Aalborg Universitet

AALBORG UNIVERSITY
DENMARK

FoxP3 mRNA splice forms in arthritis patients

Ryder, Lisa Rebekka

FoxP3 mRNA splice forms in arthritis patients

PhD thesis by L. Rebekka Ryder, MSc

The Parker Institute, Frederiksberg Hospital

and

Center for Sensory-Motor Interaction, Department of Health Science and Technology,

Aalborg University

Denmark 2011

Preface

This thesis is submitted to obtain the PhD degree at University of Aalborg. My main supervisor was Henning Bliddal, professor, MD, DMSc, The Parker Institute, Frederiksberg Hospital, and my co-supervisor was Thomas Graven-Nielsen, PhD, Associated Professor, Laboratory for Experimental Pain Research, Center for Sensory-Motor Interaction, Aalborg University.

The majority of the laboratory work on which this thesis is based has been carried out from June 2007 to August 2008 at the Parker Institute, Frederiksberg Hospital and the Tissue Typing Laboratory, Department of Clinical Immunology, Rigshospitalet. Additionally, I collected paired samples of blood and synovial fluid from February 2005 to January 2007, and Tove Riis Johannesen collected blood samples from patients before and during treatment with Etanercept, Adalimumab or Infliximab from April 2001 to November 2004.

This thesis is based on the following manuscripts:

I. **L. Rebekka Ryder**, Anders Woetmann, Hans O. Madsen, Niels Ødum, Lars P. Ryder, Henning Bliddal, Bente Danneskiold-Samsøe, Søren Ribel-Madsen, and Else Marie Bartels.
    Expression of full length and splice forms of FoxP3 in rheumatoid arthritis. (Ryder 2010)
    Scandinavian Journal of Rheumatology (2010), 39(4):279-86. (DOI: 10.3109/03009740903555374)

II. **L. Rebekka Ryder,** Else Marie Bartels, Anders Woetmann, Hans O. Madsen, Niels Ødum, Henning Bliddal, Bente Danneskiold-Samsøe, Søren Ribel-Madsen, and Lars P Ryder.
    FoxP3 mRNA splice forms in synovial CD4+ T cells in rheumatoid arthritis and psoriatic arthritis. (Submitted 2011)

III. **L. Rebekka Ryder**, Lars P. Ryder, Else Marie Bartels, Anders Woetmann, Hans O. Madsen, Niels Ødum, Bente Danneskiold-Samsøe, Søren Ribel-Madsen, and Henning Bliddal
    Differential effects of decoy receptor- and antibody-mediated TNF blockage on FoxP3 expression in responsive arthritis patients. (Submitted 2011)

    Additionally: Supplementary experiments using DNA Microarray technology.
    Gene expression profiling of paired samples of synovial and blood CD4+ T cells.

References to these publications in the text are indicated by their roman numbers given above.

Acknowledgement

I would like to thank everyone who helped me or in any way contributed to the completion of this thesis. Especially, I would like to thank Henning Bliddal and Bente Danneskiold-Samsøe, The Parker Institute, for their encouragement and support, for recruiting patients to my project, and for giving me the chance.

I owe great thanks to Tove Riis Johannesen for invaluable technical assistance especially regarding cell work, and for not retiring before finishing my samples. At the Tissue Typing Laboratory, Department of Clinical Immunology, Rigshospitalet, I thank Lars P. Ryder for his involvement from the beginning of the project, his support and enthusiasm and for numerous scientific discussions, and Hans O. Madsen for help in designing the Taqman systems, sequencing, and technical discussions. I am thankful to Else Marie Bartels for her enthusiastic support and proof reading, and Niels Ødum and Anders Woetmann for their encouragement, inspiration and collaboration on the FoxP3 project.

I am appreciative of everyone at the laboratory at Parker: Eva Jørgensen, Inger Wätjen, Else Marie Bartels, Søren Ribel-Madsen, as well as all my colleagues at the Parker Institute. Also I am grateful to everyone at ultrasound Henning Bliddal, Bente Danneskiold-Samsøe, Søren Torp-Pedersen, Etienne Qvistgaard, Lene Terslev, Jette Bing and Lars Juul for providing me with samples of joint fluid for the study, to Mette Gad for her help in recruiting patients and being my eyes and ears at the clinic, and to Christian Cato Holm for helping me with my database. I would also like to thank Lone Frier Bovin, Ingrid Alsing, Søren Ulrik Sønder, and Klaus Rieneck for their helpfulness, as well as Arne Borgwardt, Lotte Borgwardt, Klaus Harager, and Birgit Borggaard. Furthermore, I would like to thank all the patients who participated in this study.

