.

CD4 T cells are obtained by ficoll gradient centrifugation of PB and subsequent FACS sorting for CD4 cells. CD4 T cells are resuspended in RPMI with 5% HEPES, 5% GPS and 5% BSA at a concentration of 1 million cells per ml. The following cytokines are added to cells:

- IL-6 (10ng/ml)
- IL-23 (10ng/ml)
- RANTES (10ng/ml)
- MIP1-α (10 ng/ml)
- IFN-γ (10ng/ml)

- TNF-γ (10ng/ml)
- IL-10 (10ng/ml and 100ng/ml)
- IL-17 (10ng/ml)
- IL-15 (10ng/ml)
- SDF (40ng/ml)

Cells were treated with these cytokines for 6h at 37ºC. After treatment cells were washed twice in PBS/BSA. Cells were subsequently used for FACS staining or RNA extraction.

2.8 PBMC, SFMC and STMC treatment.

PBMCs, SFMCs, and STMCs were obtained from density gradient centrifugation of PB, SF and processed ST. The cells were resuspended in RPMI (with HEPES, GPS and 1% BSA) at a concentration of 5 million cells per ml. 20µl of cell suspension were pipetted into the wells of a flexiplate previously coated with FCS. Cells were treated at 37ºC for 15 minutes with hyper IL-6 (25ng/ml) IL-6 (1 µg/ml), sIL-6R (10 ng/ml), IL-2 at (50 U/ml) and IFNβ. Following treatments were washed with 100 µl of TBS. Cells were then ready for FACS staining.

2.9 Tissue sectioning

Synovial tissue sections are cut from ST surgically removed from RA patients which is first snap frozen in liquid nitrogen and then stored at -80ºC. The sections are 5 microns in depth; each section is deposited on a 4 spot glass slide. The sections are allowed to dry under a fan for 2-3 hours and then they are fixed in acetone for 20 minutes at 4ºC. After fixation the sections are stored at -80ºC in individual plastic bags.

2.10 Immunohistochemistry Staining

Immunofluorescence allows us to visualize the expression of cellular molecules in synovial tissue sections stained with primary antibodies against these molecules and secondary antibodies conjugated to fluorescent markers that specifically bind to the primary antibodies.

The fluorescent markers are excited by several lasers (351/364, 488, 543 and 633 nm lasers) using a Zeiss LSM 510 confocal scanning microscope (Carl Zeiss, Jena, Germany). This translates into images in which targeted cell molecules are seen in different colors. Confocal microscopy is an imaging technique used to obtain high resolution images. A key feature of confocal microscopy is its ability to produce blur-free images that are taken point-by-point and reconstructed with a computer. A digital camera is attached and allows obtaining digital pictures of stained tissue. Digital photos are later analyzed using the Zeiss LSM Image browser software.

Tissue sections are taken out of the freezer and allowed to reach RT inside the plastic bag where they were stored. Once at RT tissue is re-hydrated by adding 2-3 drops of PBS on top of each section. PBS is removed with a fine Pasteur pipette and 2-3 drops of Image-iTTM FX Signal Enhancer (Molecular Probes, Invitrogen detection technologies) are added to each section. The sections are incubated for 30 minutes at RT in a humid chamber. Following this incubation the Image-iTTM FX Signal Enhancer is removed and without washing, 100µl of the primary antibody mixture or matched isotype irrelevant controls is pipetted on top of the tissue sections. Primary and isotype control antibodies used are as follows:

- Primaries: CD3Biotin (1:20) (BD), CD4 (OKT4) (1:50) (University of Birmingham) and CD130 (1:50) (Santa Cruz), anti-IL-6 (1:10)(R&D Systems), anti-IL-10 (1:50) (R&D Systems), CD157 (1:50), CD19 (1:50)
- Isotype controls: Mouse IgG1, mouse IgG2b and rabbit IgG (all from DAKO)

After 30 minute incubation step at RT in a humid chamber the sections are washed for 10 minutes in PBS. 100µl of diluted secondary antibody is used to reveal the bound primary antibody. Secondary antibodies used include:

- Anti mouse IgG2b-Cy5 (1:250) (Southern Biotechnology), anti-mouse IgG1-Fitc (1:50) (Southern Biotechnology) and anti-rabbit IgG-Alexa Flour 594 (1:200) Molecular Probes, Invitrogen).

Following an incubation of 30 minutes at RT in a humid environment the tissue is washed for 15 minutes in PBS. After washing the tissue is dipped for 2 minutes in Hoechst nuclear stain (Hoechst bisbenzimide 332382, 10mg/ml with 0.05% azide) excess of nuclear stain is washed off in PBS, and slides are mounted by adding a drop of DAKO mounting media to each spot and placing a coverslip onto the slide.

2.11 Quantification of CD130 signal on CD4 T cells within and outside the perivascular infiltrates in ST sections from RA patients.

Confocal pictures were taken of ST sections stained with anti-CD3, anti-CD4 and anti-CD130 Images were further analyzed using the Zeiss LSM Image software. First the software identifies double positive cells for CD3 and CD4 by multiplying positive pixels for the two channels we were able to create a new channel (and give it a new color, yellow) that identified CD3/CD4 positive cells. A gate was drawn around the cell membrane of individual CD3/CD4 positive cells, the number of positive pixels for CD3CD4 and for CD130 and the area gated around was given by the program. Finally we obtained number of positive pixels per micrometer square.

2.12 FACS staining for phosphorylated STAT3.

25 µl of TBS plus 50 µl 3% formaldehyde in PBS was added to cytokine treated mononuclear cells were incubated for 10 minutes at RT. Cells were subsequently washed with 100 µl TBS. 50 µl of CD4Tricolor and CD45ROPE was added to the cells and cells were incubated for 20 minutes at RT. Cells were then washed with 100 µl TBS. After washing the cells are resuspended in 75 µl of TBS and 150 µl of ice-cold absolute methanol were added to the cell

suspensions, the cells are incubated in methanol for 10 minutes at 4ºC. Following incubation cells were centrifuged and washed with 150 µl of TBS 2% BSA and left overnight. 50 µl of Fitc conjugated anti-phospho-STAT3 (BD) (1:5) antibody was added to the cells and cells were incubated for 30 minutes at RT. Cells were then washed twice in TBS 2% BSA and resuspended in 400 µl of TBS 2% BSA and analysed by flow cytometry.

2.13 RNA extraction.

The RNeasy® Mini Kit (Qiagen) was used for all RNA extractions.The RNeasy® Mini Kit is based on a column containing a silica-based membrane to which up to 100µg of RNA can bind in the presence of the appropriate high-salt containing buffer. High-quality RNA is then eluted in 30–100 µl water. 100,000 to 200,000 treated cells were resuspended in 350µl of RLT buffer; the RLT buffer contains highly denaturing guanidine-thiocyanate, which inactivates RNases. The cells were then kept at -80ºC for at least 30 minutes, but at several occasions the cells were stored in these conditions for several days. When the cell suspension defrosted, 1 volume of 70% ethanol was added. Subsequently 700µl of the cells suspension were transferred to an RNeasy mini column, the column was places in a 2 ml collection tube. The cells were centrifuged at >10,000 rpm for 15 seconds, the flow through was discarded. 700µl of RW1 buffer were added to the column, the column was then centrifuged at >10,000 rpm for 15 seconds, the flow-though was discarded. The later step was repeated, then 500 µl of RPE buffer were added to the column which was subsequently centrifuged at > 5000 g for 15 seconds, the flow through was discarded. 500 µl of RPE buffer were added to the column to was membrane bound RNA and this was centrifuged at >10,000 rpm for 2 minutes, the flow though was discarded and the column was places in a clean collection tube, 50µl of RNase free-water was added to the column an the column was centrifuged at >10,000 rpm for

1 minute. Finally the column was discarded and the flow through containing the RNA was stored at -20ºC for future use.

2.14 Reverse transcription of RNA to produce cDNA.

5µg of RNA were transferred into to a PCR tube. 1µl of oligo (dT) 12-18 primer (1µg/ml) was added and finally the volume was made up to 17µl with RNA-free water. The mixture was heated in a thermocycler to 65ºC for 10 minutes. After this first incubation the mixture is allow to cool down on ice. Subsequently a mixture containing: 8µl 5x first-strand buffer (250mM Tris-HCl, pH 8.3 at room temperature;3.75 mM KCl, 15 mM MgCl2),, 4µl of 0.1M DTT, 2µl of each dNTP(10mM), 2µl of Superscript II (200U/µl) and 1µg of water was added to the tube which was then incubated at 42ºC for 2 hours. After incubation resulting cDNA is stored at -20ºC for further use.

2.15 cDNA purification.

All cDNA purifications were performed using NucleoSpin® Extract II kit (Macherey-Nagel). 2 volumes of NT buffer were added to 1 volume of unpurified cDNA. A NucleoSpin® Extract II column was placed in a collection tube and then loaded with the sample, the column was centrifuged at 11,000g for 1 minute. The flow through was discarded and 600µl of NT3 buffer were added to the column. After a 1 minute centrifugation at 11,000 g the flow though was discarded and the column was centrifuged again for 2 minutes at 11,000 g for to eliminate all excess of NT3 buffer. Subsequently the column was placed in a clean collection tube and the purified cDNA was eluted in 50 µl of elution buffer. Purified cDNA was stored at -20ºC

2.16 Real Time Polymerase Chain Reaction (RT-PCR).

All RT-PCR reactions were performed in a Stratagene Mx3000P™ RT-PCR machine. A mixture containing 1µl of GP130 Probe, 1µl GP130 Forward primer (5` CATTGGAAATGAAACTGCTGTGA 3`),1 µl of GP130 Reverse Primer (5`

GCTGCCATTCGTACCATGTACA3`), 0.5 µl of 18s probe, 0.2 µl of 18s Forward Primer (: 5` GCCGCTAGAGGTGAAATTCTTG 3`), 0.4 µl of 18s Reverse Primer (5` CATTCTTGGCAAATGCTTTCG 3`), 12.5 µl of RT-PCR TaqMan® TR-PCR master mix (Applied Biosystems), 50 ng of cDNA and water to make up a volume of 25 µl. The plate containing the PCR mixtures was sealed and centrifuged then it was placed in the RT-PCR machine and this was set to do one cycle of 2 minutes at 50ºC, one other cycle of 10 minutes at 95ºC and 45 cycles of 15 seconds at 95ºC and 1 minute at 60ºC.

2.16.1 RT-PCR Analysis.

RT-PCR was analysed using the MxPro-Mx3000P software. The fluorescence (DR) was plotted against the number of cycles on a log scale. The baseline was set at fluorescence 50. The thresholds for 18s and CD130 fluorescence were set at the exponential phase of the fluorescence curve. The Ct values are obtained and exported to Excel (Microsoft office). The Delta Ct is calculating subtracting the 18s Ct to the CD130 Ct. The Relative Quantity of CD130 mRNA is 2^{-DCt}

2.17 Isolation of Human Umbilical Vein Endothelial Cells (HUVEC) cells and cell culture. Endothelial cells were isolated from the veins of fresh human umbilical cords using collagenase as previously described (213). The vein was cannulated at each end and washed through with PBS until all the blood was removed. Then 10ml of collagenase (1mg/ml) diluted in PBS was perfused through the cord until it was visible in both cannula. The cord was then incubated for about 20 minutes at 37^{o}C. Then, the cord was massaged to loosen the cells. The cells were placed into two 15ml Falcon tubes by perfusing the cord with 30ml PBS, and then, the cells were centrifuged for five minutes at 500g. The isolated HUVEC were re-suspended in HUVEC medium and were plated into 1% gelatin-coated T25 culture flasks

(Falcon), and cultured for about four to six days until confluent, the medium was changed every day to maintain the pH.

When cells were confluent, the HUVEC medium was aspirated off and the HUVEC were bathed in 2mls EDTA (0.02%) for 1 minute. The HUVEC were then incubated with trypsin solution (2.5mg/ml) in a 2:1 ratio with EDTA. Using phase-contrast microscopy, visual exam determined when the cultured HUVEC had detached. At this point the reaction was stopped by adding 5mls of HUVEC medium. The contents of the T25 flask were transferred to a 15ml Falcon tube and spun at 500g for 5 minutes. The HUVEC were re-suspended in HUVEC medium, plated out and incubated at 37^{o}C. These first passage HUVEC were plated onto low-density 0.4μm pore polycarbonate Transwell filters, which were placed in matching plates (six or twenty-four well format; from BD Pharmingen and referred to as filters in future text). Seeding densities were chosen to yield confluent monolayers within 24 hours, and cultures were maintained for up to five days. Each experiment used first passage cells from a single, different cord. The isolation and culture of HUVEC cells was done by Dr. Helen MacGettrick.

2.18 Transmigration of sorted CD45RO positive CD4 T cells through a monolayer of HUVEC cells

First passage HUVEC were cultured on filters designed for 24-well Falcon plates for 24 days in a 5% CO_2 incubator. HUVEC were stimulated for 24 hours with TNF and IFNγ (100u/ml and 10ng/ml respectively) after which, the medium in the lower chamber was replaced with fresh medium M199 (SAF Biosciences) + BSA, and medium in the upper chamber was replaced with PBL ($2x10^6$cells/ml in M199+BSA).Freshly FACS sorted CD4RO Positive CD4 Positive T cells were allowed to settle, adhere to, and migrate at 37^{o}C for 24h. The experiment was stopped by transferring the filter into a fresh well. The PBL in the upper chamber were transferred to a fresh well and the filter was washed twice. The cells from the

washes were pooled with the upper sample and were taken to represent non-adherent PBL. The PBL in the lower chamber were re-suspended and washed with fresh medium. These were taken to represent the transmigrated PBL. The transmigrated cells and non transmigrated cells were collected in different tubes for further FACS staining.

1.18.1 Flow cytometry to determine CD130 expression on CD45R0 positive CD4 T cells following HUVEC transmigration.

CD45R0 positive CD4 T cells are obtained by FACS sorting from PBMCs. Sorted cells are places on top of a HUVEC monolayer and are incubated for 24h. Cells on top and under the HUVEC cells are collected in different tubes and used to determined CD130 expression via flow cytometry. CD45R0 positive CD4 T cells are stained with CD130PE (1:5) (BD Pharmingen^{TM}) for 30 minutes in a flexiplate. Cells are then washed and resuspended in 500ul of PBS/BSA and read in the flow cytometer.

2.19 Statistical Analysis

To analyze and compare data two non parametric statistic tests were used: the Kruskal Wallis test and the Mann Whitney tests. The Kruskall Wallis test is used for testing the equality of population medians between different groups. The Mann Whitney test is often used as an alternative to t test when the data are not normally distributed; this test also compares population medians of unpaired groups. The result of statistical tests applied is a P value, and when the P value is < 0.05 the difference in groups of data compared is statistically significant (214).

Results

Chapter 3

3. Expression of IL-6 receptor by T cells infiltrating the rheumatoid joint.

3.1 Introduction:

The complete IL-6 receptor complex including both CD126 and CD130 chains is only expressed in only a few cell types such as leukocytes and hepatocytes. In contrast, CD130 is expressed ubiquitously (58). As I have previously explained IL-6 can initiate a signal through two distinct mechanisms: direct signaling and transsignaling. Transsignaling, when IL-6 forms a complex with the soluble form of the IL-6R (IL-6/sIL6R) and this complex bind to CD130. Transsignaling allows IL-6 to signal to a wide array of cells that expressed CD130 but not CD126. IL-6 transsignaling is important in inflammation as stromal cells of inflammatory sites are IL-6R negative and CD130 positive; therefore transsignaling mediates many of the IL-6 proinflammatory effects (reviewed in (58)). It is clear that IL-6 contributes to RA pathology as illustrated by the high levels of IL-6 found in RA SF and serum (181,215-217), the promising results of blocking IL-6 in clinical trials in RA (208) and the clear correlation of IL-6 level and joint damage (217). Some experimental work has shown that some of the effects of IL-6 in RA are mediated via trans-signaling (reviewed in (218)). IL-6 deficient mice are resistant to AIA and reconstitution with IL-6 is not sufficient to trigger the disease, in the other hand disease symptoms do appear as you inject the mice with sIL-6/IL-6 (reviewed in (218)). In addition IL-6 transsignaling promotes osteoclast formation in vitro (215), it triggers collagenase-3 secretion by osteoblasts and proteoglycan synthesis by chondrocytes contributing to joint destruction (219,220).

In addition IL-6 has various important effects on CD4 positive T cells. IL-6 promotes CD4 T cells survival (50). IL-6 protects CD4 T cells from activation induced cells death (AICID) by downregulation of FasL (221). The expansion of antigen specific CD4 T cells is increased by IL-6 (222). In addition to support of survival and proliferation of CD4 T cells, IL-6 also modulates the differentiation of T cells into Th2 and Th17 cells (reviewed in (223)).

In the work shown in the following chapter we investigated the expression of both CD130 and CD126 in the different compartments of the rheumatoid joint and whether they respond to IL-6 directly or via transsignaling.

3.2 RESULTS

3.2.1 Divergent patterns of CD130 and CD126 expression in the different compartments of the rheumatoid joint.

We investigated the expression of CD126 and CD130 on CD4 positive T cells from PB, SF and ST of RA patients to determine the expression of the two chains of the IL-6R in these three different compartments of RA patients.

PBMCs and SFMCs were obtained by density gradient centrifugation. STMCs were obtained by mechanical degradation of ST followed by density gradient centrifugation. PBMCs, SFMCs and STMCs were stained with the appropriate fluorescent labeled antibodies and then read in the flow cytometer. An example of the staining in PB cells is shown in figure 9. The expression of CD126 was lower on CD4 positive T cells from RA SF and ST compared with paired PB samples. CD130 was expressed at low levels on CD4 T cells from RA SF when compared to PB. However, it was expressed at higher levels in ST CD4 positive T cells, at levels similar to those found in PB cells (Figure 10). The level of CD126 on SF and ST CD4 T cells is significantly lower than their PB CD4 T cells ($p<0.0001$, Mann-Whitney test). The level of CD130 on CD4 T cells from SF is also statistically significantly lower than on PB CD4 T cells ($p<0.005$, Mann-Whitney test). Because most cells found in the rheumatoid synovium are primed CD45RO positive T cells we repeated the experiments comparing CD126 and CD130 expression on CD45RO positive CD4 T cells from ST, SF and PB. An example of the staining is shown in figure 9. We observed that the results were consistent and CD126 expression was low in SF and in ST, while CD130 was only low in SF but expressed at relatively high levels in ST (figures 10, 11 and 12). So we have defined 3 different phenotypes on CD4 positive T cells regarding IL-6R chain expression. CD126high CD130 high in PB, CD126low CD130low in SF and CD126low CD130 high in ST.

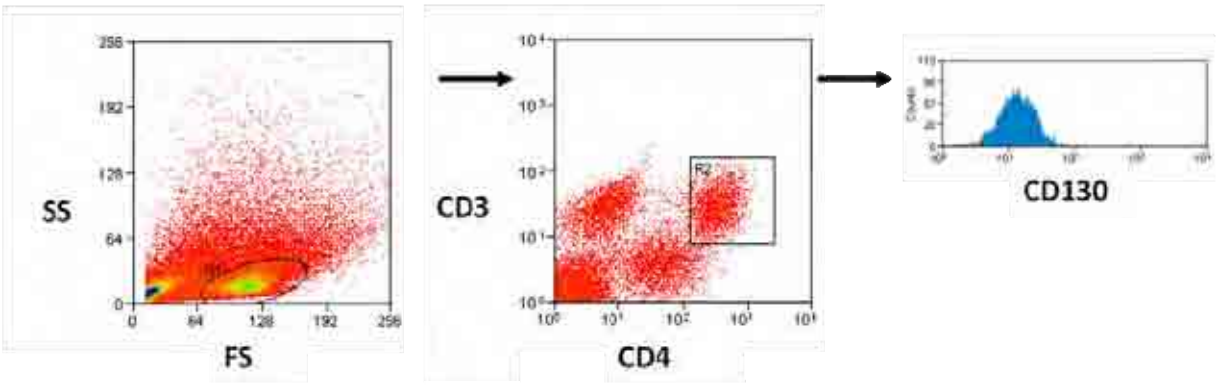


Figure 9. CD130 on CD4 T cells from PB. Figure 9, from left to right shows: first a FACS plot of the FS/SS, secondly a FACS plot of CD3 versus CD4 straining and finally a histogram showing the CD130 expression on CD3/CD4 double positive cells.

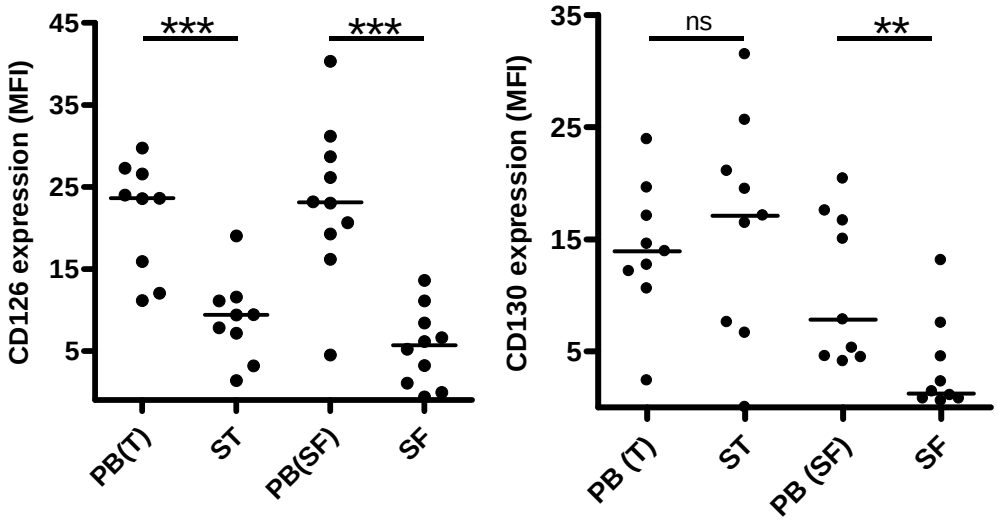


Figure 10. CD126 and CD130 expression on CD4 positive T cells in SF, ST and PB of RA patients. Figure 10A shows the expression of CD126 on CD4 positive T cells obtained from SF, ST and PB samples from RA patients. Figure 10B shows the expression of CD130 on CD4 positive T cells of SF, ST and PB from RA patients. P values as calculated using a Mann-Whitney test. *** $p<0.0001$, ** $p<0.005$.