In November 2006 and May 2007 I was fortunate enough to spend some time at the Center of Experimental Rheumatology, Department of Rheumatology and Institute of Physical Medicine, University Hospital Zurich in Switzerland. I am grateful for the opportunity to work in an international laboratory, and I would like to thank Haiko Sprott, Valeriu Toma, and Steffen Gay for making my stay an enjoyable one.

Most of all I would like to thank my husband Rasmus and my parents for their never ending support and for believing in me. And finally I would like to dedicate this thesis to Rasmus.

L. Rebekka Ryder, København 2011

Table of contents

Abstract

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease leading to destruction of joint tissue. Malfunction of regulatory T cells (Treg) has been implicated in a number of diseases including RA, and several studies indicate an increased presence of Tregs in synovial fluid (SF) from RA patients. The marker best defining Treg cells is FoxP3, and two low molecular splice forms, FoxP3Δ2 and FoxP3Δ2Δ7, have been identified lacking exons 2 and 7. FoxP3 is an intracellular transcription factor involved in the development and function of Tregs. The mechanism of function and the importance of the splice forms are not fully understood.

The major objective of this PhD project was to examine the expression of Treg relevant molecules CD4, CD25, CTLA-4 and the different splice forms of FoxP3 in both peripheral blood (PB) and SF CD4+ T cells from RA patients compared to PB CD4+ T cells from healthy controls (HC), and to study the effect of anti-TNF drugs on the expression of the splice forms in PB CD4+ T cells. A quantitative real-time PCR method was developed to measure the amount CD4, CD25, and CLTA-4, as well as full length and low molecular splice forms of FoxP3 mRNA.

In a study including 50 RA patients and 10 HC an increase in expression of FoxP3fl and CD25 mRNA was observed in RA when compared to HC, but no corresponding increase in CTLA-4. FoxP3Δ2 was slightly increased in patients compared to HC, and was more abundant than FoxP3fl in almost all our patients and in every HC. However, the increase of FoxP3Δ2 was less pronounced than that of FoxP3fl, indicating that FoxP3fl is more relevant in relation to the immunopathogenesis of RA. FoxP3Δ2Δ7 was barely detectable in patient samples and not at all in HC. These data suggested an increase in Treg cells with a compromised expression of CTLA-4 in RA patients. Since CTLA-4 is thought to be important for contact-dependent suppression by naturally occurring Tregs (nTreg), we propose that the nTregs are unable to suppress the ongoing inflammation due to CTLA-4 deficiency. However, based on our data we cannot exclude the possibility of an increase in Treg cells induced in the periphery (iTreg) rather than dysfunctional nTregs.

In a second study involving paired samples of PB and SF from 17 RA patients we observed a large increase of 21-fold in expression of FoxP3fl in SF and a 6-fold increase in PB compared to HC. When comparing the SF to the paired PB sample from the same individual, the difference in expression between PB and SF appeared to be ranked according to sub-diagnosis: RApos < RAneg < PsA, reflecting a high expression in both PB and SF from RApos patients. This observation was interpreted as a reflection of an involvement of blood as part of the systemic nature of this disease and supports previous reports of an accumulation of Tregs in inflamed joints of RA patients. Notably, the FoxP3fl expression in SF CD4+ T cells was highly increased in all patient groups indicating that it is a common feature of arthritis and not linked to the aetiology of the arthritis in question. Additionally, a gene expression analysis using DNA Microarray was performed on six paired samples (12 arrays) of PB and SF CD4+ T cells form three RAneg and three PsA patients. The array data indicated that the increase in expression of FoxP3 and CD25 mRNA was not due to the presence of activated T cells.

In a third study 45 RA patients were followed during 12 weeks of anti-TNF treatment, and the data suggest that the TNF decoy receptor Etanercept and the antibody based therapies Adalimumab and Infliximab differ in their

effect on FoxP3 expression in responsive patients. Only the decoy receptor Etanercept had an influence on the expression of Treg-relevant markers and reduced the expression of FoxP3fl and CD25 in the responders, though no normalization was achieved. This may indicate a reduction in the number of Treg cells. As Etanercept binds both TNF-α and Lymphotoxin (LT-α) while the antibodies only target TNF-α, one can speculate that LT-α regulates FoxP3 expression in a subset of RA patients. Further it may be speculated that individual patients may experience different treatment effect depending on whether the TNF receptor ligand is mainly of monocyte/macrophage origin (TNF-α) or T cell origin (LT-α), perhaps adding to the disease heterogeneity. Overall, however, the present findings support the view that anti-TNF treatment is mainly symptomatic and not a cure for RA.

Dansk resumé

Leddegigt (reumatoid artritis, RA) er en autoimmun inflammatorisk sygdom, der fører til ødelæggelse af vævet i leddene. Funktionssvigt af regulatoriske T celler (Tregs) har været impliceret i en række sygdomme, herunder RA, og flere undersøgelser tyder på en øget tilstedeværelse af Tregs i synovialvæsken (SF) fra RA patienter. Den markør, der bedst definerer Treg celler, er FoxP3. FoxP3 er en intracellulær transkriptionsfaktor involveret i udviklingen og funktionen af Tregs, og to lavmolekylære splejsningsformer der mangler exon 2 og 7, FoxP3Δ2 og FoxP3Δ2Δ7, er blevet identificeret. Funktionsmekanismen og vigtigheden af spliceformerne er ikke klarlagt.

Formålet med dette PhD projekt var at undersøge ekspressionen af de Treg relevante markører CD4, CD25 og CLTA-4 samt de forskellige FoxP3 splejseformer i CD4+ T celler fra både blod og SF fra RA patienter og sammenligne disse med CD4+ T celler fra blod fra raske kontrolpersoner (HC). Yderligere var det formålet at undersøge effekten af anti-TNF gigtmedicin på ekspressionen af FoxP3 splejseformer i CD4+ T celler fra blod fra RA patienter i behandling. En kvantitativ real-time PCR metode blev udviklet for at måle mængden af CD4, CD25 og CTLA-4 samt fuldlængde (FoxP3fl) og de lavmolekylære splejseformer af FoxP3 mRNA.

I et studie med 50 RA patienter og 10 HC blev der observeret en stigning i ekspressionen af FoxP3fl og CD25 mRNA i CD4+ T celler fra blod fra patienterne i forhold til de raske kontroller, men vi så ingen tilsvarende forøgelse af CTLA-4 mRNA hos patienterne. FoxP3Δ2 mRNA var lidt øget hos patienter, sammenlignet med HC, og var til stede i større mængder end FoxP3fl hos de fleste af patienterne og i alle HC. Men stigningen i FoxP3Δ2 var mindre udtalt end FoxP3fl, hvilket indikerer, at fuldlængde FoxP3 er mere relevant i forhold til patogenesen af RA. FoxP3Δ2Δ7 kunne knapt detekteres i patientprøverne, og slet ikke hos HC. Disse data tyder på en stigning i antal Treg celler med en kompromitteret ekspression af CTLA-4 hos RA patienter. Eftersom CTLA-4 menes at være vigtig for de naturligt forekommende Tregs (nTreg) cellekontaktafhængige inhibering, foreslås det, at nTregs ikke er i stand til at inhibere den igangværende inflammation pga CTLA-4 mangel. Baseret på de foreliggende data kan muligheden for tilstedeværelsen af et øget antal Treg celler induceret i periferien (iTreg) snarere end dysfunktionelle nTregs ikke udelukkes.