SF: Synovial Fluid

ST: Synovial Tissue

PB (T): Peripheral Blood sample paired with a synovial tissue sample.

PB (SF); Peripheral Blood sample paired with a synovial fluid sample.

Ns: Non Significant

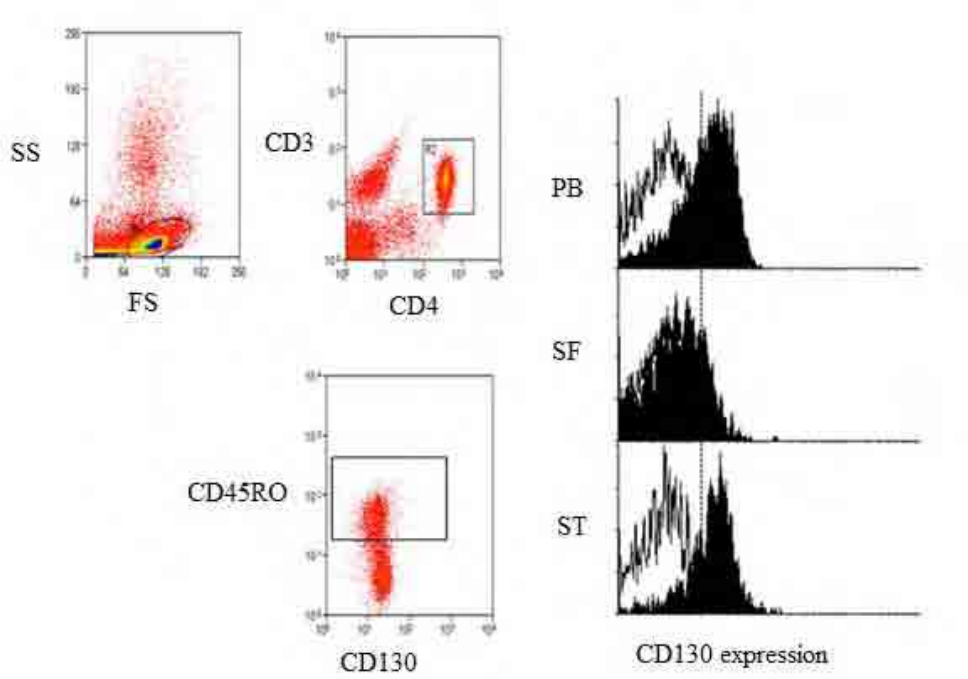
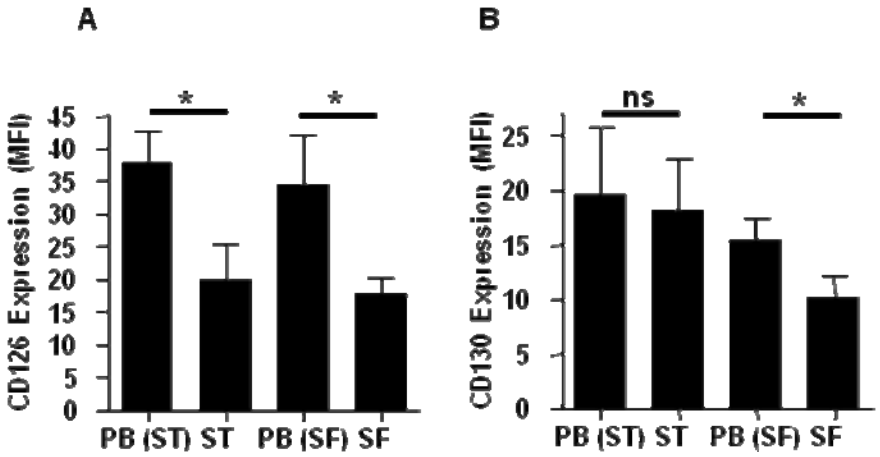


Figure 11 Expression of CD130 on CD45RO positive CD4 positive T cells from RA SF, ST and PB. This graph shows on the left hand side FACS plots of PBMCs, SFMCs and STMCs stained for CD3, CD4, CD45RO and CD130. On the right, the three histograms show the expression of CD130 (black) on CD45RO positive CD3 positive and CD4 positive cells compared to an isotype control (white).



n=4 for ST and PB (ST)

n=6 for SF and PB (SF), SD

Figure 12. CD126 and CD130 expression on CD45RO/CD4 double positive T cells. Figure 12A shows the level of expression of CD45RO positive CD4 positive T cells in SF, ST and PB. Figure 12B shows the expression of CD130 on CD45RO positive CD4 positive T cells from SF, ST and PB from RA patients. Statistical significance of these differences was determined by Mann-Whitney test. * $p<0.05$.

SF: synovial fluid

ST: synovial tissue

PB (ST): peripheral Blood paired with a synovial tissue

PB (SF): peripheral blood paired with a synovial fluid sample

3.2.2 Tissue distribution of CD130 expression on CD4+ T cells

So far we have demonstrated high expression of CD130 on CD4 positive T cells form RA ST and low expression in the SF compartment by flow cytometry. At this point we decided to investigate how CD130 expression is distributed on CD4 T cells localized in different areas of the synovium; the perivascular cuff and scattered throughout the tissue. To investigate this we took ST sections and we stained them with labeled anti-CD3, anti-CD4 and anti-CD130 antibodies using immunohistochemistry techniques. Following staining we observed the tissue using confocal microscopy and took images of the stained tissue (Figure 13A). We analyzed the expression of CD130 on CD4 single cells clustering around the perivascular cuffs and single cells located distant from the cuffs. For this purpose we created a channel that identified double positive CD3 and CD4 (Figure 13B). Once CD4 positive T cells could be identified we drew a gate around the cell membrane of single CD4 positive T cells, with the CD130 channel switched off, then we switched it back on, and quantified CD130 expression measured as the number of positive pixels of intensities of above 20 per micrometer square. We found that CD130 expression in the perivascular cuff was high, at comparable levels to CD3/CD4 expression. However CD130 expression was lower in cells localized distant from the perivascular infiltrate (Figures 14 and 15). These observations suggest that a higher level of CD130 expression is found on CD4 positive T cells located in the perivascular areas in ST and that as CD4 positive T cells migrate away from the perivascular cuff the expression of CD130 may be gradually lost.

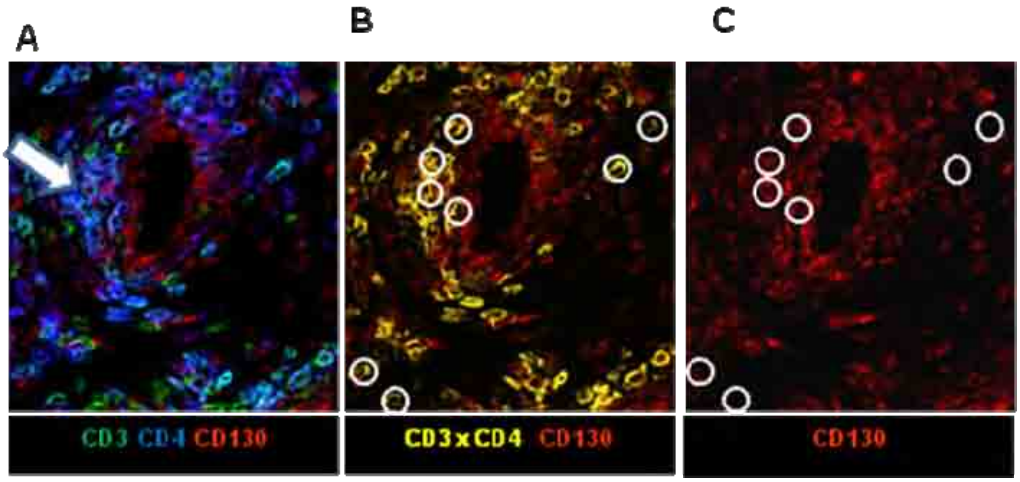


Figure 13. Confocal images of RA synovial tissue stained with anti-CD3, anti-CD4 and anti-CD130.

Figure 13A shows a confocal image of RA synovial tissue stained with anti-CD3 (green), anti-CD4 (blue) and anti-CD130 (red). In the centre of the image there is a perivascular cuff, shown with a white arrow, and some CD4 positive cells can be seen clustering around this perivascular cuff or located further away from it. Figure 13B shows the same image after being processed to identify double positive cells for CD3 and CD4. A new channel is created identifying all double positive cells and these cells can be seen in yellow. Several CD3, CD4 positive cells have been selected in this image from the perivascular cuff area and away from the cuff. Figure 13C is the same confocal image showing only CD130 signal. The circles show the level of CD130 expressed on the selected CD3CD4 double positive cells within the perivascular cuff and at a distance from it. The arrow points at the perivascular cuff.

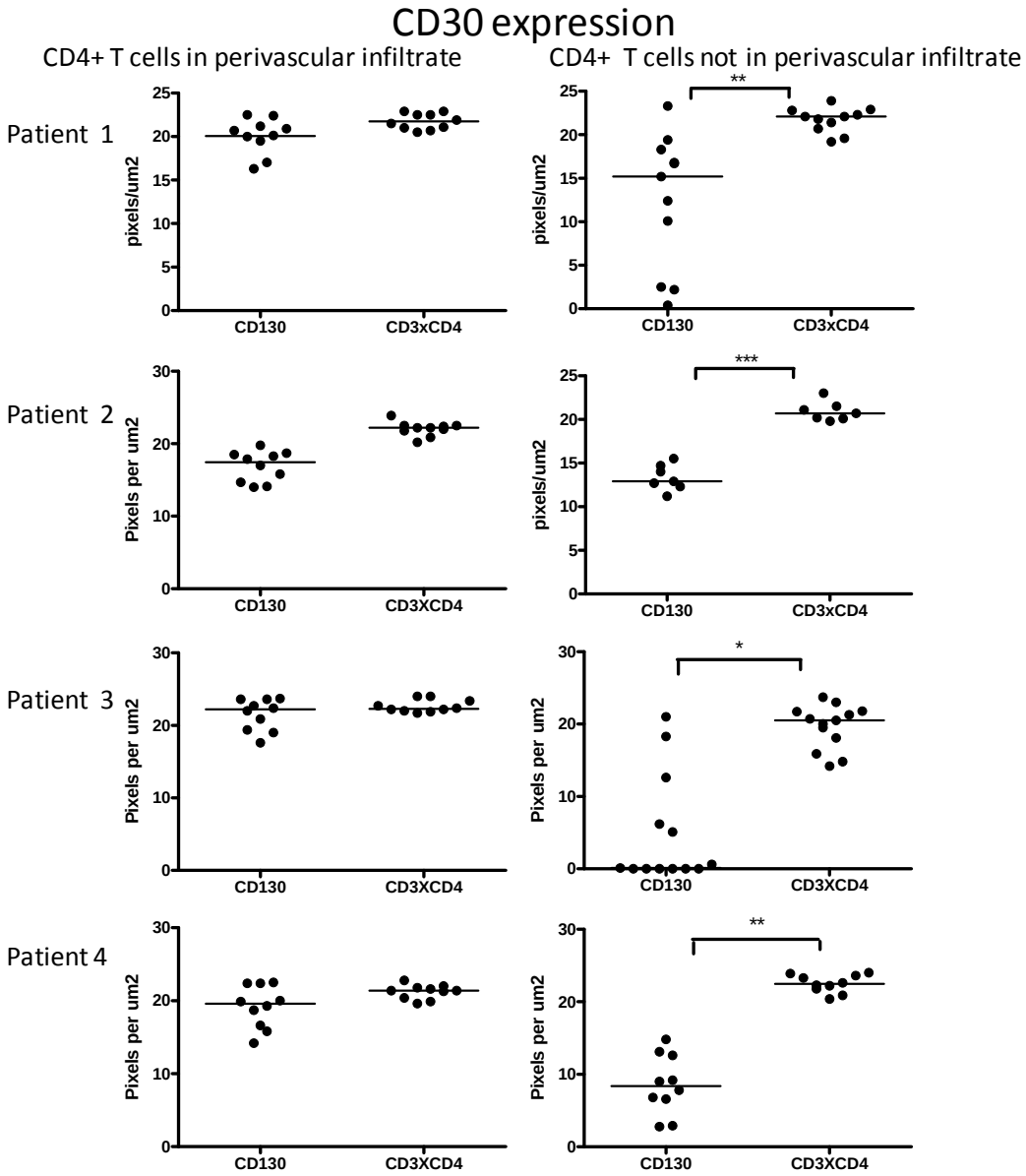
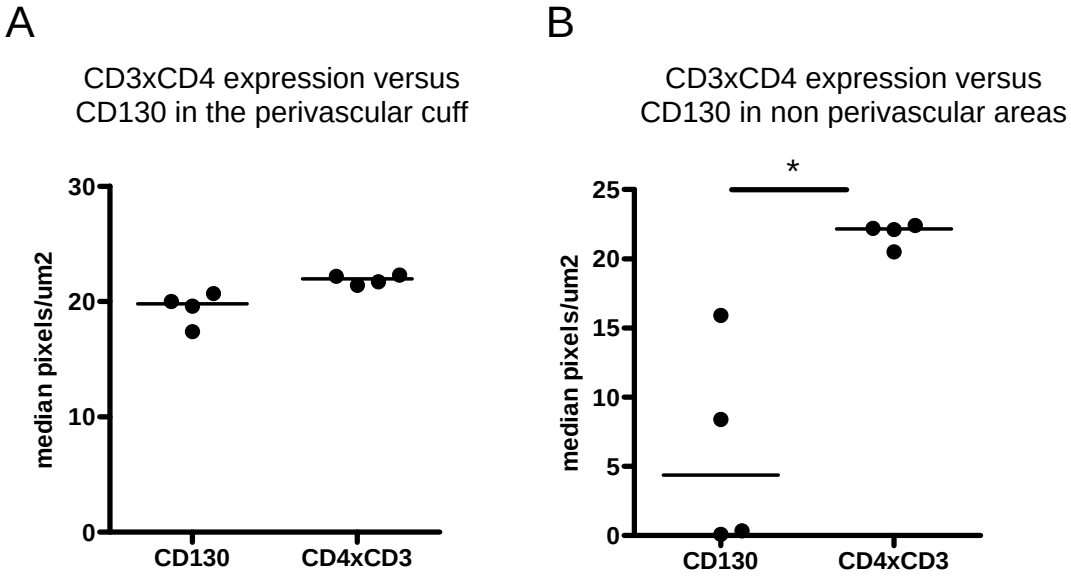


Figure 14. CD130 expression on CD4 positive cells located in the perivascular cuff and at a distance from the perivascular infiltrate.

Graphs 14 shows the expression of CD130 on CD4 positive cell located within and away the perivascular cuff areas in the ST of four different RA patients.



n=4

* p value = 0.0286

Figure 15. CD130 expression on CD3CD4 positive cells in perivascular cuffs and away from the perivascular cuffs.

Figure 15. Shows the median expression of CD130 on 10 CD3CD4 positive cells located in the perivascular cuffs of four different patients. Figure 15B shows the median expression of CD130 on 10 CD3/CD4 double-positive cells located distant to the perivascular cuffs of four different patients. The statistical analysis was performed using a Mann-Whitney test.

3.2.3 Biological significance of high CD130 expression on CD4 T cells in the rheumatoid joint.

CD4 T cells express low levels of CD126 and CD130 in SF but a different pattern of the IL-6R expression is seen in the ST where CD126 expression is low but CD130 expression is high. As we found this different IL-6R pattern in SF and ST we decided to investigate whether there was a functional consequence to this expression pattern. We obtained PBMCs and SFMCs by ficoll gradient centrifugation and STMC by mechanical degradation of the tissue followed by ficoll gradient centrifugation. We treated cells with IL-6 and IL-6/sIL-6R, after treatment we stained the cells intracellularly for Phospho-STAT-3 at the Tyrosine 705. The presence of Phospho STAT-3 on CD4 positive T cells was then assessed by flow cytometry gating on the lymphocyte population. We looked at STAT-3 as readout of IL-6 signaling as STAT-3 is phosphorylated following exposure of cells to IL-6. It is activated both by direct signaling and transsignaling. Example of the raw data can be seen in figures 16 and 17. We found that CD4 positive cells from PB respond well to both IL-6 and IL-6/sIL-6R treatment and show high level of STAT-3 phosphorylation following treatment with either IL-6 or sIL-6R/IL-6 (Figure 18). CD4 T cells from SF did not respond to IL-6 or IL-6/sIL-6R treatment and showed little STAT-3 phosphorylation (Figure 18). CD4 positive T cells from ST did not respond to IL-6 treatment, showing little STAT-3 phosphorylation, however, tissue CD4 T cells did respond to IL-6/sIL-6R treatments by STAT-3 phosphorylation (Figure 18). ST CD4 T cells were responsive to IFN-β (data not shown).

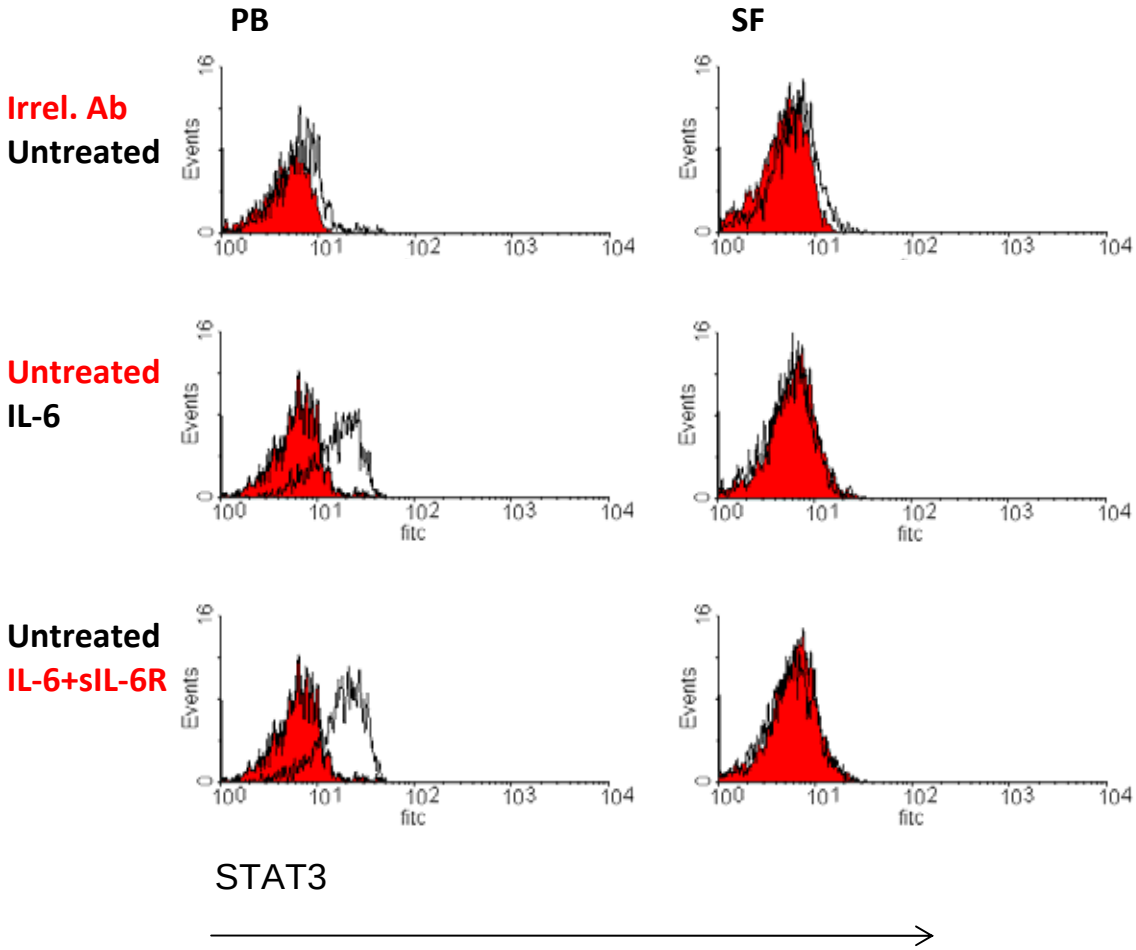


Figure 16. Expression of Phospho-STAT3 on CD4 T from PB and SF. On the left hand side the histograms show the expression of phospho-STAT3 on CD4 T cells from PB following treatment with medium, IL-6 and IL-6 plus sIL-6R. On the right hand side histograms show the expression of phospho-STAT3 on SF CD4 T cells following the same treatments. IL-6 was used at 10ng/ml for 1h and sIL-6R was used at 1000ng/ml for 1h together with IL-6 at 10ng for 1h.