I et andet studie med parrede prøver af blod og SF fra 17 RA patienter blev der observeret en stor stigning i ekspressionen af FoxPfl på 21-fold i SF og en 6-dobling i PB i forhold til HC. Når man sammenligner ekspressionen i SF med den parrede blodprøve fra samme individ, ser forskellen ud til at være rangordnet efter subdiagnose: RApos < RAneg < PsA hvilket afspejler en høj ekspression i både blod og SF hos RApos patienter. Denne observation tolkes her som en afspejling af, at den systemiske karakter af denne sygdom også involverer blodet, og den støtter tidligere rapporter om en ophobning af Tregs i inflammerede led hos RA patienter. Navnlig den voldsomt forøgede ekspression af FoxP3fl i SF CD4+ T celler hos alle patientgrupper antyder, at dette er et fælles træk ved RA og ikke er knyttet til ætiologien af den specifikke subdiagnose. Derudover blev en genekspressionsanalyse ved brug af DNA Microarray udført på seks parrede prøver (12 arrays) af blod og SF CD4+ T celler fra tre RAneg og tre PsA patienter. Fra disse array data kan det konkluderes, at stigningen i ekspression af FoxP3 og CD25 ikke skyldtes tilstedeværelsen af aktiverede T celler.

I et tredie studie blev 45 RA patienter fulgt under 12 ugers anti-TNF behandling, og data tyder på, at TNF

decoy receptoren Etanercept og de antistofbaserede behandlinger Adalimumab og Infliximab er forskellige i deres effekt på FoxP3 ekspressionen i de responderende patienter. Kun decoy receptoren Etanercept påvirkede ekspressionen af Treg relevante markører og reducerede ekspressionen af FoxP3fl og CD25 i de responsive patienter, selvom ingen normalisering blev opnået. Dette indikerer en reduktion i antallet af Treg celler. Eftersom Etanercept binder både TNF-α og lymphotoxin-α (LT-α), mens antistofferne kun binder TNF-α, kan man overveje om LT-α påvirker FoxP3 ekspressionen i en delmængde af RA patienterne. Yderligere kan man spekulere om enkelte patienter kan opleve forskellig virkning af behandlingen, afhængig af om TNF receptor liganden hovedsageligt er af monocyt/makrofag oprindelse (TNF-α) eller T celle oprindelse (LT-α). Overordnet set støtter de foreliggende resultater det synspunkt, at anti-TNF behandling primært er symptomatisk og ikke en helbredelse for RA.

List of abbreviations

Anti-CCP	Anti-cyclic citrullinated protein antibodies
CD	Cluster of differentiation
CRP	C-reactive protein
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DAS28	Disease activity score 28 based on CRP (mg/L)
FoxP3	Forkhead box protein 3
FoxP3fl	Full length FoxP3 mRNA
FoxP3Δ2	FoxP3 mRNA lacking exon 2
FoxP3Δ2Δ7	FoxP3 mRNA lacking exon 2 and 7
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HC	Healthy controls
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
iTreg	Treg induced in the periphery
LT-α	Lymphotoxin-α
MNC	Mononuclear cells
MonoA	Monoarthritis
mRNA	Messenger RNA
MTX	Methotrexate
nTreg	Naturally occurring Treg
PB	Peripheral blood
PCR	Polymerase chain reaction
PsA	Psoriatic arthritis
QRT-PCR	quantitative real-time RT-PCR
RA	Rheumatoid arthritis
RAneg	Seronegative RA
RApos	Seropositive RA
SF	Synovial fluid
TCR	T cell receptor
Th1	T helper 1 cell
Th2	T helper 2 cell
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell

Short Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with autoimmune manifestations (Aletaha 2010). RA is characterised by inflammation in multiple joints leading to destruction of joint tissue and, consequently, serious impairment of joint function. The inflammation in the joint capsule of RA patients is often accompanied by an increased amount of synovial fluid (SF) enriched with cells (Feldmann 1996). Among these cells are regulatory T cells (Tregs) which are engaged in the maintenance of immunological self-tolerance by direct or indirect suppression of self-reactive T and B cells (Fontenot 2003; Hori 2003; Shevach 2006; von Boehmer 2005). A paradoxical accumulation of Tregs has been reported both in a number of tumours (Yu 2006), and at sites of inflammation such as the synovial fluid of RA patients (Cao 2004; Raghavan 2009), and malfunction of Tregs may play a role in the pathogenesis of RA (Cao 2006; Flores-Borja 2008), making Treg cells an interesting topic for further research. Are they different in RA? Why do they not suppress the ongoing inflammation?