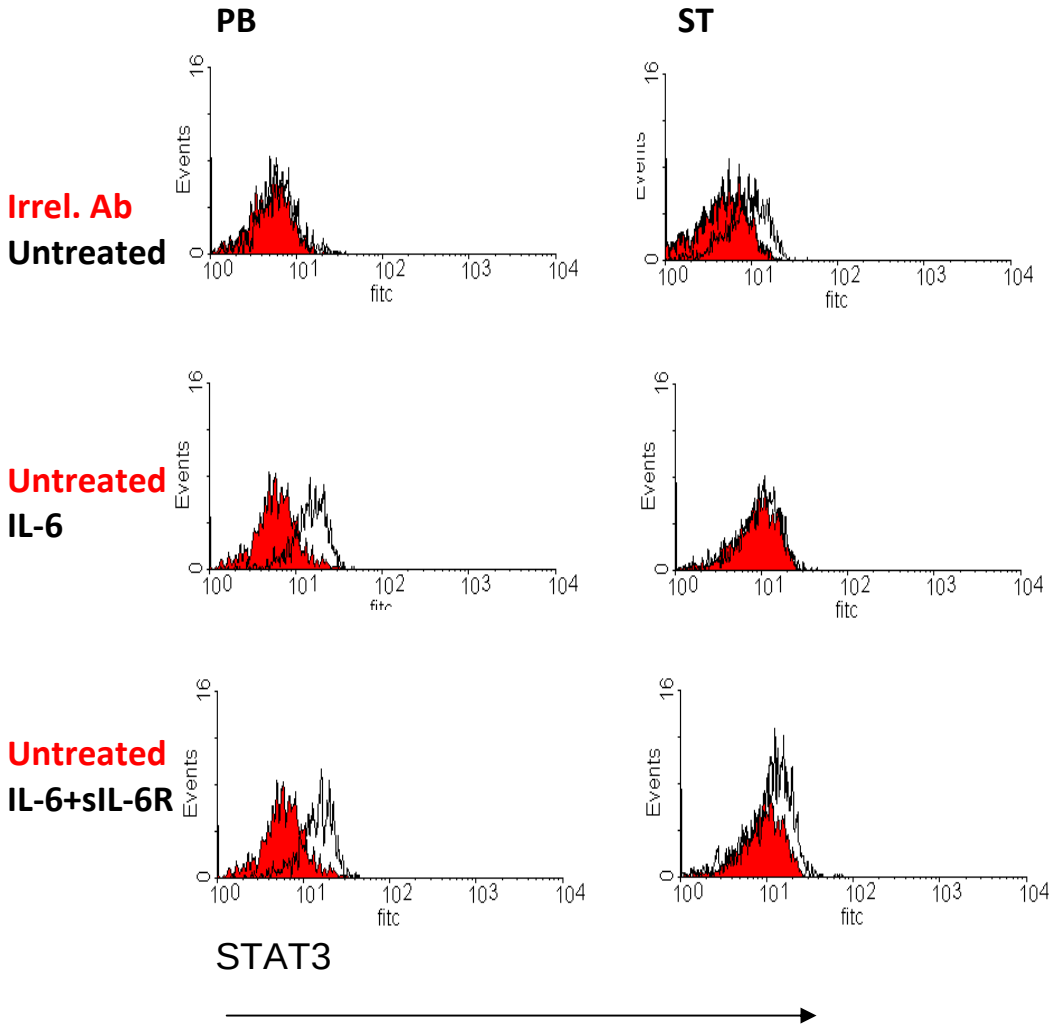
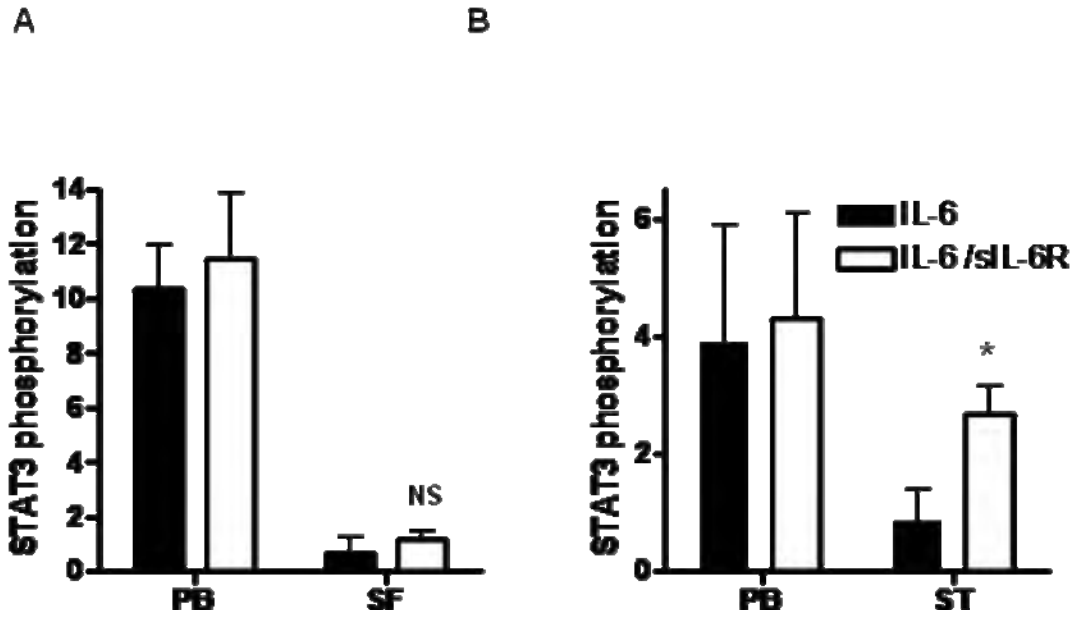


Figure 17. Expression of Phospho-STAT3 on CD4 T from PB and ST. On the left side, the histograms show the expression of phospho-STAT3 on CD4 T cells from PB following treatment with medium, IL-6 and IL-6 plus sIL-6R. On the right side histograms show the expression of phospho-STAT3 on ST CD4 T cells following the same treatments. IL-6 was used at 10ng/ml for 1h and sIL-6R was used at 1000ng/ml for 1h together with IL-6 at 10ng for 1h



n=4

Figure 18. Level of STAT-3 phosphorylation on CD4 T cells from PB, SF and ST from RA patients following IL-6 and IL-6/sIL6R treatments.

Graph 16 shows the levels of STAT-3 phosphorylation in CD4 T cells from patients with rheumatoid arthritis PB, SF and ST treated with IL-6 and IL-6/sIL-6R. IL-6 was used at 10ng/ml for 1h and sIL-6R was used at 1000ng/ml for 1h together with IL-6 at 10ng for 1h. SEM error bars p= 0.042 (T Test)

3.3 DISCUSSION

Our data suggests that expression of the two components of the IL-6 receptor, CD126 and CD130, is differentially regulated in different compartments of the rheumatoid joint. While both CD126 and CD130 expression is downregulated in $CD4^+$ T cells that infiltrate the SF, in the ST, we observed $CD4^+$-T cells with a low level of CD126 expression, but with CD130 expression comparable to that found in PB.

This differential regulation of receptor expression cannot be explained by different proportions of CD45R0 positive T cells as T cells in both SF and ST are predominantly CD45RO positive. The differential expression independently of CD45R0 expression was confirmed in 4 color staining experiments in which we co-labelled for CD4, CD3, CD45R0 and CD126 or CD130 respectively. There is a wide range of expression of CD130 on ST CD4+ T cells is wide. It is normal to find variation when using human samples, various factors may help explain the wide range of CD130 expression. The fact that CD130 is already expressed at very different levels within the perivascular cuff and away from the perivascular cuff, may help explain this differences as some patients could have different proportion of cells away from the in and away from the cuffs. It is also a factor to take into account when looking at the expression of CD130 in confocal images that there is no way to investigate if underneath or above single cells there was also a blood vessel, perhaps making these cuff cells rather than non cuff cells. And finally a very important factor potentially affecting CD130 expression would be the different amounts of IL-10 present in the tissue of the different patients used in the study. The observation that the ST CD4+ T cells express CD130, but only low levels of CD130 suggested that these cells may be a target for IL-6 transsignaling. To test this, we performed an experiment treating SF, ST and PB cells from RA patients with IL-6 and IL-6/sIL-6R to investigate whether the cells are able or not to

respond to IL-6 directly, via transsignaling or whether they do not respond to IL-6 at all. As functional readout we measured STAT3 phosphorylation following the treatments. We saw that while ST CD4 T cells could not respond to IL-6 direct signaling, as expected from their higher expression of CD130, they did respond to IL-6 via transsignaling. This is an important observation, suggesting that CD4 T cells not only show a different phenotype in ST and SF, but that this phenotype also has a biological consequence. The use of STAT3 phosphorylation as a readout of IL-6 mediated signal transduction has been used extensively by others (224) . Since STAT3 signaling is activated by a number of different cytokines such as IL-10 and Type I interferons it is not a reliable readout for ex vivo assessment of Il-6 signaling, but in our experiments only IL-6 and its soluble receptor were present in the short term cultures. Previous work has shown that while some of the functions of IL-6 are mediated via direct signaling, (reviewed in (51)) ubiquitous expression of CD130 enables transsignaling to mediate many of the effects of IL-6 on a wider range of cell types. IL-6 transsignaling promotes the growth of diverse cells types such as hematopoietic progenitors (225) and synovial fibroblasts (226). It also triggers osteoclast differentiation in vitro (180,215,227) and neuronal survival and differentiation (228,229). IL-6 transsignaling has also been implicated in the switch from innate to acquired immune responses, crucial for an efficient inflammatory response, its termination and the restoration of tissue homeostasis (reviewed in (58)).

In RA IL-6 has been proposed to play an important role, the first evidence for this is the high level of IL-6 found in SF and serum of RA patients (230,231). IL-6 causes some of the local and systemic signs and symptoms of RA such as fever, autoantibody production and joint inflammation (177,181). In addition, IL-6-/- mice are protected from antibody induced arthritis and the addition of IL-6 is not sufficient to restore disease, however when mice are injected with Hyper-IL-6 they do show signs and symptoms of arthritis as well as an

increased recruitment of CCR2+ mononuclear leukocytes, this strongly suggests that of antibody induced arthritis depend on IL-6 transsignaling rather than direct signaling (reviewed in (218)). Transsignaling has also been proposed to play a role human RA. Elevated levels of sIL-6R have been demonstrated in serum and SF of RA and Juvenile arthritis (91,215,216). In addition, the levels of sIL-6 and IL-6 have been correlated with the degree joint destruction. Furthermore, RA SF containing sIL-6R and IL-6 promotes osteoclast formation in vitro (215). So it is clear that many of the effects of IL-6 in RA are mediated by transsignaling. There is a natural occurring antagonist of transsignaling, the soluble form of CD130 or sgp130. sgp130 can bind to sIL-6R/IL-6 complexes and prevent transsignaling. In RA the levels of sgp130 are not elevated (232). This may suggest the need for sgp130 regulation of transsignaling in RA, indicating a good therapeutic approach (reviewed in (218)).

As I mention earlier a very distinctive characteristic of T cells in RA is their ability to escape apoptosis and remain alive in the joint for a long period of time in the joint environment. IL-6 is a survival signal for lymphocytes but in the synovial tissue T cells are chronically exposed to this cytokine and they seem to become refractory to its effects due to their low expression of CD126. High expression of CD130, however, described on CD4 T cells in the rheumatoid ST may allow IL-6, when completed with sIL-6R, to act as a survival signal via transsignaling.

Chronic exposure to a cytokine can lead to the down regulation of receptor expression, rendering the target cell refractory to this cytokine. However, our data supports that in the inflamed synovium, with its high level of IL-6, the expression of CD130 is maintained, even though CD126 expression is down regulated. Intriguingly, we observed that CD130 expression in T cells infiltrating the ST is not distributed homogenously throughout the inflamed tissue. We found higher levels of CD130 expression in the T cells in the perivascular

infiltrates. There many possible reasons for such a distribution. The higher level of CD130 in this microenvironment may de due to selective migration of a subset of T cells to this site, alternatively it may also be reflecting local induction of CD130 gene expression. It may also reflect differentiation of a specific T cell subset, we showed that the synovial tissue T cells do not become refractory to IL-6 but can respond to sIL-6R/IL-6 complexes binding to CD130. The next important step is to identify factors that regulate the expression of the components of the IL-6R in the different compartments of the rheumatoid joint.

Chapter 4

4. Mechanism of regulation of CD130 expression on T cells in the synovium.

4.1 Introduction

In the previous chapter we have shown that the expression of CD130 is maintained high in T cells in the rheumatoid joint that are chronically exposed to IL-6. These cells have also been shown to respond to IL-6 via transignaling.

The immune system uses cytokines for a large proportion of cell to cell communication. Cytokines bind to receptors found on the surface of cells and they stimulate signaling pathways that ultimately lead to the increase or decrease of gene transcription. The production of cytokines can be triggered by several stimuli such as complement, immune complexes, bacterial and viral components, acute phase proteins and other cytokines themselves among others (reviewed in (233)). Many cytokines have been identified in the SF and synovial tissue of RA patients, as well as in animal models of RA (234-236). In addition, T cells from the RA joint share similarities with T cells that have been activated in vitro using a cytokine cocktail including: IL-2, IL-6 and TNF-α, highlighting the importance of cytokines in the pathophysiology of RA (115). In established RA, there are several key events contributing to the disease, the high presence of inflammatory cells in the joint, angiogenesis that provides oxygen and nutrition to the enlarged synovium and bone and cartilage destruction. Cytokines play an active role in all of these processes (reviewed in (155)). Not only do cytokines mediate some of the local effects seem in RA, they are also involved for systemic symptoms such as the production of acute phase proteins triggered by IL-6 or the cachexia triggered by TNF (155). We have observed high expression of CD130 on CD4 T cells clustering around the perivascular cuffs in ST. We wanted to study possible inducers of CD130 expression in the joint so we picked a panel of cytokines including: IFN-γ, IL-1α, IL-6, IL-10, IL-12, IL-15, IL-17, IL-23, MIP-1α, RANTES, SDF-1, TNF-α and TGF-β. We selected this panel of cytokines on the basis that they have all been detected in the rheumatoid joint and are known

regulators of T cell function or differentiation. For practical reasons we were not able to extend these experiments to all cytokines present in the joint. We investigated if any of these cytokines had an effect in CD130 expression on CD4 T cells in vitro.

IL-1α and β have been known for some time to enhance T cells activation and (237)proliferation. It is expressed at high levels in many inflammatory conditions. IL-6 has been shown lead to internalization of both CD130 and CD126, the changes in IL-6 receptor protein expression on the cell membranes induced by IL-6 therefore could be considered as a positive control for the experiments(64). IL-10 is typically an anti-inflammatory cytokine that prevents antigen presentation to T cells. It does, however also have direct effects on T cells (238). IL-10 has been detected in the RA joint by and its overproduction is linked with RA joint destruction (239). The main growth factor for T cells in the synovium is thought to be IL-15 (112). In the synovium IL-15 is produced by macrophages, endothelial cells and fibroblasts (240). IL-15 acts as a chemotactic factor for CD45RO positive T cells. It also triggers the secretion of proinflammatory cytokines: IL-6, TNF-α and IL-1 by macrophages (241).

TNF-α has been identified in most synovial biopsies of RA, its inhibition in RA mice models results in suppression of arthritis, its overexpression induces spontaneous arthritis (200). TNF-α induces the activation of leukocytes, endothelial cells and synovial fibroblasts. TNF-α is also a synovial fibroblast survival factor and promotes angiogenesis (reviewed in (155)). As a part of a cytokine cocktail it is able to activate T cells proliferation in the absence of cognate interaction with MHC bound antigenic peptides(113).

SDF1 or CXCL12 has previously been shown by our group to contribute to accumulation of T cells in the rheumatoid synovium (242). MIP1-α (CCL3) and RANTES (CCL5) are major

regulators of T cell migration (243). TGF-b, IL-23 and IL-12 were include in this study because they are important regulators of T cell subset differentiation and plasticity (244).

The differential expression of CD130 by T cells in different compartments of the rheumatoid joint may also be explained by selective migration or induction during transmigration through the endothelium. T cells infiltrating the rheumatoid joint are recruited from the circulation. In order to infiltrate the synovium T cells must transmigrate through the endothelium. It was been described in the literature that in a HUVEC model of endothelial transmigration activated CD4 positive cells are able to acquired endothelial cell markers including: CD31, CD49d, CD61, CD54 and CD62E (245). We have described the differential expression of CD126 and CD130 on CD4 positive T cells in the rheumatoid synovium in the previous chapter. In this chapter we wanted to investigate the factors present in the SF and ST that contribute to the differential expression of CD126 and CD130. In addition we wanted to investigate if CD130 high expression in the RA ST was locally induced or there was a possibility that endothelial transmigration has an effect on the expression of CD130 on CD4 positive T cells.

4.2 RESULTS

4.2.1 IL-6 present in SF is responsible for the low expression of CD126 and CD130 on CD4 positive T cells from RA SF.

So far we have observed a different IL-6R expression phenotype on CD4 T cells from SF and ST. We have also observed that differential expression of CD126 and CD130 influences CD4 T cells response to IL-6 and IL-6/sIL6R. At this point we decided to investigate the cause of CD126 and CD130 low expression in SF CD4 T cells. An obvious candidate to be tested was IL-6, as IL-6 can be internalised together with its receptor and can therefore down regulate its surface expression if present at high enough level (reviewed in (218). Therefore, to test whether IL-6 in SF is responsible for the low expression of CD126 and CD130 on CD4 T cells, we isolated PBMCs from PB from healthy donors by density ficoll gradient centrifugation and then sorted CD4 positive T cells using the MoFlow cell sorter. As shown in figure 17, We treated CD4 T cells with SF in the presence and absence of an IL-6R blocking antibody and with IL-6 as a positive control and medium as a negative control for 1 hour. Then we assessed the expression of CD126 and CD130 on the treated cells by flow cytometry. An example of raw data in shown in figure 19. We observed that SF was able to downregulate CD126 and CD130 at the same level as IL-6. When anti-IL6R antibody was added to the SF before incubation CD126 and CD130 expression was only partially downregulated compared to IL-6 or SF treatments (figure 20).

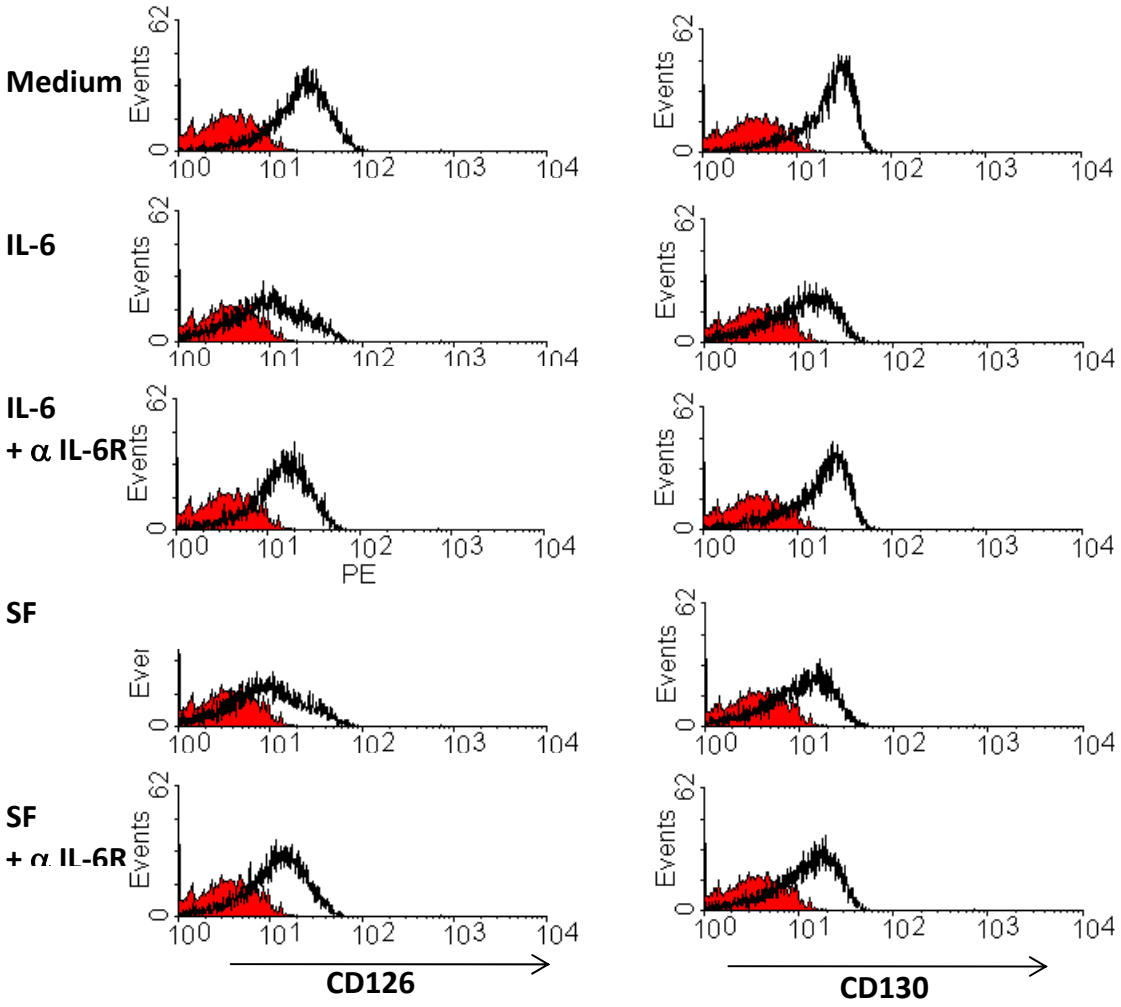
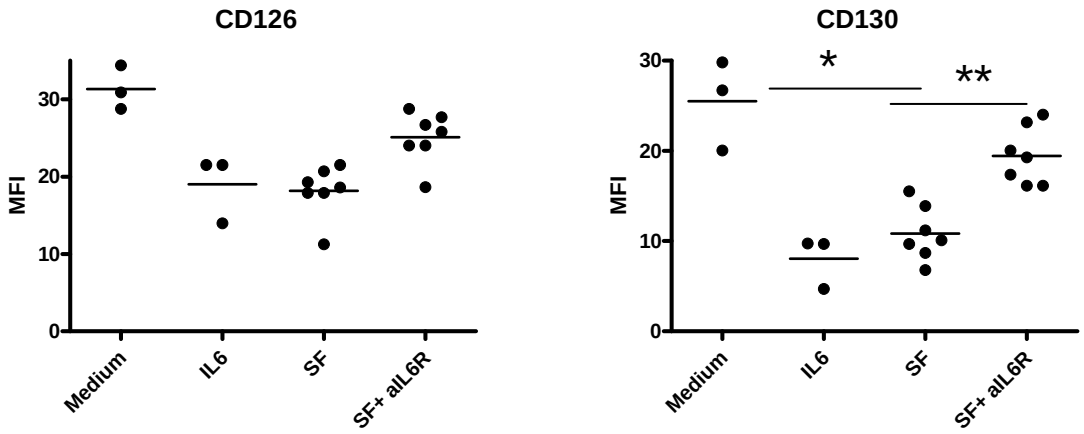


Figure 19. Expression of CD126 and CD130 on CD4 T cells treated with IL-6, SF and SF combined with an anti-IL-6R blocking antibody. On the left, the histogram shows the expression of CD126 and on the right the expression of CD130 following treatment with IL-6 (10ng/ml for 30 minutes at 37ºC), SF treatment (30 minutes at RT) and SF plus anti-IL-6R antibody; anti-IL-6R antbody added at 1 µg/ml for 30 minutes incubated on ice and then SF added and further incubated 30 minutes at 37ºC. The black curve represents CD126 and CD130 on the left and right respectively and the red curve represents the irrelevant control.



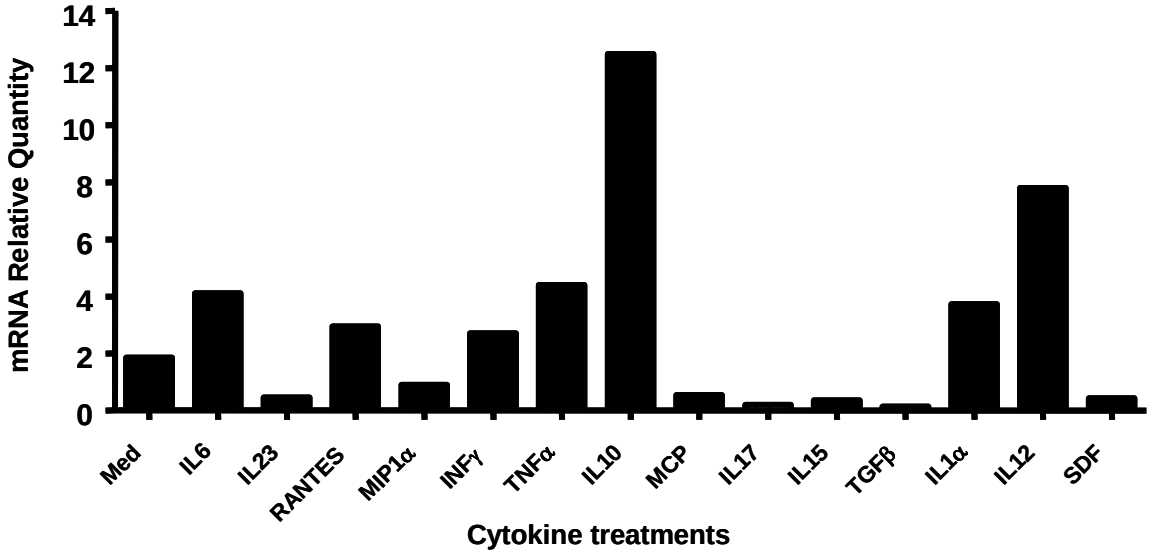
* p value = 0.0167

** p value = 0.0041

Figure 20. CD126 and CD130 expression on CD4 positive T cells following SF treatment and SF plus anti-IL-6R blocking antibody. Graph 17A shows the expression of CD126 on freshly isolated CD4 positive T cells following IL-6 treatment (10ng/ml for 30 minutes at 37ºC), SF treatment (30 minutes at RT) and SF plus anti-IL-6R antibody anti-IL-6R antobody added at 1 µg/ml for 30 minutes incubated on ice and then SF added and further incubated 30 minutes at 37ºC..Graph 17 B shows the expression of CD130 on CD4 positive cells after IL-6, SF and SF plus anti-IL-6R treatment (same incubation conditions as 17A). P values were calculated using a Mann Whitney test

4.2.2 IL-10 may contribute to the high level of expression of CD130 demonstrated on CD4 positive T cells in ST.

CD4 positive T cells in ST express high levels of CD130. We wanted to investigate the mechanism causing CD130 upregulation or maintenance of CD130 expression. For this purpose we obtained PBMCs from healthy donors by ficoll gradient centrifugation, and sorted CD4 positive T cells using a MoFlow cell sorter. Sorted CD4 T cells were treated with a panel of cytokines for 6 hours. Following treatment, the expression of CD130 was measured by flow cytometry and CD130 mRNA levels measured by RT-PCR. RNA was extracted from cytokine-treated cells. RNA was then reverse transcribed to obtain cDNA and cDNA was used to determine the level of CD130 mRNA by RT-PCR. Cytokine treated CD4 T cells were used to assess CD130 protein levels by flow cytometry. We found that IL-10 treated CD4 positive cells have higher levels of CD130 mRNA (fig 21). We also found a concentration dependent upregulation of CD130 at the protein level on CD4 positive T cells treated with IL-10 (fig 22).



n=1

Figure 21: Relative quantity of CD130 mRNA in cytokine-treated CD4 T cells. This graph shows the relative amount of CD130 mRNA present in CD4 positive T cells following 6 hour treatment at 37ºC with the following cytokines : IL-6, IL-23, RANTES, Mip1α, INFγ, TNFα, IL-10, MCP, IL-17, IL-15, TGFβ, IL-1α, IL-12 and SDF, all cytokines were used at a concentreation of 10ng/ml, except SDF which was used at 40ng/ml.

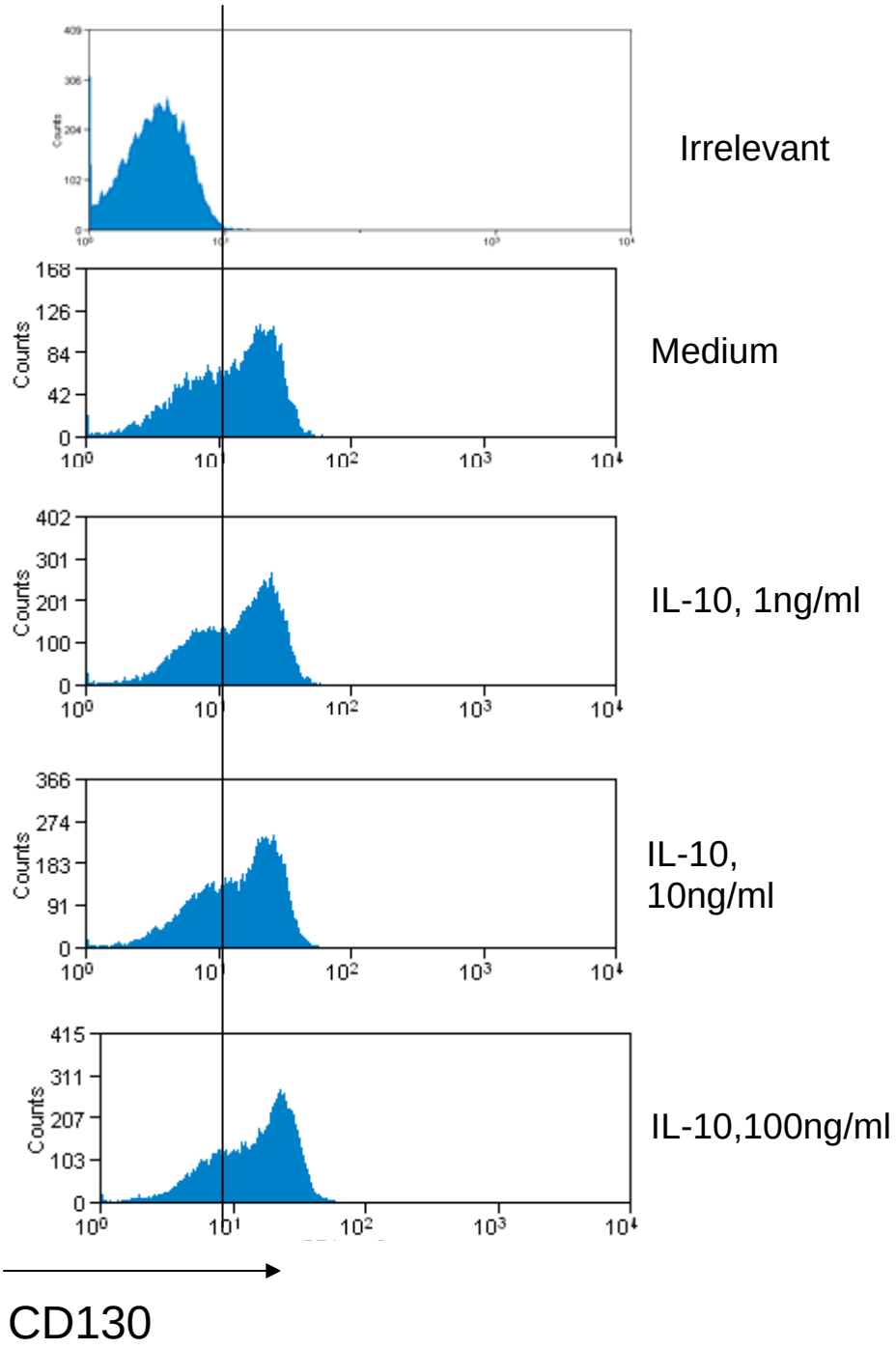
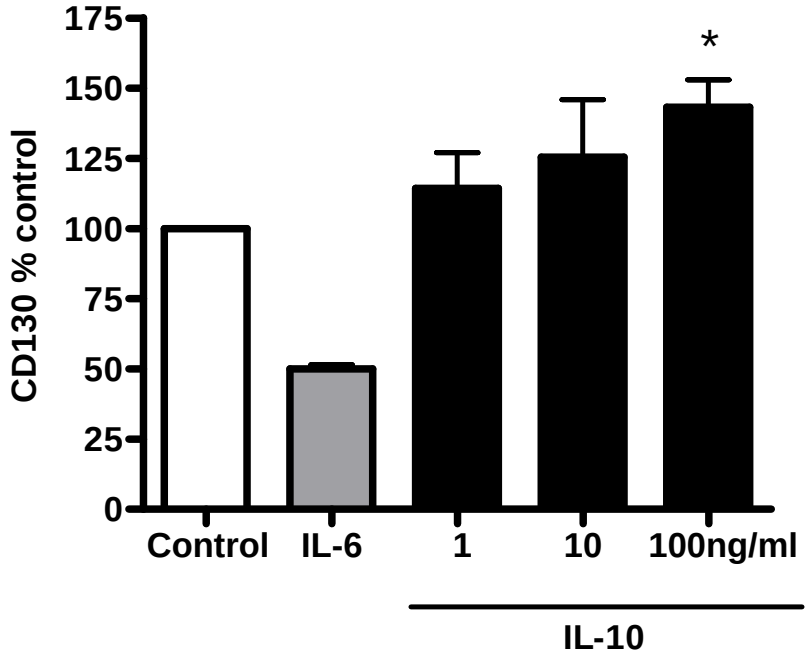


Figure 22 . Expression of CD130 on CD4 T cells treated with IL-10. The histograms show the expression of CD130 on CD4 T cells that were treated with IL-10 at 1, 10 and 100 n/ml for 6 hours.



* p value = 0.0165

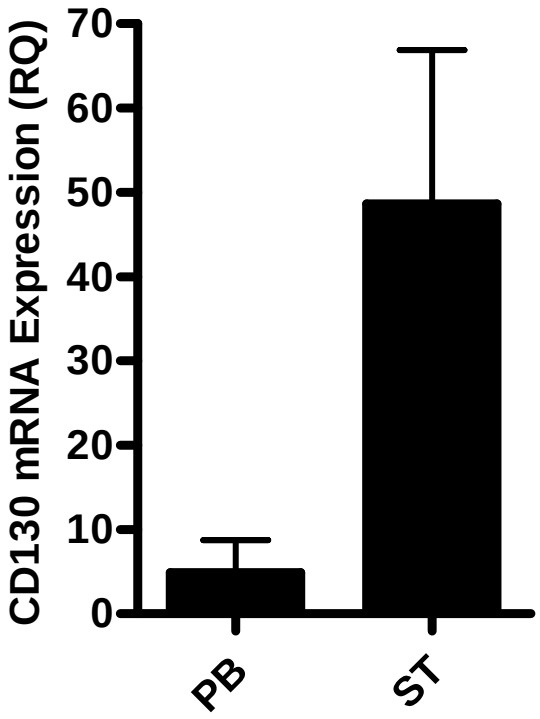
n=3

SD error bars

Figure 23. Protein expression of CD130 induced by IL-10. Figure 23 shows the CD130 protein expression on CD4 positive T cells following treatment with IL-10 at 1, 10 and 100 ng/ml for 6 hours at 37ºC.

4.2.3 High levels of CD130 mRNA have been found in CD4 T cells in RA ST.

We described earlier that CD4 positive T cells in the perivascular areas of RA ST show high levels of expression of CD130 at the protein level. We wanted to determine if this was reflected in the CD130 mRNA expression. For this purpose, we obtained PBMCs by ficoll gradient centrifugation and STMCs by tissue mechanical degradation followed by ficoll gradient centrifugation. Then CD4 positive T cells were sorted by flow cytometry. Sorted cells were used for RNA extraction followed by reverse transcription to obtain cDNA. cDNA was used for RT-PCR to determine the relative quantity of CD130 mRNA present in CD4 positive cells from ST compared to PB. The level of CD130 mRNA was higher in ST CD4 T cells compared to PB (figure 24).



n=3

SD error bars

Figure 24. CD130 mRNA levels on CD4 positive T cells in RA ST. This graph shows the relative quantity of CD130 mRNA present in CD45RO positive CD4 T cells obtained from PB and ST paired samples of RA patients.

4.2.4 In vitro endothelial transmigration does not have an effect on CD130 expression on CD4 positive cells.

CD130 is expressed at high levels on CD4 positive T cells in the perivascular areas of ST. We wanted to investigate if endothelial transmigration was responsible for this. To test this we used a model established in the Department of Physiology of the University of Birmingham.

We grew monolayers of HUVEC cells and sorted CD4/ CD45R0 double positive cells from PB of healthy donors. We stimulated HUVEC cells with TNF-α and IFN-γ (100u/ml and 10ng/ml respectively) for 24 hours and rinsed off. HUVEC and freshly isolated T cells were co-cultured with CD4 T cells on top of the HUVEC cells for 24h hours in the presence and absence of IL-10 at 10ng/ml. After incubation we collected the CD4 T cells that had crossed the HUVEC monolayer and the cells that did not cross the HUVEC monolayer and remained on top of the monolayer. Finally we looked at the expression of CD130 of transmigrated and non-transmigrated CD4 T cells by flow cytometry. Examples of raw data are shown in figure 25. The expression of CD130 was similar in migrated and non-migrated cells and similar to freshly isolated cells that were not exposed to the HUVEC cells. CD130 expression was slightly higher on both non transmigrated cells and on transmigrated cells in the presence of IL-10 (figure 26). So we conclude that transmigration through a monolayer of HUVEC does not change the expression of CD130 on CD4 positive T cells in vitro, making it unlikely transendothelial migration in vivo has an effect on CD130 expression.

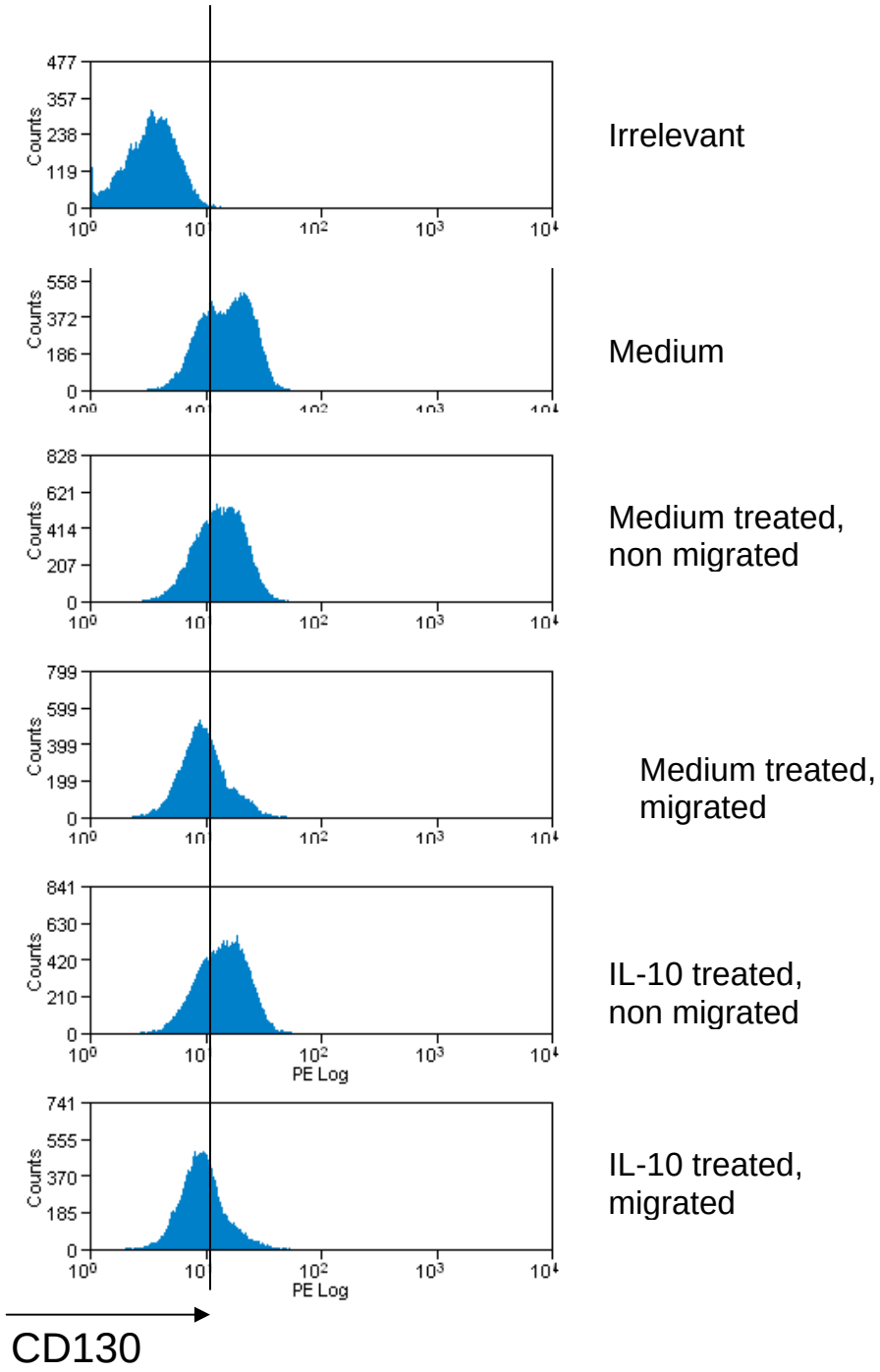
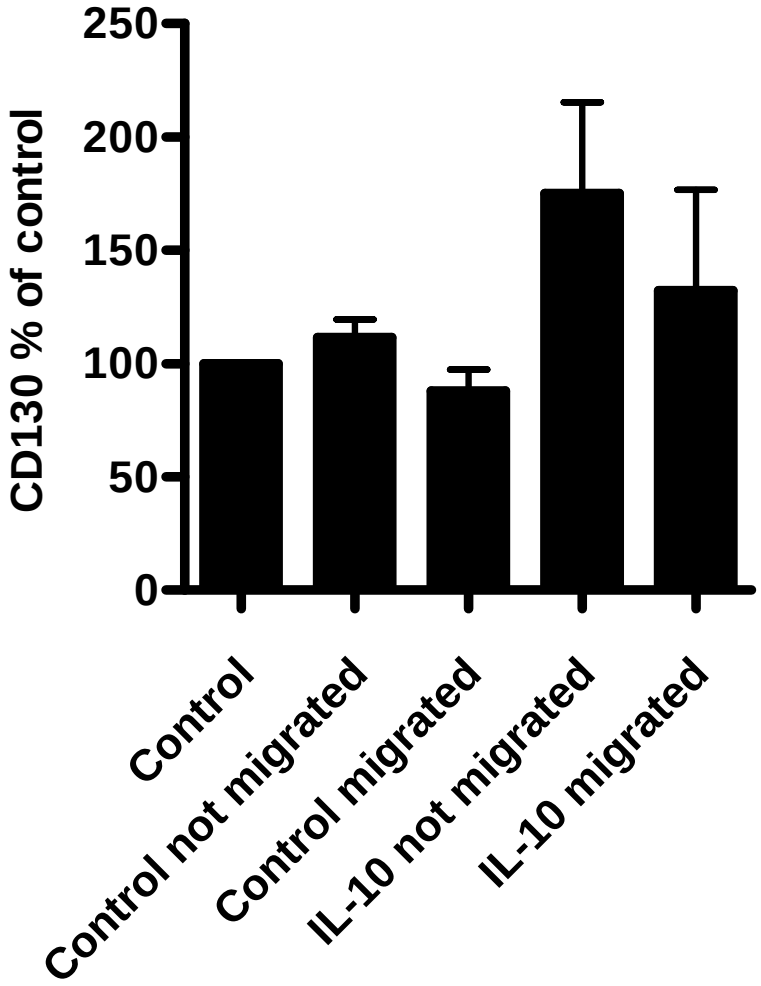


Figure 25. CD130 expression on CD45RO positive CD4 T cells following exposure to a HUVEC monolayer of cell. The histograms show the expression of CD130 on CD45RO positive CD4T cells from healthy donors before and after transmigration through a monolayer of HUVEC cells. HUVEC cells were stimulated with TNFα (100u/ml) and IFNγ (10ng/ml) for 24 hours and CD4+ T cells were incubated on top of the HUVEC monolayer for 24h at 37ºC.