A relatively new and very expensive therapy of RA consists of targeted intervention against processes in the immune system, most often by blockade of proinflammatory cytokines, e.g. TNF-α (Doan 2005; Fox 2000). Treatment with these drugs is limited to patients who are not responding sufficiently to other treatments. Only about two thirds of the patients achieve an acceptable effect of the treatment (Papagoras 2010) and ideally, patients should be screened in advance to sort out who will benefit the most from the treatment. The personal, social, and economic consequences of RA are enormous (Albers 1999) as the patients live with pain and disabilities affecting their quality of life, functional capacity and working ability. Many questions still remain unanswered regarding the pathogenesis of RA - the origin of the disease and the chain of events leading to the disease - how is joint inflammation initiated? Why does it persist and become destructive? Is specific treatment possible? Currently there is no cure for RA, and it is therefore important to keep studying the disease mechanism in order to optimise and possibly individualize treatment for the patients.

….

Aim

The overall aim of this study was to gather information on the role of key factors in the pathogenesis of RA in order to improve the molecular understanding of the disease.

The specific objectives of the present study were:

- To establish a quantitative real-time RT-PCR method to measure the relative amount of Treg-related mRNA including alternative splice forms of FoxP3 as well as CD4, CD25 and CTLA-4 mRNA.
- To compare the presence of alternative splice forms of FoxP3 mRNA in blood CD4+ T cells from RA patients and healthy controls.
- To investigate the presence of alternative splice forms of FoxP3 mRNA in CD4+ T cells in synovial fluid from RA patients and compare the results to paired samples of peripheral blood CD4+ T cells.
- To clarify the effect of anti-TNF drugs on the expression of the different splice forms of FoxP3 mRNA in RA patients.
- To investigate if the patients who did benefit from the anti-TNF drugs displayed a different expression pattern of FoxP3 mRNA splice forms during therapy.
- To correlate the relative amount of the different splice forms of FoxP3 mRNA to disease activity and clinical parameters.

On the hypotheses that:

i) A difference in the distribution of FoxP3 mRNA isoforms reflects selective recruitment and activation of Tregs.

ii) The expression profile of FoxP3 mRNA isoforms in peripheral blood Tregs differs in arthritis patients when compared to healthy controls.

iii) The expression profile of FoxP3 mRNA isoforms in peripheral blood Tregs is normalized after 12 weeks of anti-TNF treatment.

iv) We are able to identify patients who will benefit the most from anti-cytokine drugs based on its effect on the expression of FoxP3 mRNA isoforms present in Treg cells in peripheral blood during therapy.

v) The distribution of FoxP3 mRNA isoforms in synovial fluid of all three types of arthritis is abnormal compared to the blood counterpart and specifically to that of peripheral blood of healthy controls.

Background

Rheumatoid arthritis and psoriatic arthritis

Rheumatoid arthritis (RA) is a chronic disease affecting 0.5-1 % of the adult population – women more frequently than men (Aletaha 2010). RA is a systemic autoimmune disease, characterised by inflammation of the synovial joints ultimately leading to erosion and destruction of cartilage and bone, causing pain and disability. RA primarily affects the joints, but other organs and tissues such as skin, lungs, and the cardiovascular system are also known to be affected. The patients have increased mortality rate from cardiovascular disease, infection, pneumonia, and non-Hodgkin's lymphoma (Naz 2007; Sokka 2008).

There is no definite diagnostic test for RA, and the diagnosis is based on clinical symptoms, serological changes, and imaging. The current study used the 1987 American College of Rheumatology criteria for RA (Arnett 1988), shown in table 1, which have been criticized for their lack of sensitivity in early disease. A newer set of criteria, the 2010 Rheumatoid Arthritis Classification Criteria, have in the meantime been introduced (Aletaha 2010), focusing on diagnosis at earlier stages of disease rather than defining the disease by its late-stage features (Aletaha 2010). In the papers this thesis is based on, the patients all had established diagnoses of seropositive (RApos) or seronegative (RAneg) RA based on the 1987 classification criteria and the presence or absence of rheumatoid factor, or the differential diagnoses psoriatic arthritis (PsA) or monoarthritis (MonoA).

Table 1. The 1987 American College of Rheumatology revised criteria for the classification of RA. At least four criteria must be satisfied for classification as RA.

Criteria	Definition
Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour
Arthritis of > 3 joint areas	Soft tissue swelling or fluid in at least 3 of 14 possible areas: right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
Arthritis of hand joints	Swelling of the wrist, MCP, or PIP joint
Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
Rheumatoid nodules	Subcutaneous nodules present
Rheumatoid factor (RF)	Abnormal concentration of RF as detected by a method that produce positive results in < 5% of healthy controls
Radiographic changes	Erosions or unequivocal bony decalcification localised to the joints of the hand and wrist

PIP: proximal interphalangeal; MCP: metacarpophalangeal; MTP: metatarsophalangeal

RA is a heterogeneous disease which may be divided into two major subgroups based on the presence or absence of autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated protein antibodies (anti-CCP) (Nishimura 2007; Quinn 2005). Seronegative RA (RAneg) is characterised by less systemic manifestations, and a better prognosis than seropositive RA (RApos) (da Mota 2009). Anti-CCP are more specific than RF for diagnosing rheumatoid arthritis and may better predict erosive disease (Nishimura 2007; Quinn 2005). In this thesis, however, RApos refers to the presence of RF only and not anti-CCP. The correlation between the

concentrations of the two autoantibodies can be seen in figure 1.

It is obvious by the distribution, that the two tests correlate, however, due to defined reference ranges, some tests are positive in one while negative in the other. Of the 42 patients (those included in paper I and III), represented in figure 1, only five were anti-CCP positive *and* RF negative.
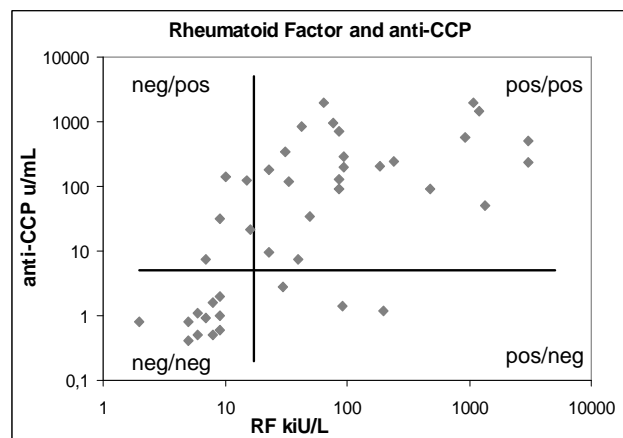
Figure 1. The correlation between RF and anti-CCP. Data from 42 patients (from paper I and III), where actual numbers were available around the time of sample collection, are shown. The lines differentiate between positive and negative samples at 17 kiU/L for RF and 5 kU/L for anti-CCP, respectively.

Psoriatic arthritis (PsA) (Veale 1994) is another joint disease that resembles RAneg regarding the absence of RF and anti-CCP. Where RA can affect other organs and tissues such as skin, lungs, and the cardiovascular system, psoriasis primarily affects the skin, but give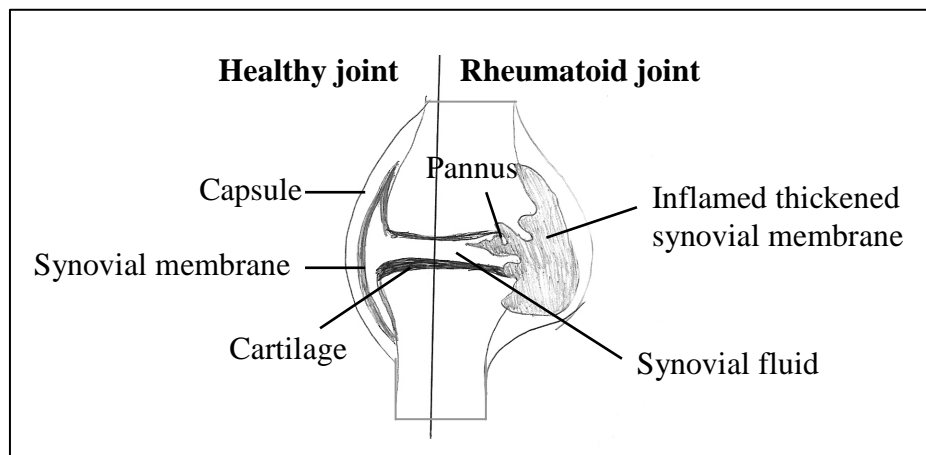s rise to joint disease, PsA, in up to 30% of the patients. On average PsA appears about 10 years after the first signs of psoriasis. The classification criteria for PsA are: clinical evidence of joint inflammation involving at least one joint for more than 3 months; the presence of at least one psoriatic skin lesion and/or dystrophy defined as > 20 nail pits and/or onycholysis; seronegative for RF. If the patient is seropositive, he or she is considered to have RA with psoriasis (Veale 1994). However, despite these differences all three types of arthritis present themselves with similar inflammatory reactions in the joints and may be difficult to distinguish clinically from each other (Quinn 2005). PsA patients are given a similar treatment to RA patients with anti-TNF drugs.