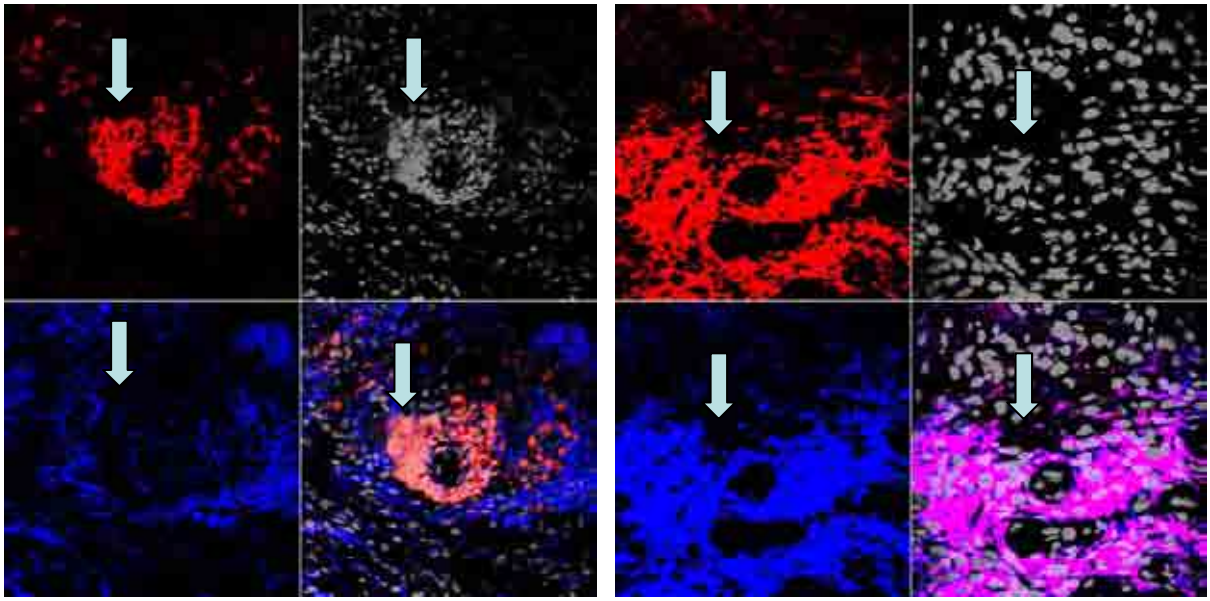
n=3

SD error bars

Figure 26. CD130 expression on HUVEC transmigrated and non-migrated CD4 T cells in the presence and absence of IL-10. This graph shows the expression of CD130 on CD45RO positive CD4T cells from healthy donors before and after transmigration through a monolayer of HUVEC cells. HUVEC cells were stimulated with TNFα (100u/ml) and IFNγ (10ng/ml) for 24 hours and CD4+ T cells were incubated on top of the HUVEC monolayer for 24h at 37ºC.

4.2.5 Localization of IL-10 and IL-6 expression within the rheumatoid joint.

The data shown above suggest that both IL-6 and IL-10 may have a role in the regulation of the expression of both chains of the IL-6 receptor in patients with rheumatoid arthritis. We therefore decided to study their localization in ST. While the localization of IL-10 in the perivascular infiltrate has been described before, we wanted to be able to investigate its localization in the tissue samples in which we had investigated the distribution of IL-6R receptor components. We stained frozen sections of synovial tissue with anti-IL-6 and anti-IL10 to asses the presence and distribution of the two cytokines. The images shown in figure 27 clearly demonstrate the presence of IL-6 and IL-10 in the joint. IL-6 was localized mainly in not heavily infiltrated areas while IL-10 was found mostly in the perivascular cuff areas with heavy presence of inflammatory infiltrate.



n=3

Figure 27. Confocal images of IL-6 and IL-10 in ST sections.

Figure 22 is a confocal picture of a synovial tissue sections, on the left: stained with anti-IL-6, anti-CD3 and a nuclear counterstain DAPI (grey). On the right a confocal image of a synovial tissue stained with anti-IL-10 and a nuclear counterstain DAPI. The arrows in the picture point the perivascular cuffs.

4.3 DISCUSSION

In the previous chapter we have described the different IL-6R phenotypes found in SF and ST CD4 T cells and we showed a biological function derived from these different phenotypes. In the work shown in this chapter we further investigated the potential mechanisms that may explain these two different phenotypes in SF and ST CD4 T cells. From the literature we knew that IL-6 is present in very high levels in the SF of RA patients (246), and that that IL-6 can downregulate the expression of both chains of its receptor (64). We decided that IL-6 present in SF would be a good candidate to explain CD126 and CD130 downregulation in SF. We treated fresh CD4 T cells with SF and SF combined with an anti-IL6R blocking antibody and observed that indeed SF downregulates CD126 and CD130. This downregulation was prevented by an anti-IL6R blocking antibody. We therefore suggest that the IL-6 present in SF of RA patients may cause the low expression of the IL-6R chains on CD4 T cells. However, IL-6 is also expressed in the synovial tissue, raising the question why CD130 is not downregulated on T cells in the tissue. Therefore we looked for mechanisms that could explain why CD130 remains highly expressed in ST CD4 T cells. We did not have a very obvious candidate for this so we used a panel of cytokines, present in the RA SF and representative of all cytokine families, to treat CD4 T cells and analyze their effect on expression of CD130 on the protein and gene transcription level. To our surprise we found that IL-10 treated CD4 T cells showed high levels of CD130 mRNA and also showed an upregulation of CD130 at the protein level. IL-10 is a homodimeric cytokine produced by a wide variety of cells, the major sources of IL-10 include: Th2, Th1, a type of Treg known as Tr1, CD8T cells, monocytes, macrophages, some subsets of DCs, B cells, some granulocytes and some non-immune cells such as epithelial cells or tumor cells (80). New data from our laboratory, however, suggests that most of the IL-10 in the RA synovium is produced by

macrophages. IL-10 is traditionally seen as an anti-inflammatory cytokine, it inhibits the activation of monocytes, macrophages and dendritic cells which in turn results in a reduced production of proinflammatory molecules such as cytokines and chemokines necessary for CD4 T cell activation and stimulation. In contrast, IL-10 has some proinflammatory or stimulatory effects on other cell types. It induces recruitment of CD8 T cells and stimulates their proliferation and cytotoxic activities. It also stimulates the proliferation of B cells, enhancing their antibody production (86). The presence of IL-10 in the rheumatoid joint has been demonstrated in by immunohistochemistry and PCR(182). It has been shown to be produced in the rheumatoid joint by T cells and macrophages. It has been associated with a reduction in the release of anti-inflammatory cytokines in the joint and therefore it is thought to have a proinflammatory effect. However, IL10 has also been associated with an increased of autoantibody production by B cells, and its mRNA level has been correlated to joint damage. This observation is supported by the results of a two year retrospective study which showed that the synovial membrane mRNA level of IL-10 together with other cytokines is predictive of a high level of joint destruction in RA (185). Another study revealed that IL-10 genotype associated with higher production of IL-10 was observed in patients showing higher rate of joint destruction (184).

The anti-inflammatory effect and immunomodulatory action of IL-10 have been highlighted experimentally using animal models of human disease. IL-10 knockout mice spontaneously develop an inflammatory lethal condition affecting their intestine which can be reversed by adding IL-10 (247). Following this observation, IL-10 was administrated to treat animals with various animal models of colitis successfully (reviewed in (248)). IL-10 treatment was beneficial in various experimental models of inflammation including: experimental autoimmune encephalomyelitis (249), pancreatitis (250), diabetes mellitus (251), and some

models of arthritis (252). However, the use of IL-10 in experimental allergic encephalitis showed controversial results being successful in some studies and unsuccessful in others (253,254). Clinical trials conducted to test the use of rIL-10 as an anti-inflammatory agent in RA, however, did not lead to clinical improvements. In a study conducted by van Roon and colleagues, 10 RA patients were treated with rIL-10 without convincing therapeutic effect according to the ACR criteria, in addition rIL-10 treatment caused the adverse effect of thrombocytopenia and the upregulation of the Fc gamma RI and Fc gamma RIIa on circulating monocytes/macrophages and an increase in TNF-α production**.** The upregulation of Fc gamma R may contribute to immune complexes formation and may therefore counteract the potential anti-inflammatory effects of IL-10 (255). Similarly, the administration of rIL-10 to sufferers of Crohn's disease in clinical trials showed disappointing results with no clinical remission or symptom reduction (254). However, the use of IL-10 in clinical trials to treat psoriasis has been very promising showing improvement of the condition as well as reducing relapse (256,257). Psoriasis is a T cell mediated autoimmune disease affecting the skin. It is believed that specialized APC present psoriatic antigen to T cells, IL-10 has been suggested to interfere with APC activities and triggers a shift from Th1 like to Th2 like response (reviewed in (248)). Taken together, these studies do suggest that there is no straightforward clinical application for recombinant IL-10 in inflammatory disease, and that it's pro and anti-inflammatory functions are more complex than anticipated from the studies in murine models.

It is not understood to date whether IL-10 is expressed in the synovium as a counterregulatory factor, produced by the immune system in an attempt to down-modulate the inflammatory process or whether it is functioning as a proinflammatory agent. Whatever the case is, our experiments suggests that IL-10 may contribute to upregulate or maintain CD130 expression on CD4 T cells in the ST allowing IL-6 to remain a survival and differentiation

signal to these cells via transsignaling. So whatever the mission of IL-10 in the synovium is, this cytokine may become an accomplice of IL-6 in T cells survival and RA chronic inflammation.

Even though we have identified IL-10 as a candidate factor that could cause the upregulation or maintenance of expression of CD130, this does not provide evidence of its role in vivo. There are several possibilities, one is that PB T cells that express high levels of CD126 and CD130 infiltrate the joint and CD126 expression goes down in the presence of IL-6 but CD130 remains high due to the presence of counter-regulatory factors, such as IL-10 that may induce de novo production of CD130. This interpretation is supported by the high levels of CD130 mRNA in ST CD4 T cells compared to PB. Increased mRNA expression in the tissue T cells is compatible with our hypothesis that it is induced locally. However, CD130 expression can also be explained by selective migration of CD130 high cells from the blood. Low expression mRNA expression in these cells would have indicated that it is unlikely to be induced locally. There is little information regarding the regulation of CD130 at the gene level in the literature, CD130 mRNA levels have been described to be upregulated in rat brains in response to LPS but not in response to rIL-1β (258) . Falus and colleges studied the effect of various compounds on the levels of CD130 mRNA on several human cell lines. They found that CD130 mRNA is downregulated in U266 and BMNH by TPA. CD130 mRNA is also downregulated in U937 by DB-cAMP and upregulated in U266 by dexamethasone (259). Another reasonable hypothesis would be that endothelial transmigration triggers CD130 expression on CD4 T cells when they travel through the blood vessels and into the synovium. We tested this in vitro, by assessing the effect of endothelial transmigration on the expression of CD130 on CD4 T cells and found that CD130 expression is not affected in the presence and absence of IL-10. Obviously it is very hard to extrapolate in vitro experiments to the

actual situation in vivo where there are may be additional factors that are absent in-vitro. It is therefore possible that endothelial transmigration does influence CD130 expression in RA but the in vitro data suggests that that is not the case.

We stained RA ST sections for IL-6 and IL-10 to confirm the presence of these cytokines in the joint and to determine the distribution of the two cytokines in the rheumatoid synovium. We found, in agreement with previous publications, that IL-6 and IL-10 are both present in the synovium. IL-6 appears to be particularly high in non infiltrated areas, but is present throughout the synovial tissue. In turn IL-10 is expressed mostly in perivascular cuff areas with high numbers of infiltrating cells. We did not investigate the source of IL-6 and IL-10 in the RA joint but it would be interesting to determine which cells are secreting these two cytokines. The presence of IL-6 in the ST explains low expression CD126 on CD4 T cells, However, it this case it would have been expected to see loss of CD130 expression as well. IL-10 may explain or at least in part the higher levels of CD130 expression on CD4 T cells in the perivascular areas of the ST. IL-10, probably together with other factors that may cause CD130 upregulation by de novo production. The long term exposure of CD4 T cells to IL-6 in the RA joint makes them downregulate the IL-6 receptor and they probably would become insensitive to IL-6 if CD130 expression was not upregulated, caused by high levels of IL-10 (and may be other factors) in the perivascular cuff making these cells sensitive to IL-6 via transsignaling. So IL-6 may be a survival factor for CD130 high expressing CD4 T cells in the rheumatoid joint contributing to their ability to escape apoptosis and keep contributing to the chronic inflammation seen in RA.

Chapter 5

5. T cell activation status and presence of iNKT cells in very early rheumatoid arthritis.

5.1 Introduction

In earlier work done in our research group, Raza et al described a unique and transient cytokine panel in very early rheumatoid arthritis patients, this are patients that have suffered the symptoms for no longer than three months. The panel of cytokines described by Raza et al included: IL-4, IFNγ, IL-2, IL-5, IL-6, IL-13, IL-13, IL-17, IL-21, TNF-α, TGF-β and GM-CSF. Since iNKT cells are a source of IL-4 and IL-13 we hypothesized that iNKT cells may be enriched in the synovium of very early RA patients and therefore in this chapter we investigated the presence of iNKT cells in the SF of established RA patients with more than 3 months symptom duration compared to very early RA patients. Samples of Very Early arthritis were collected at the very Early RA clinic run by Dr. Karim Raza in City Hospital in Birmingham, where patients present with undefined synovitis. Full diagnosis of the patients would be then obtained overtime a shown in figure 28. In the very early RA group it has been described a transient cytokine profile Raza et al from which we conclude that T cells may have different roles in the different stages of the disease. Therefore their activation status could be different in the different stages of RA, so we investigated the activation status of T cells in the SF of very early RA patients compared to established RA patients.

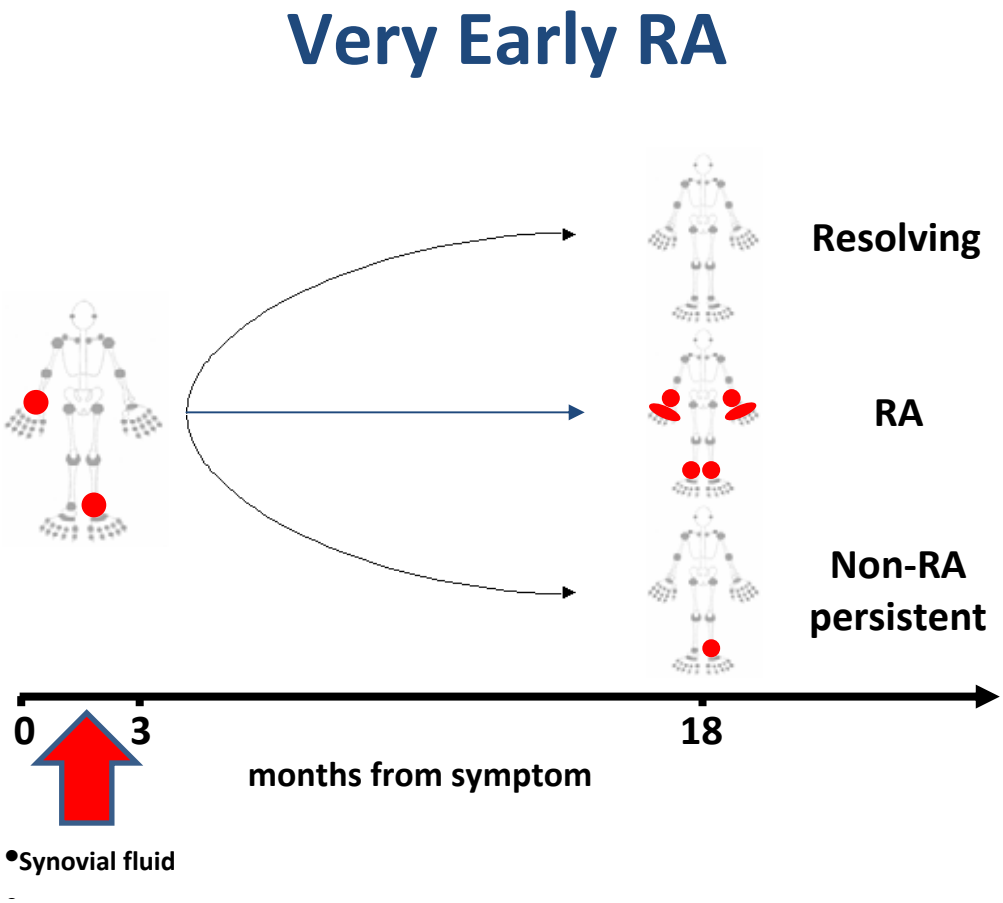


Figure 28. The different outcomes of very early synovitis

iNKT cells are a T cells subset with which have the particular characteristic to modulate the immune system by secreting a different range of cytokines in different situations being able to trigger both Th1 and Th2 responses. The role of iNKT cells in rheumatic disease has been extensively studied in the past few years. Reduced numbers of iNKT cells and impaired functionality have been reported in animal models genetically susceptible to various autoimmune diseases including type I diabetes, SLE and RA reviewed by (169). The involvement of iNKT cells in murine models of RA has been reported in several studies. iNKT cells appear to be necessary for the induction of mice CIA. It has been reported that CIA is substantially less severe in iNKT cell KO mice and mice in which iNKT cells have been blocked with anti-CD1d antibodies compared to WT mice (260). CIA is characterized by

pro-inflammatory Th1 type cytokines, OCH, an analogue of αGal-Cer, stimulated iNKT cells to produce Th2 cytokines. When administered to mice OCH collagen induced arthritis was ameliorated (reviewed by (169) while the same effect could not be achieved in iNKT cell deficient mice (168) In another model of RA, antibody induced arthritis, in which arthritis is triggered by transfer of serum from K/BxN mice, which spontaneously develop joint autoimmune inflammation, to healthy mice, this leads to auto-antibody deposition in the joint and subsequent inflammation triggered by activation of complement and Fcgamma receptor pathways (reviewed by (169). Joint inflammation was deficient in the absence of iNKT cells (261). Since iNKT cells seem to be a potential contributor to the development of arthritis we decide to study the presence of this cell type in the PB and SF in the early stages of RA compared to other inflammatory conditions and with later stages of RA. While there is a considerable body of literature on the presence of activated T cells in patients with rheumatoid arthritis (262-264), there has not been a systematic study of the level of T cell activation in the very early stages of disease. Since our previous work on cytokine expression has suggested a different role in the stages of RA development, we investigated the level of expression of activation markers in RA.

5.2 RESULTS

5.2.1 T cells in very early arthritis RA SF express the activation markers CD25, CD69, CD71 and HLA-DR.

We investigated patients recruited from the Birmingham Early Arthritis Clinic. These patients were recruited within the first 3 months of joint swelling and at 18 months depending on disease outcome, were classified as having RA, resolving arthritis, or chronic arthritis other than RA. Patients with established RA were either recruited from the early Arthritis Clinic after 3 months of symptoms or from other arthritis clinics in Birmingham. Cells in whole SF and whole blood were stained with the appropriate directly labeled antibodies for flow cytometry, red blood cells were lysed and cells were read in the flow cytometer to determine the level of expression of CD25, CD69, CD71 and HLA-DR in CD3 positive T cells from PB and SF samples, an example of the staining is shown in figure 29.

All activation markers are upregulated in SF compared to blood but no significant differences are observed in the different patient groups. The percentage of CD25 positive T cells in PB of all patients is slightly lower than in healthy controls but still at comparable levels. In SF CD25 is lower in early arthritis patients but the difference is not statistically significant CD69 and CD71 are expressed at very low levels in PB of all patients and controls but significantly higher in SF of all patients. HLA-DR is very low in PB but higher in SF (figure 30). In summary, while we detected an increased number of CD69, CD71 and HLADR positive T cells in the SF of most patients, there was no significant difference in the expression of activation markers in the groups of arthritis patients investigated here.

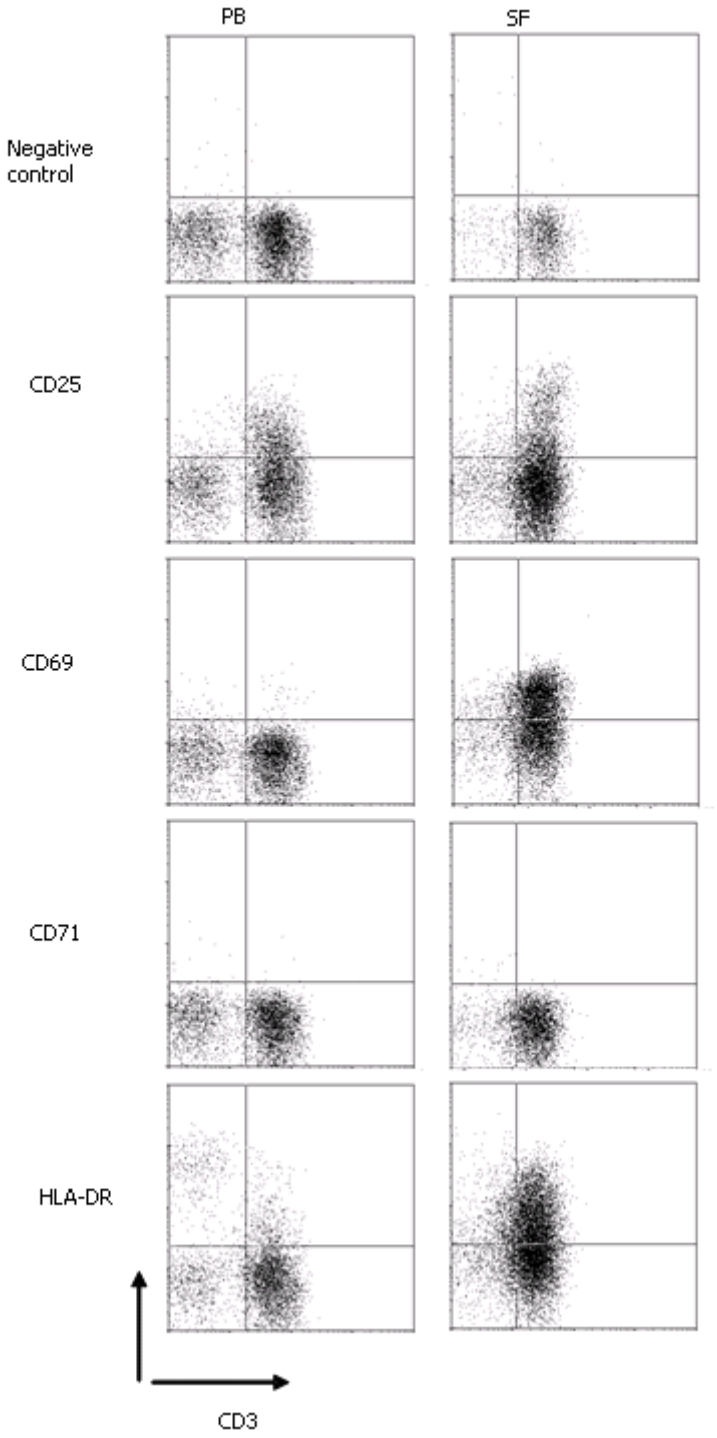
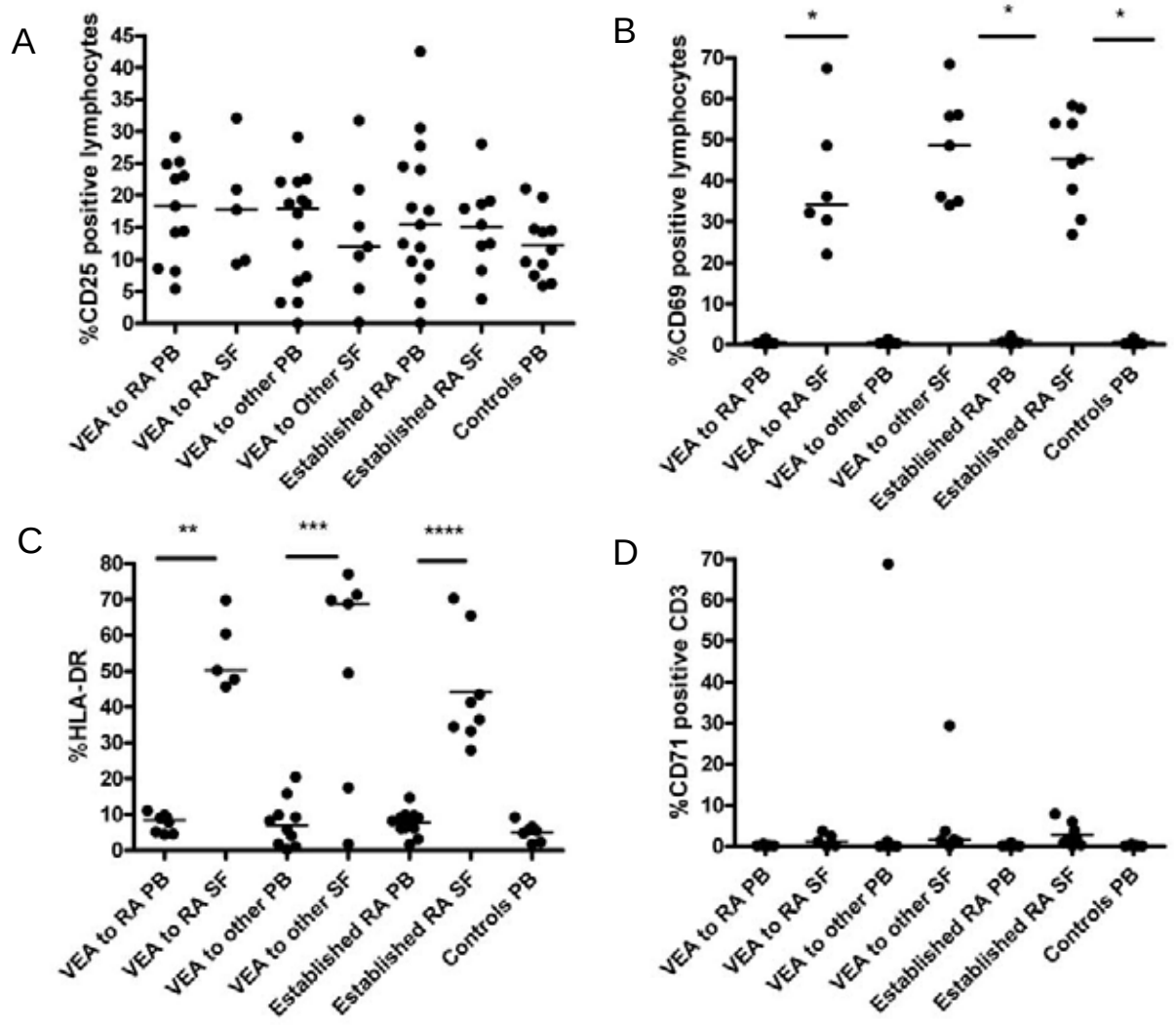


Figure 29. CD25, CD69, CD71, HLA-DR expression on CD3 positive cells from PB and SF. Figure 23 shows the number of CD3 positive cells that are also positive for the activation markers CD25, CD69, CD71 and HLA-DR in PB and SF samples of RA patients.



* p value <0.05 ** p value = 0.0006 *** p value = 0.0041 **** p value = 0.0001

(Kruskal-Wallis Test)

Figure 30. Expression of T cell activation markers on PB and SF.

The graphs show the expression of CD25 (figure 24A), CD69 (figure 24B), CD71 (figure 24D), and HLA-DR (figure 24C) on T cells in PB and SF of very early RA patients, Very early synovitis (other than RA) patients, established RA patients and healthy controls.

VEA to RA: patients with very early synovitis that become RA patients

VEA to Other: Very early synovitis patients that develop a condition other than RA and patients that resolve the synovitis.

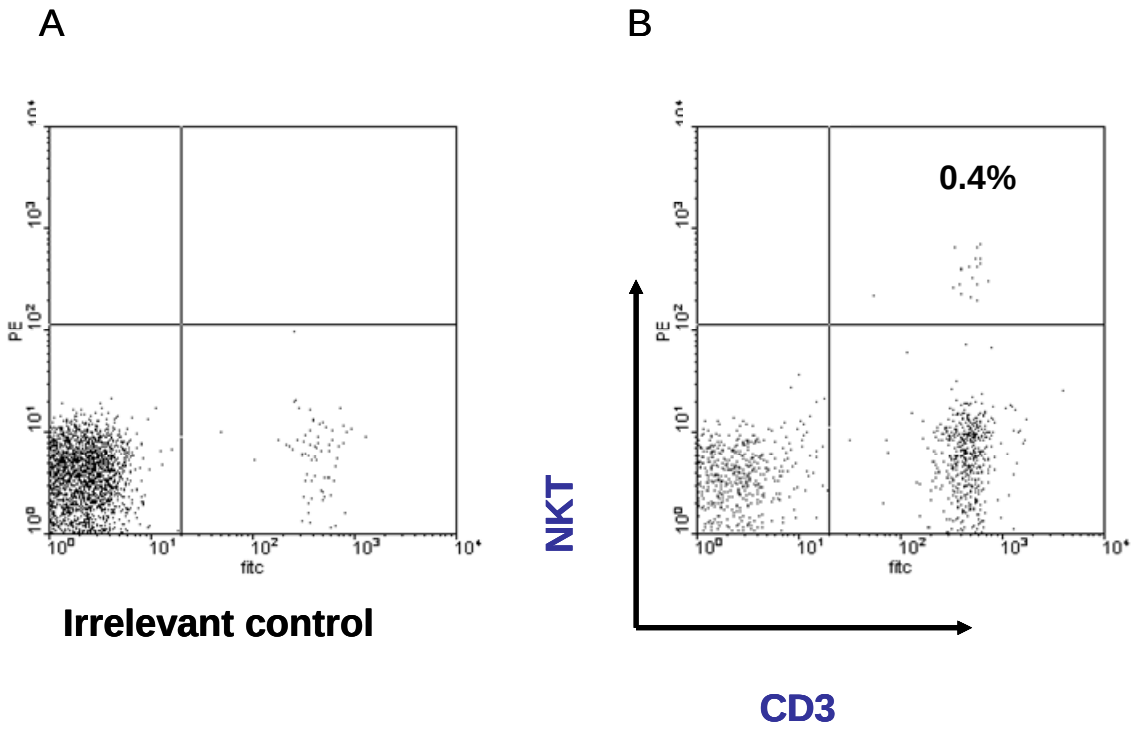
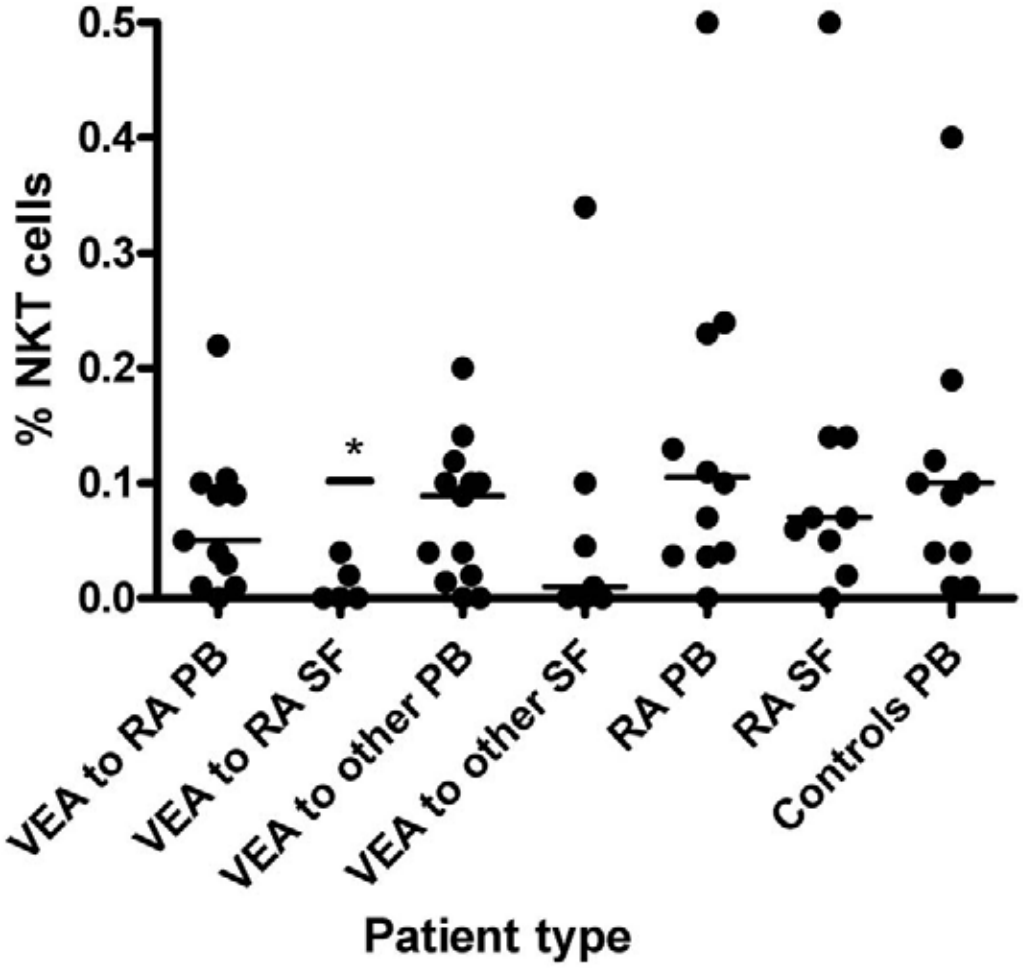


Figure 31. CD3 versus iNKT.

An example of a staining of a healthy control PBMC with anti-CD3 versus anti-iNKT. On the left an irrelevant control for iNKT and on the right the actual staining CD3 versus iNKT gated on the lymphocyte population in the FS/SS



*** p value = 0.019 (Mann Whitney test)**

Figure 32. iNKT cells in PB and SF.

This graph shows the percentage of iNKT cells amongst CD3 positive cells in PB and SF from Very early RA and non RA patients, Established RA patients and healthy controls. 50,000 events were run in total per sample. The absolute numbers of iNKT cells found are extremely low between 0 and 30 cells.

5.2.2 The levels of iNKT cells in PB and SF of Early arthritis (RA and non RA), established arthritis and healthy controls.

The percentage of iNKT cells was quantified in the PB and SF of early arthritis and established RA patients as well as healthy controls by flow cytometry. PBMCs and SFMCs were obtained by ficoll gradient centrifugation. Cells were stained with anti-CD3, anti-iNKT and anti-CD25/CD69/CD71/HLA-DR and the percentage of positive iNKT cells was obtained gating on the CD3 positive lymphocyte population. 50,000 events were run per sample. The level of iNKT cells in the PB of early arthritis and established RA does not significantly differ from the level in the PB of healthy controls. The percentage of iNKT cells is lower in SF compared to paired PB samples. All groups of patients show reduced numbers of iNKT cells in the SF but very early RA patients show the lowest number and it is significantly lower than the number of iNKT cells in the SF of established RA patients (Mann-Whitney test, p value = 0.019) (figure 32)

5.3 DISCUSSION

T cells are thought to contribute to disease pathology in RA; they are present infiltrating the synovial tissue of RA patients in RA SF. Their role and interaction with other cells in RA has been studied extensively. While the pattern of activation markers expressed by T cells in established RA has been subjected to considerable research, the activation status of T cells in PB and SF of patients with very early stages of RA has not been investigated so far. We found that the frequency of CD69 and HLA-DR positive cells are upregulated in SF of all patients. The frequency of CD25 expressing cells is slightly but not significantly increased in both blood and SF, being slightly lower in the SF of early RA patients. The frequency of CD71 positive cells is slightly higher in SF compared to blood in all patients but the differences are both significant. The marked upregulation of CD69 and HLA-DR suggests that T cells not only are present in the rheumatoid synovium but they are also activated and therefore most likely contributing to the chronic inflammatory process seen in RA. CD69 is an activation marker which gets upreglated very early upon activation, even though we showed high frequency of CD69 expressing T cells in the SF we cannot know is whether these cells become activated once in the joint or if they were previously activated outside the joint and are then selectively recruited. Previous work shows that Influenza specific T cells can be detected in the joint of RA patients after vaccination, suggesting that a proportion of the T cells found in the synovium are activated outside the joint (reviewed in (265)). It is noteworthy as well the fact that T cell activation could happen independently of antigen encounter in RA, it was shown that synovial T cells resemble cells activated by a cocktail of cytokines without TCR engagement (Brennan et al. 31-41) The positive results from the

abatacept trials indicate that T cell activation is important even in late stage disease, as interfering with T cell activation proves beneficial for RA patients (reviewed in (212)).

We can conclude from these experiments that there is no difference in the activation status of SF T cells in very early and established RA. In both cases SF T cells show high expression of activation markers CD69 and HLA-DR. We can also conclude that our activation markers (CD25, CD69, CD71 and HLA-DR) have no value as prognostic markers as they show no differences in the different patient groups and stages of RA. As I mention in the results chapter, iNKT cells are present in PB of early RA, early arthritis and established RA at a normal level that compares very well with our healthy control group. These findings are in disagreement with previous publications that claimed reduced frequency of iNKT cells in the PB of RA patients as well as other rheumatic patients such as SLE or systemic sclerosis patients (266). We also compared the levels of iNKT cells in the SF of the different patient groups and interestingly, we found that at a very early stage of RA (less than three months since the first symptoms began) the numbers of iNKT cells in SF are significantly reduced compared to established RA patients and early arthritis patients that develop a disease other than RA. However, the total number of iNKT cells was very small, making it very difficult to identify a biological significance. And due to small numbers of cells we could not sort iNKT cells or asses their activation status.

One possibility is that iNKT cells have an anti-inflammatory effect trying to dampen down inflammation at a very early stage of RA by promoting Th2 responses and that there are not sufficient numbers of iNKT cells to achieve clearance of inflammation, especially at the very early stage of RA. Alternatively it is possible that iNKT cells are pro-inflammatory in the context of RA and at very early stages they promote Th1 responses contributing to the development of RA and their lower numbers at this stage could be explained as iNKT cells

undergo apoptosis following cytokine secretion (reviewed in (17)). iNKT cells could be recruited to the joint in very early RA, They could secrete pro-inflammatory cytokines and undergo apoptosis, then they would be lost for a period of time until more iNKT cells are generated and recruited to the joint. An additional explanation could be that iNKT are actually present in the RA synovium in greater numbers but they have downregulated their TCR and we could not identify them.

Clearly we have disproved the hypothesis that enrichment of iNKT cells in the synovium leads to the upregulation of IL-4 and IL-13 in very early RA.

Chapter 6.

DISCUSSION

Cells chronically exposed to cytokines will respond by the initiation of counterregulatory mechanisms to limit the extent of the signal received. Thus the target cells are often rendered refractory to that cytokine. These mechanisms include downregulation of receptor expression, downregulation of signalling molecules as well as upregulation of molecules that are having an inhibitory effect on cytokine signal transduction such as SOCS and PIAS.

However, our data suggests that in the inflamed synovium, with its high level of IL-6, the responsiveness to IL-6 is maintained by transsignaling. The success of IL-6 targeting therapy underlines the importance of IL-6 in RA pathology. While it affects many cell types, for example B cells and fibroblasts, its role in the biology of T cell responses is particularly intriguing. It is one of the factors that regulate differentiation of T cells into Th-17 or Treg cells and therefore can affect the outcome of an inflammatory response. Furthermore it is known to be a T cell survival factor. To study the potential responsiveness of T cells in the inflamed rheumatoid joint, we investigated the differential expression of the two components of the IL-6 receptor CD126 and CD130. We have shown that expression of CD130 is maintained in T cells in the synovial tissue, even though CD126 expression is downregulated. In agreement with this observation, although synovial tissue T cells are refractory to IL-6 on its own, they will respond to soluble IL-6/IL-6R complexes. Induction of CD130 expression by IL-10 may be an explanation for this observation.

IL-6 is an important part of the network of proinflammatory cytokines found at sites of acute and chronic inflammation. (267), TNF-α (268), (reviewed by (48). Specific cell types respond differently to cytokine stimulation in regards of IL-6 production. For example, bone marrow cells produce little IL-6 when stimulated by IL-1 but high levels when stimulated by

IL-3, on the other hand fibroblasts stimulated with IL-3 produce low amounts of IL-6 but much higher amounts when stimulated with IL-1 (reviewed by (48). The IL-6 receptor as a complete CD126/CD130 complex is expressed by a limited number of cell types including hepatocytes and lymphocytes. CD130 can also be expressed independently of CD126 and is found in almost all cell types. Transsignaling is important as it allows IL-6 to influence cells that are not expressing the IL-6 receptor. As CD130 is expressed ubiquitously, IL-6 has the potential to signal to many cell types. The response of a cell to IL-6 signalling can be regulated through a number of pathways described in the literature; STAT3 activation enhances the expression of various genes, amongst others the genes coding for SOCS1 and SOCS3. SOCS3 inhibits the JAK-STAT pathway and therefore acts as an intracellular negative feedback for IL-6 signaling. Furthermore, IL-6 signalling can be limited by the downregulation of the IL-6 receptor caused by internalization of the receptor following IL-6 binding. These negative feedback mechanisms limit the amount of IL-6 signal that a cell can receive. (51). IL-6 has several pro-inflammatory functions that affect both local and systemic inflammation. It contributes to local inflammatory processes by activating endothelial cell production of IL-8 and monocyte chemoattractant proteins, and also by triggering the expression of adhesion molecules which in turn facilitate the recruitment of leukocytes to the site of inflammation. IL-6 also stimulates the proliferation of synoviocytes and the activation of osteoclasts. Furthermore, it contributes to systemic inflammation by its involvement in the acute phase response. It triggers the production of acute phase proteins such as CRP by hepatocytes. Acute phase proteins cause complement activation which leads to induction of proinflammatory cytokines and neutrophil chemotaxis. IL-6 is also associated with the induction of fever and the secretion of hepcidin, an iron regulatory protein. Important effects of IL-6 on immune cells include the differentiation and stimulation of B cells to produce

antibodies, T cell activation and differentiation and macrophage differentiation (reviewed by (269). In addition, IL-6 favors the differentiation of CD4 T cells into proinflammatory Th17 cells. CD4 T cells have the potential to differentiate into an ever increasing number of T cell subsets. Among these are Treg and Th17 cells. Tregs are associated with self tolerance (reviewed by(9)) but Th17 cells are considered as proinflammatory cells and have been proposed to play an important role in RA (reviewed by (7)). In mice, TGF-β is required for the differentiation to both Treg and Th17 but IL-6 is thought to favor the differentiation of T cells toward Th17 (270).

There is clear evidence derived from animal models and patients that IL-6 is a key factor in the pathology of RA. Elevated levels of IL-6 and sIL-6R have been demonstrated in both serum and SF of RA patients (217). Even though IL-6 has been found also in OA the levels are not as high as in RA (246). In RA synovial fibroblast show an increased production of IL-6 (271). IL-6 levels have been reported to correlate well with markers of disease activity such as RF or CRP (272) and clinical manifestations of the disease such as morning stiffness or number of inflamed joints (173). Intriguingly, there is even a correlation of severity of symptoms that follows the circadian rhythm of IL-6 production. IL-6 production peaks in the morning when RA symptoms, particularly joint stiffness are particularly severe. Conversely, IL-6 levels are lower in the afternoon and evening where discomfort for RA patients is less severe (reviewed in (273)). Treatment with disease modifying anti-rheumatic drugs is associated with a decrease in IL-6 which in turn correlates with improvement in symptoms (274). In animal models it has been observed that reduced levels of IL-6 are protective against arthritis while excess IL-6 signaling is linked to arthritic disease development (reviewed by (269). IL-6 deficient mice are resistant to the development of CIA (275) while mice with a CD130 mutation that enhances IL-6 signaling results in spontaneous development of an

inflammatory disease of the joints (276). In addition to this, IL-6 deficient animals resistant to CIA do only develop arthritis when injected with IL-6 when it is given as a soluble complex with, sIL-6R (181). This observation strongly suggests an important role for IL-6 transsignaling in the joint. Taken together, a clear importance for IL-6 in arthritis has been demonstrated in a range of human and animal studies. IL-6 is also known as an important regulator of lymphocyte differentiation and survival. The relative importance of direct and transsignaling is largely regulated by the relative expression of the two components of the IL-6 receptor and by the availability of IL-6 and the soluble receptor. sIL-6R has been demonstrated to be produced by naïve and memory CD4 T following TCR engagement, activated CD4 T cells shed the IL-6R of their cell membrane(277). The production of sIL-6R by autoreactive CD4T cells may be an important source of sIL-6R in autoimmune disease making CD126 lacking cells, such as synoviocytes, a target for IL-6 transsignaling (62). Secretion of the sIL-6R has also been reported in neutrophils and macrophages(reviewed in(58)

In this study, we investigated the expression of CD126 and CD130 on CD4 positive T cells from the SF and ST of RA patients. We have found three different phenotypes in the three different compartments studied. In PB of our patients, CD126 and CD130 are both expressed at fairly high levels. In the SF both CD126 and CD130 have almost disappeared from the cell surface of CD4 positive T cells and interestingly in the ST CD4 T cells express very low levels of CD126 but higher levels of CD130. This is a very interesting finding as high expression of CD130 makes ST CD4 T cells a potential target for IL-6 transsignaling. The same pattern of CD126/CD130 expression was reproduced when we compared CD45RO positive CD4 positive T cells from PB, SF and ST. We confirmed the high expression of CD130 on CD4 positive T cells in the ST using immunohistochemical staining and confocal

microscopy, in addition we observed that high expression of CD130 was particularly found on CD4 T cells clustering together around the perivascular cuffs and that CD130 expression on CD4 positive T cells becomes lower as CD4 T cells move away from the perivascular cuff areas, until they reach the SF where the expression of CD130 is virtually lost. So we observed a phenotype on CD4T cells in ST, particularly in the perivascular cuff areas that suggests that these cells do not become insensitive from chronic exposure to IL-6, but maintain sensitivity to IL-6 transignalling. In addition, the observation that CD130 expression is lower in cells dispersed throughout the tissue when compared to those in the perivascular cuff, suggests that a factor present in the cuff upregulates or maintains the expression of CD130 on CD4 T cells. Alternatively, high expression of CD130 may keep the cells clustering together in the cuff and when the expression of CD130 drops cells are able to move away from the cuff. Oberg et al investigated the expression of CD126 and CD130 on PBMCs from healthy individuals. They found high expression of both CD126 and CD130 on CD14 positive monocytes, lack of CD126 and CD130 expression on CD56 positive NK cells, CD19 positive B cells expressed no CD130 and CD126 only to a certain extent, CD8 positive T cells were shown to express high CD126 and varying levels of CD130. CD4 positive T cells expressed high levels of both CD126 and CD130, in agreement with our data, although they also found a small proportion of CD4 T cells with high CD126 and low CD130 expression (278). Interestingly, this group also looked at the expression of CD126 and CD130 in tonsil and spleen (of mice) and they found that CD126 and CD130 were slightly higher in tonsil compared to PB but lower in spleen on CD4 T cells (253). Betz and colleagues investigated the expression of CD126 and CD130 on T cells in their different developmental stages. They showed that thymocytes express CD130 all throughout thymic development but only acquired CD126 expression when they reached either the CD8 or CD4 single positive stage. In peripheral blood, this

group found that T cells express both CD126 and CD130, which does agree with our own results. They also demonstrated that both CD126 and CD130 are downregulated upon TCR engagement and that CD126 low, CD130 low T cells in the PB show markers typically found on memory T cells (279).

It has been reported that the loss of IL-6R expression on CD4T cells during inflammation gives transsignaling a role in the maintenance of Th17 cells (280). In addition STAT3 activation via transsignaling in various models of arthritis contributes to neutrophil and T cell retention, including IL-17A producing T cells in the synovium (277). Our own work is in agreement with the low expression of IL-6R, and we showed functional contribution of IL-6R expression on T cells of RA patients. Gathering these studies together we could postulate that in RA maintenance of CD130 expression, brought about by IL-10, which allows for IL-6 transsignaling could be an important mechanism for maintenance, retention and viability of Th17 cells, clear contributors to autoimmunity and inflammation. In contrast Tregs have been reported to express low normal levels of CD126 and low levels of CD130(278). If we looked at Tregs in the synovium they would have downmodulated CD126 due to the high level of IL-6 available and they would have no potential power to respond to IL-6 via transsignaling so we would be left with Tregs that could not benefit from IL-6 signalling, the possible regulatory effects of Tregs in the joint are limited while Th17 cells are favored by CD130 expression and transignalling.

Having established the three different CD126/CD130 phenotypes found on CD4 T cells from PB, SF and ST of RA patients, we then addressed the question whether there is a biological consequence derived from the differential expression of CD126 and CD130 in the different compartments of RA patients. We know that IL-6 can signal directly by binding to CD126 and then associating with the signal transducer CD130 or via transsignaling when IL-6/sIL-6R

complexes binding to CD130. We demonstrated that CD4 T cells from blood are able to respond to both IL-6 and IL-6/sIL-6R complex. This capacity of PB CD4 T cells to respond to IL-6 directly and via transsignaling is likely to be mediated by their high expression of CD126 and CD130. On the other hand, SF CD4 T cells, lacking expression of CD126 and CD130, did not respond to either IL-6 or IL-6/sIL-6R complex. In contrast, CD4 T cells from ST with their low levels of CD126 and high levels of CD130 cannot be stimulated by IL-6 alone. However, they are able to respond to IL-6/sIL-6R complex treatment. In conclusion, the responsiveness to either IL-6 or IL-6/sIL-6R complexes is in agreement with the receptor expression and suggests that the differential receptor expression has a biological significance. In agreement with our finding that IL-6 signals to CD4+ T cells from ST via transsignaling , Oberg *et al* reported that Tregs with low CD130 expression, lacked response to IL-6/sIL-6R complex when compared to T cell populations with higher levels of CD130 expression (reviewed in (269).

Data derived from the experiments by Teague and colleagues suggests that IL-6 can rescue mouse T cells from apoptosis in vitro by inducing or maintaining high Bcl-2 expression levels (50). Our own data suggests that CD4 T cells in the ST can recognize IL-6 via transsignaling and since IL-6 has the potential ability to rescue T cells from apoptosis. We suggest that IL-6 transsignaling provides a potential survival mechanism of T cells in the rheumatoid joint which in turn contributes to the ongoing inflammation and chronicity of the disease.

The role of transsignaling in the joint has previously been demonstrated by Jones and coworkers (181). They demonstrated high levels of sIL-6R in the SF of RA patients when compared to OA. This elevation was correlated positively with IL-6 levels. Although the major source of sIL-6R is membrane shedding, they also demonstrated the presence of low levels of ds-sIL-6R in the RA joint (reviewed by (218). Furthermore, their experiments

showed the involvement of IL-6 transsignaling in a murine model of arthritis. While IL-6 KO (F2 mice C57Bl/6_129Sv) mice were resistant to the induction of AIA, reconstitution of the animals with IL-6 was only partially able to induce arthritis like symptoms. Reconstitution with Hyper-IL-6, a fusion protein consisting of human IL-6 and the human sIL-6R connected by a flexible polypeptide chain, however, led to joint inflammation at a level similar to wild type animals. In addition, they demonstrated that IL-6 transsignaling through Hyper-IL6 in AIA mice induces leukocyte recruitment through CCL2 expression. They further confirmed the important role of transsignaling in RA like disease in this animal model by showing that AIA in wild type mice could be blocked by the administration of sgp130 which binds to IL-6/sIL-6 receptor complexes preventing IL-6 transsignaling (181).

In summary, there is a considerable amount of evidence that IL-6 makes a strong contribution to the RA pathology, both via direct signaling and transsignaling. This is highlighted by the use of anti-IL-6R antibodies in clinical trials for RA (reviewed in (281)). Tocilizumab is an anti-IL-6R monoclonal antibody (206) which recognizes IL-6R and its soluble form, sIL-6R (282). The administration of Tocilizumab causes an increase in IL-6 in serum due to the inability of cells to consume IL-6 as its receptor has been blocked in both forms, membrane bound and soluble form. Even though there is an increase in serum IL-6, its effects are efficiently blocked by the drug (283). Several clinical trials have been run internationally. Phase III clinical trials have resulted in around 40% ACR 50 responses. The use of Tocilizumab is safe and efficient, but there are other anti-cytokine drugs that work with a similar efficacy, such as anti-TNF-α. There is therefore the need to predict patient response to a particular drug in order to administer to each patient the drug that would be most efficient for him/her (reviewed by (281).

IL-6 is not the only survival factor for T cells in the RA joint. In 1999 our group reported that type I interferons rescue activated T cell blasts and converts them to the G0/G1 memory state resulting in inappropriate survival of T cells (139). T cells can also be potentially rescued from apoptosis by excess level of IL-15 found in the RA joint (112), IL-15 is an stimulatory cytokine for T cells that uses the gamma chain of the IL-2R to signal (284) and upregulate genes that code for antiapoptotic proteins such as Bcl-2 (284).

Continuing our studies, we investigated the regulation of CD126 and CD130 expression by CD4 T cells in SF and ST. In SF both CD126 and CD130 were found in very low levels. Since IL-6 has been reported to be able to downmodulate the expression of its receptor (reviewed by (51) and IL-6 is present at high concentration is the rheumatoid joint we had a very obvious candidate to be responsible for CD126 and CD130 low expression in SF. Indeed, our findings suggest that CD126 and CD130 expression on CD4 T cells from healthy donors can be downregulated following SF treatment and that this effect can be reversed by blocking IL-6 mediated signals in the SF. The next challenge was to find an explanation for the CD126 and CD130 phenotype seen on CD4 T cells from ST. CD130 expression on CD4 T cells in the perivascular areas of RA ST is high. First we decided to investigate if there is a possibility that CD130 is being induced locally, so we demonstrated that level of CD130 mRNA present in ST CD4 T cells was higher compared to PB CD4 T cells. This does not provide comprehensive evidence for CD130 being induced locally, but on the other hand if there had been low expression of CD130 mRNA, local induction of CD130 expression would have been very unlikely. It is, however, possible that CD130 expression is induced at other sites and that these cells selectively migrate to the inflammatory infiltrate in the inflamed synovium. We realize that the difference, though statistically significant is not very high. However, discrepancies between protein and mRNA data are frequent and can often be

explained by posttranslational regulatory processes. When comparing the CD130 expression between PB and ST T cells, we found no significant difference. More importantly, the functional difference between SF and ST T cells in their response to transsignaling suggests a potential biological role for CD130 expression in ST T cells. Also ST T cells, while they may express CD130 *de novo* are permanently in contact with IL-6/sIL-6R complexes which counterregulate the surface expression. We are therefore in a real –life situation looking at a dynamic equilibrium of mechanisms that continuously up and down regulate CD130. So the actual role of IL-10 may be to maintain the CD130 expression at the rate that counteracts loss from exposure to IL-6/IL-6R complex..

To identify factors potentially involved in the upregulation of CD130, we investigated the effect of a large panel of cytokines found in the rheumatoid synovium on CD130 mRNA expression. We showed that CD4 T cells treated in vitro with IL-10 have higher levels of CD130 mRNA compared to untreated cells and cells treated with other cytokines. We also showed that IL-10 treatment triggers CD130 upregulation on CD4 T cells at the protein level and that the upregulation is titratable. This result was initially surprising as IL-10 is generally viewed as an anti-inflammatory cytokine. IL-10 can be produced by Th1, Th2, Th17, CD8 positive T cells and some Tregs, monocytes, macrophages, dendritic cells, B cells, eosinophils, mast cells, keratinocytes, epithelial cells and some tumor cells. Data from our own research suggest that in the rheumatoid joint IL-10 is mainly produced by T cells and macrophages. (Personal communication, Yeo, L.). The two chains of the IL-10R are regulated independently. IL-10R2 is expressed by most cell types while IL-10R1 is expressed on most hematopoietic cells, epithelial cells and fibroblasts at very low levels but is upregulated upon many stimuli. The expression of the IL-10 receptor was demonstrated to be higher on CD4 positive cells rather than CD8 T cells from PB of healthy controls by Schultz and coworkers

(285). If that is the case in an inflammatory setting CD4 T cells in the perivascular cuffs of RA ST are able to respond to IL-10 and upregulate of CD130. To date there is no information regarding IL-10 receptor expression by inflammatory cell populations in the rheumatoid synovium available. IL-10 binds to IL-10R1 and this triggers the recruitment of IL-10R2 which enables signal transduction. Following IL-10 binding Jak1 is recruited to IL-10R1 and Tyk2 is recruited to IL-10R2, they phosphorylate the intracytoplasmic tails of the receptor chains. These results in STAT3 recruitment subsequent phosphorylation and finally its homodimerization and release from the receptor. It then translocates into the nucleus where it binds to specific DNA sequences. It is interesting how two cytokines that activate the same signaling pathway like IL-6 and IL-10 can have such diverging effects. A possible explanation for this is that SOCS-3 inhibits STAT3 activated through the IL-6 receptor but not by IL-10, because SOCS3 does not bind to the IL-10R (89).

IL-10 has various anti-inflammatory functions, it inhibits antigen presentation by downregulation of MHC class I molecules and co-stimulatory molecules CD80 and CD86 on DCs and Macrophages. Furthermore, it inhibits monocyte differentiation into DCs and DC maturation. IL-10 actions can result in inhibition of cytokine and chemokine production(286). However, IL-10 also has very important pro-inflammatory functions. IL-10 causes B cell activation and serves as a B cell survival factor; it also promotes class switching in B cells (reviewed in (87). It also exerts its proinflammatory effects on other cells types. NK cells proliferate and produce cytokines under the influence of IL-10 and IL-10 acts as a growth factor for CD8 positive T cells (reviewed by (79) in. Importantly, Cohen et al. demonstrated in 1997 that IL-10 can rescue T cells from apoptosis *in vitro,* by Bcl-2 upregulation (287). If this finding was also confirmed *in vivo* and under inflammatory conditions IL-10 could well a role rescuing CD4 T cells from apoptosis in the context of RA directly by upregulating Bcl-2

in addition to the indirect pathway through triggering CD130 upregulation which in turn allows IL-6 anti-apoptotic signal to prevent cells death via transsignaling.

The regulation of IL-6R and CD130 genes has been studied in several cell lines. In rat hepatocytes CD126 mRNA but not CD130 is upregulated by dexamethasone. IL-1 and IL-6 can cause the downregulation of CD126 mRNA (288). In the brain, upregulation of CD126 and CD130 mRNA in selective areas of the brain was described following treatment with LPS or IL-1β(258). In line with our observation that protein levels of CD126 and CD130 are regulated differently, Falus et al showed that the gene regulation of CD126 and CD130 can be independent (259).

IL-6 signaling is not only regulated by the availability of its receptor, but also by SOCS molecules. SOCS molecules inhibit STAT-mediated signal transduction acting as negative feedback inhibitors (reviewed by (51). The expression of SOCS in RA is altered. SOCS1 is upregulated in PB T cells and SOCS3 is upregulated in PB monocytes from RA patients compared to controls. In SF macrophages the expression of SOCS 1 and 3 is high but SF T cells show no expression of SOCS (289). Therefore, SOCS deregulation in RA could contribute to excess IL-6 signaling that contributes to the overall inflammation.

Since IL-6 and IL-10 both appear to be involved in the regulation of responsiveness to IL-6 we decided to study the distribution of these two cytokines in the inflamed synovium. The expression of both of these cytokines has been described before in the rheumatoid joint (230,290) however we wanted to test how its localization within the tissue compares to IL-6 receptor expression. We observed that IL-10 seems to be located mainly the perivascular cuff areas; this is exactly the location of high CD130 expression by CD4 T cells. We are not the first group to find a detrimental role for IL-10 in the context of RA. Huizinga and coworkers found an association between IL-10 promoter polymorphism -2849 that causes high IL-10

production and predicts more rapid development of joint damage in RA (184). Stonek F *et al* identified a further IL-10 promoter polymorphism, (-1082 G/A) in the IL-10 promoter which was associated with the production of autoantibodies such as RF in RA patients (291) and finally a possible mechanism triggering IL-10 production in the RA context was revealed by Xiao's group in China, they found that heat protein 70, present in RA SF, triggers the production of IL-10 by fibroblast-like synoviocytes (292). Further evidence for a pathogenic role of IL-10 in RA derives from studies investigating recombinant IL-10 as a potential anti-inflammatory treatment in RA. There was no clear therapeutic benefit from the administration of rIL-10 in clinical trials. In a study conducted by Keystone et al RA patients were treated for 28 days with rIL-10 showing no adverse effects but also limited benefits(293). The use of rIL-10 as treatment of autoimmune disease remains controversial as it does not show benefits in various cases such as RA or Crohn's disease it has, however, been shown to be beneficial in psoriasis (reviewed by (248)). At the time, given the known anti-inflammatory properties of IL-10, the lack of efficiency was surprising. But if we consider the pro-inflammatory components of the effect of IL-10 on the immune system, it is possible that these counteract some of the anti-inflammatory properties. One of these proinflammatory properties may be the maintenance of sensitivity of T cell to IL-6 signalling by upregulation of CD126.

To explore possible mechanisms that maintain IL-10 levels high in the rheumatoid joint, is worth thinking of IL-27. IL-27 has been demonstrated to induce IL-10 secretion by CD4 T cells and it has been shown to do so in combination with TGF-β too, in process dependent on STAT1 and STAT3 activation. In the other hand IL-6 combined with TGF-β also triggers IL-10 secretion by CD4 T cells but in a STAT3 only dependent manner (294). This ability of IL-27 and IL-6 to induce IL-10 would provide a possible mechanism for the high levels of IL-10

found in the rheumatoid joint. If the production of IL-10 in RA was dependent on IL-6 signalling it would not be very high as we have shown the low expression of the IL-6R and the low responsiveness of CD4 T cells to IL-6 via direct signaling, but IL-27 could provide an alternative mechanism by with IL-10 production could be stimulated in CD4 T cells in the rheumatoid joint, IL-27 would stimulate CD4+_ T cells to produce IL-10 which in turn in our model would increase expression of CD130, making CD4 T cells responsive to IL-6 via transignaling and providing an extra mechanism to maintain IL-10 levels as IL-6 transignaling through CD130 could also result in more IL-10 production. And even if SOCS3 was activated to limit the extent of IL-6 signalling, IL-10 production induced by IL-6 and IL-10 dependent on STAT3 transcription factor would be reduced but IL-10 production induced by IL-27 through STAT1 would not be affected.

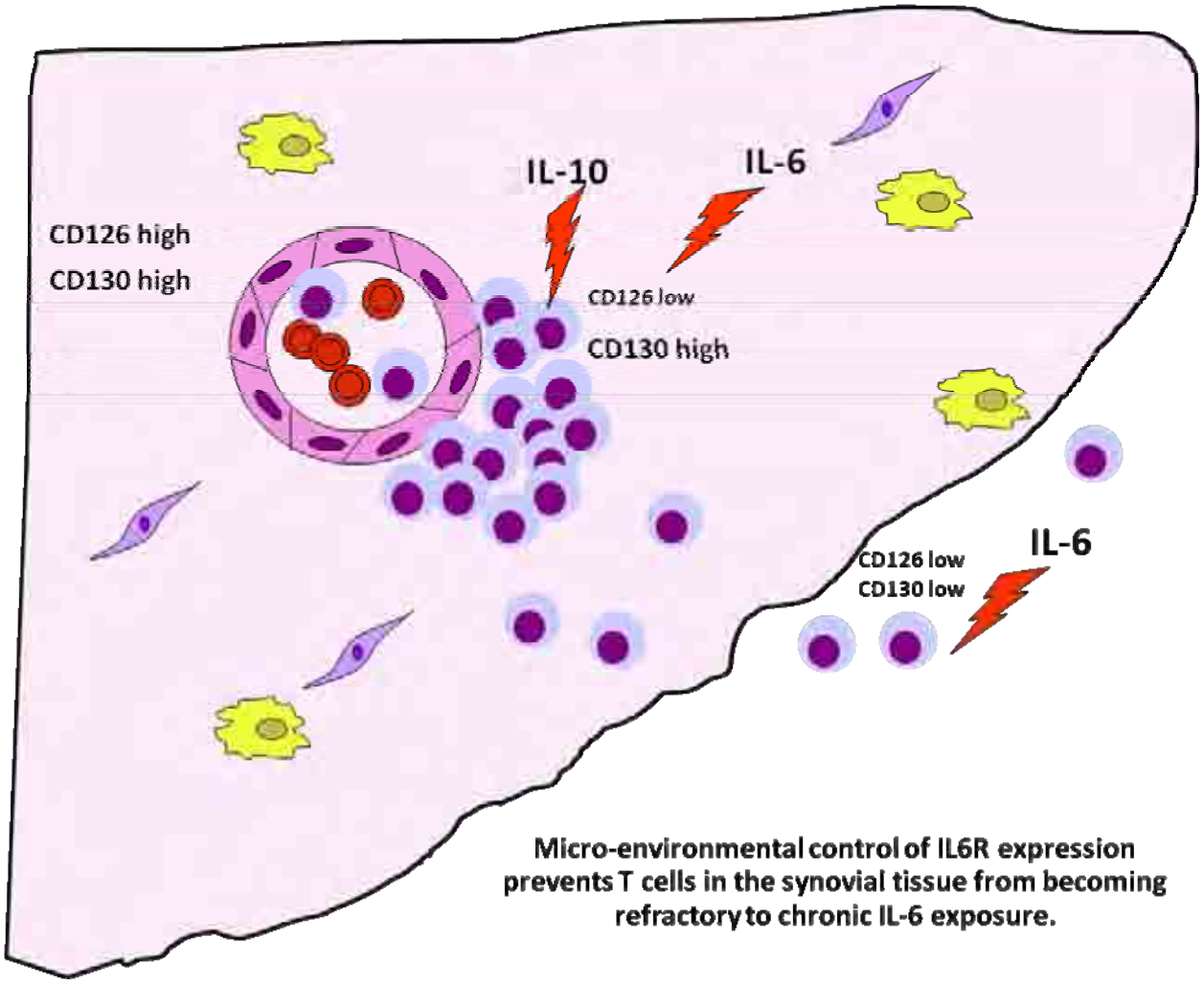


Figure 33. Model of CD130 expression and its consequences on T cells in the rheumatoid joint. This figure shows schematically the differential expression of CD130 on T cells in the different compartment of the joints. In addition shows a possible mechanism of CD130 expression being regulated by IL-10 in the RA joint.

In summary, as it can be seen in figure 33, our data suggest high expression of CD130 on CD4 T cells infiltrating the synovial tissue of the rheumatoid joint, which allows these cells to be signal by IL-6 though a process known as transsignaling. Our further investigations suggest that CD130 expression is triggered by the inflammatory micro-environment, possibly by the high presence of IL-10 in the perivascular infiltrate of the ST. We have shown that CD130 mRNA expression is likely to be locally induced and is not influenced by

transendothelial migration. All this suggests that transsignaling by IL-6/sIL-6R complexes may be an important survival and differentiation factor for T cells in the inflamed joint.

In this study we have shown that there is no difference in the expression of CD25, CD69, CD71 and HLA-DR on T cells in early versus established arthritis.

The expression of activation markers such as CD69, CD44 and HLA-DR on RA synovial T cells was previously shown by other groups (Cush and Lipsky 1230-38;Katschke, Jr. et al. 1022-32). It has been difficult to demonstrate that T cells from the rheumatoid joint are producing cytokines, even after stimulation with anti-CD3 and anti-CD28 (Simon, Seipelt, and Sieper 8562-66;Dolhain et al. 1961-69), leading to controversial discussions of their relevance in inflammation. From our activation marker data we can only conclude that T cells are not different in activation status in early and established RA, therefore the hypothesis that T cells may have different roles in the different stages of RA is no supported.

We also hypothesized that iNKT cells could be enriched in the very early stages of RA compared to established RA and therefore be a possible source SF cytokines found in very early RA, such as IL-4 and IL-13.. We showed that iNKT cells in PB are present in low numbers in all patients at similar levels to healthy controls. In SF iNKT cells are present in very low numbers in RA and early non RA at similar levels but in early RA there are virtually no iNKT cells. It is therefore unlikely that these cells make a major contribution to the cytokine profile found in the synovial fluid.

Our results suggest that the numbers of iNKT cells in PB of patients with varying disease outcomes are very similar and also similar to healthy controls. Generally, in accordance with the literature, we found lower numbers of iNKT cells in SF compared to PB. Interestingly, the frequency of iNKT cells appears to be lower in SF of patients with VEA who eventually

progressed to RA. It is not easy to draw conclusions from these results because the actual number of cells analyzed is very low. . Linsen et al described a low frequency of iNKT cells in peripheral blood of patients with established RA (295) Our own observation of normal numbers of iNKT cells disagree with this finding. Possible explanation for this could be technical, the number of cells read in the flow cytometer, as so little iNKT cells are found in the blood we had to read a large number of cells to show a population of iNKT cells, another possible explanation may be the patients used for the study, which treatments they were on and how this treatments may affect the numbers of iNKT cells in PB. An additional explanation may be the characteristic of the control group used in the study; we compared our patients with age matched controls. It seems that iNKT cells may have a pathogenic role in RA as derived from the animal and human data, iNKT cells are indeed necessary to induce RA-like disease in animal models and seems to be present in the RA joint. Intriguingly, our data shows lower numbers of iNKT cells in very early stages of RA compared to later stages of disease. With the extremely low numbers of iNKT cells found in the SF of patients with VEA, it is impossible to assess their cytokine production. If indeed iNKT cells have a pathogenic role it may be possible that iNKT cell are recruited to the joint early in RA, are activated, secrete cytokines and undergo apoptosis after stimulation and even though they could contribute to the disease they are no longer seen in the SF. On the other hand, if we hypothesize a protective role of iNKT cells in RA, lower levels of iNKT cells may support the progression of early inflammation of the joint to RA.

The most logical conclusion to be drawn from the lack of iNKT cells in SF is that these T cells subset is never recruited therefore not present and a very unlikely source of cytokines. IL-4 and IL-13, both cytokines present in SF in very early RA may be synthesized by other

cells present in the rheumatoid joint. Activated CD4 positive T cells may be a source of both IL-4 and IL-13, while CD8 T cells may be a source of IL-13.

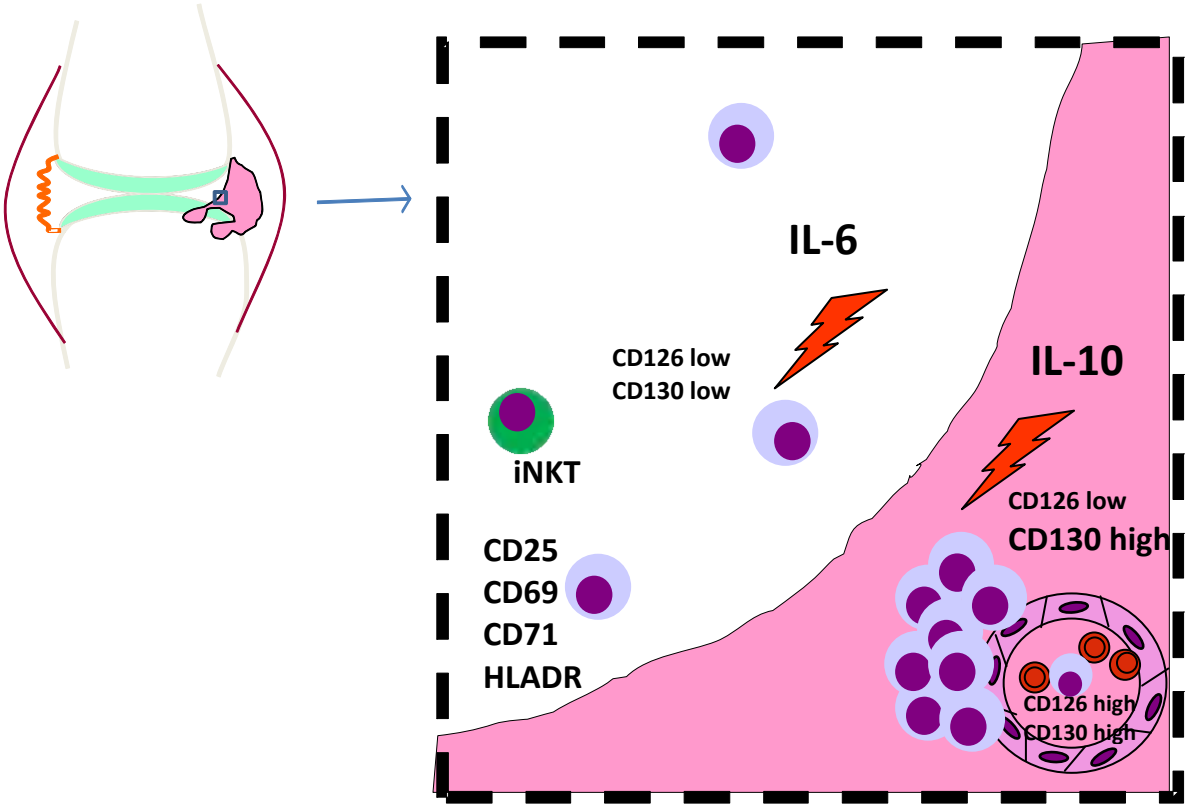


Figure 34. T cells in the RA joint.

In summary, we studied the presence of T cells subsets and T cells activation in different stages of RA, finding no significant differences in T cell subset presence or T cell activation status in early and late RA. Not supporting a different role for T cells in the different stages of RA. We also studied a possible mechanism contributing to T cell viability in RA; finding that high expression of CD130 on T cell in the rheumatoid joint caused by the local microenvironment may facilitate IL-6 transignaling and therefore T cell survival.

The IL-6 data highlights the importance of IL-6 and T cells in RA and it gives IL-10 a possible pro-inflammatory role in the context of RA. These data suggests to me that interfering with IL-6 may be a powerful tool for treatment but it is not enough to target IL-6

direct signaling but also IL-6 transsignaling through CD130, in fact selective targeting to block IL-6 transsignaling only may have added benefits, it seems that there is not a lot of IL-6 direct signaling taking place in the tissue and that many of the detrimental effects of IL-6 are mediated via transignalling. The added benefit for patients would be that IL-6 siganling would still be available to help fighting immune challenges through the acute phase response mediated by IL-6. . In addition the role of IL-10 should be looked at as the presence of IL-10 in the joint may not have any anti-inflammatory properties at all, in fact it may have a pathological role. As RA is such a multifactorial disease, in the sense that many factors are present contributing to the disease I think treatment should be equally aimed to several factors, targeting IL-6 or T cells or TNF separately may only lead to some improvement in patients whereas targeting various factors at once could be more effective so a combination of treatments targeting different cells and cytokines could be an option, always taking into account the overall immunity of the patient and not compromising his/her defense mechanism. There is still a long way to go and we have only made a tiny contribution to the puzzle that is RA but hopefully this information would be useful in the future and will contribute to the understanding of the disease.

Future Work.

To take the project forward it would be interesting to identify the source of IL-10 in the rheumatoid joint. This work is currently in progress in our team. IL-10 is part of a family of related cytokines, it will be intriguing to test whether other members of the IL-10 family of cytokines have similar effects on CD130 expression. It will be important to determine whether IL-10 can upregulate CD130 expression in other cell types. In this context particularly B Cells are interesting, as IL-10 is an important growth factor for these cells. So far we have focused our research on arthritis. However, in SLE high levels of IL-10 have been described in the serum. It would therefore be interesting to study whether this affects expression of CD130 in this disease. Furthermore the relation between IL-10 and CD130 expression so far is only associative, there is no direct proof for a link in the rheumatoid synovium. This could either be tested in animal models, by using Il-10 deficient mice or by using blocking anti IL-10 antibodies in cultures of cell suspensions derive from synovial tissue.

While the work on activation markers and iNKT cells in very early arthritis currently does not appear to be a very promising line to pursue, we are now investigating the cytokine profiles of sorted immune cell populations in early and established RA using quantitative PCR on low density arrays.

Appendix 1: List of patients used for the T cell activation and iNKT studies

Patient	Age	Gender	Symptom Duration	CCP	CRP	RF	Medication	Diagnosis
1	58	F	12 weeks	negative	21	Negative	Indomethacin	PA
2	66	F	10 weeks	negative	30	Negative	Etoricoxib	RA
3	75	F	5 months	NA	10	positive	Diclofenac	RA
4	41	F	6 weeks	positive	8	Positive	Diclofenac	RA
5	41	F	6 weeks	positive	8	Positive	Diclofenac	RA
6	66	F	NA	NA	NA	NA	Mtx	RA
7	59	F	>10 years	NA	111	Positive	Etanercept	RA
8	41	F	10 weeks	negative	0	Negative	Ibuprofen	RA
9	44	M	5 weeks	negative	12	Negative	Celecoxib	SLRA
10	72	F	>10 years	NA	33	Positive	Etanercept	RA
11	33	F	4 weeks	Negative	0	Negative	None	SLUA
12	64	F	7 weeks	positive	34	Negative	Arthrotec	RA
13	75	F	NA	NA	NA	Negative	Mtx	RA
14	63	M	10 weeks	Negative	44	Negative	Prednisolone	RA
15	75	F	8 years	NA	30	Positive	None	RA
16	59	M	8 months	Positive	11	Positive	Mtx	RA
17	51	F	2 years	Positive	NA	NA	Naproxen	RA
18	66	F	5 months	NA	36	Positive	Etodolac	RA
19	33	F	2 years	NA	21	Positive	Naproxen	RA
20	77	M	7 weeks	Negative	38	Negative	Etoricoxib	RA (self-limiting)
21	41	F	7 weeks	Negative	0	Negative	None	Unknown
22	58	F	7 years	NA	88	Positive	Mtx, ssa arthrotec	RA
23	73	M	5 years	NA	NA	NA	None	RA
24	69	M	NA	NA	NA	Positive	NA	RA
25	40	M	1 year	NA	NA	NA	Mtx	RA
26	68	F	12 weeks	Positive	77	Negative	Diclofenac	RA
27	26	F	12 weeks	Positive	20	Negative	Arthrotec	RA
28	60	M	10 weeks	Positive	63	Positive	Diclofenac	RA
29	66	M	4 weeks	NA	102	NA	Naproxen	Gout, OA
30	72	F	19 years	Positive	NA	Negative	Mtx, ssa	RA
31	68	M	11 weeks	Positive	6	Positive	Ibuprofen	RA
32	71	F	30 years	NA	NA	Positive	Meloxicam, adalimumab	RA
33	23	F	8 weeks	Negative	35	Negative	Ibuprofen	PUA
34	72	F	19 years	Positive	NA	Negative	Mtx, ssa	RA
35	53	F	30 years	NA	NA	Positive	Hydroxychloroquine	RA
36	68	F	8 weeks	Positive	15	Positive	Ibuprofen	RA
37	23	F	11 weeks	Negative	7	Negative	Ibuprofen	PUA
38	45	M	6 months	NA	NA	NA	Mtx	RA
39	36	F	6 weeks	Negative	5	Negative	Etoricoxib	PUA
40	47	M	NA	NA	NA	NA	Allopurinol, prednisolone	Gout
41	70	M	11 weeks	Negative	79	Negative	Ibuprofen	RA
42	38	F	8 weeks	Negative	0	Negative	Diclofenac	SLUA
43		M	NA	NA	NA	NA	NA	NA
44	42	F	20 years	Negative	NA	Negative	Gold, prednisolone	RA
45	36	F	NA	NA	31	NA	Mtx, leflunomide	RA
46	39	F	4 weeks	Negative	34	Negative	None	Persistent SLE
47	79	M	NA	NA	NA	NA	Prednisolone, mtx	RA
48	18	F	12 weeks	Negative	26	Negative	Etodolac	Persistent SLE
49	64	F	5 weeks	Positive	71	Positive	Etoricoxib	RA
50	46	M	3 weeks	Negative	38	Negative	None	Reactive arthritis
51	49	F	9 years	NA	NA	Positive	Diclofenac, adalimumab, leflunomide	RA
52	77	M	7 years	Positive	45	Positive	Azathioprine	RA
53	63	F	13 weeks	Positive	43	Positive	Etoricoxib	RA
54	39	M	4 years	Positive	26	Positive	Prednisolone, etanercept	RA
55	47	F	3 weeks	Negative	228	Negative	Diclofenac	Sarcoid
56	36	M	3 weeks	Negative	0	Negative	Ibuprofen	SLUA

57	50	M	4 weeks	Positive	26	Positive	Diclofenac	RA
58	72	F	4 months	Positive	91	Positive	Prednisolone	RA
59	23	M	3 weeks	Negative	0	Negative	Ibuprofen	Persistent PA
60	49	M	1 year	Positive	10	Positive	Mtx, ssz	RA
61	56	F	4 years	Positive	9	Positive	Nabumetone, prednisolone, adalimumab, leflunomide	RA
62	63	F	11 weeks	Negative	27	Negative	Doclofenac	NA
63	50	F	5 weeks	Positive	77	Positive	NA	RA
64	40	M	5 years	Positive	35	Positive	Prednisolone, etanercept	RA
65	64	M	6 weeks	Negative	15	Negative	None	SLUA
66	75	F	NA	Positive	77	Positive	Adalimumab, prednisolone	RA
67	56	F	NA	Positive	3	Positive	NA	RA

Appendix 2: list of patients used for the CD130 studies

Patient	Age	Gender	RF	medication	Diagnosis
1	72	F	NA	Sulphasalazine	RA
2	74	F	Positive	None	RA
3	69	F	Positive	Sulphasalazine	RA
4	71	F	NA	Mtx	RA
5	71	F	NA	NA	RA
6	61	F	Positive	NA	RA
7	57	F	Positive	MTX	RA
8	57	F	Positive	None	RA
9	43	F	Positive	None	RA
10	36	F	Positive	Mtx, infliximab	RA
11	76	M	NA	Pred	RA
12	72	M	NA	None	RA
13	76	M	NA	Pred	RA
14	73	F	NA	Azathioprine	RA
15	62	F	NA	Pred	RA
16	62	F	NA	NA	OA
17	77	M	Positive	Nabumetone leflunomide	RA
18	73	M	NA	None	RA
19	53	F	Positive	Hydroxychloroquine	RA
20					RA
21	38	M	Positive	Prednisolone, etanercept	RA
22	67	F	Positive	Etanercept, mtx	RA
23	66	M	Positive	Mtx	RA
24	70	F	Positive	Mtx	RA
25	53	F	Negative	Meloxicam, sulfasalazine	RA
26	52	M	Positive	Mtx, ssa, ohchl, pred 10, diclofenac	RA
27	57	F	Positive	Mtx, ssa, arthrotec	RA
28	58	F	Positive	Mtx, ssa, arthrotec	RA
29	49	M	Positive	None	RA
30	77	M	Positive	Azathioprine	RA
31	NA	F	NA	NA	RA
32	39	M	Positive	Prednisolone, etanercept	RA
33	19	F	Positive	ibuprofen	RA
34	60	M	Positive	Doclofenac	RA
35	82	M	Positive	Arthrotec	RA
36	79	M	NA	Prednisolone, mtx	RA
37	36	F	NA	Mtx, leflunomide	RA
38	40	F	Positive	Doclofenac, adalimumab, leflunomide	RA
39	19	F	Positive	Ibuprofen	RA
40	73	F	Positive	Prednisolone	RA
41	33	F	Positive	Naproxen	RA
42	71	F	Positive	NA	RA
43	67	F	Positive	Etanercept, mtx	RA
44	78	F	Positive	Sulphasalazine	RA
45	NA	F	Negative	None	Psoriatic Arthritis

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