The aetiology of RA is unknown and there are indications of both genetic and environmental factors of importance for the development of RA. The main genetic risk factor for RA is "the shared epitope" of the human MHC class II, HLA-DR (Gregersen 1987), but since women are affected three times as often as men, estrogens may also be associated with increased risk of RA. The genetic association with HLA-DR suggests a classical immunological mechanism involving CD4+ T cells at some stage in the pathogenesis of RA. The environment plays a role, and the main environmental risk factor for RA is smoking (Costenbader 2006; Padyukov 2004), but also infectious agents may be a factor (Kobayashi 2008). Certainly, the low concordance rate of 15% between identical twins (Silman 1993) suggest that environmental factors are the major determinants for the onset of disease. On the other hand, the concordance rate of 4% in dizygotic twins (Silman 1993) shows that genetic factors do contribute.

In figure 2 a schematic picture of a healthy and an inflamed joint is presented. In a healthy joint the amount of SF is usually very small and contains few cells, if any, while the RA joint often contains increased volume of SF

enriched with cells, predominantly neutrophils and granulocytes, but also CD4+ T cells, B cells, macrophages and synoviocytes (Feldmann 1996).

Synovial fluid is routinely aspirated guided by ultrasound at the Parker Institute (Bliddal 2000; Qvistgaard 2001; Terslev 2003). There is an increase in cellularity of the synovial membrane, where the thickness is increased from 1-2 cells to 6-8 cells comprised mostly of activated macrophages with an underlying layer of fibroblast-like synoviocytes. This expansion of synovial membrane forms a structure called pannus that penetrates the cartilage and invades the subchondral bone, causing the subsequent erosion of these tissues (Feldmann 1996). This process occurs under the influence of a large number of cytokines. The micro-environment in the inflamed synovium of RA patients has been compared to that of tumours with regards to cytokine and chemokine profiles, and to neoangiogenesis in the formation of pannus tissue (Rudolph 2005). In PsA there is an increased vascularity of the skin, and angiogenesis is a prominent event. T lymphocytes are the most common inflammatory cells in the skin and joints of PsA.

Tumour necrosis factor (TNF) has proven to be a key player in the autoimmune reaction. It is considered to be of major importance in arthritis and is a therapeutic target (Doan 2005; Feldmann 1996; Fox 2000; Rubbert-Roth 2009). The TNF family of cytokines include TNF-α and lymphotoxin-α (LT-α) both binding and signalling through the same receptors TNFR1 and TNFR2. TNF-α is a pleiotropic cytokine with multiple actions, and in arthritis TNF-α induces e.g. the production of other pro-inflammatory cytokines such as IL-1 and IL-6 and chemokines. It also induces the expression of matrix metalloproteinases and prostaglandin E_2 by synoviocytes and activates osteoclasts, resulting in destruction of cartilage and bone resorption (Brennan 2008; Fox 2000). Furthermore, TNF-α activates the T cells, enhancing T cell infiltration, and augments angiogenesis and the proliferation of keratinocytes in psoriatic plaques (Veale 2005). The receptors TNFR1 and TNFR2 occur in various combinations in different tissues, and the binding of TNF-α to cell surface TNFR1 can also initiate signalling pathways that lead to apoptosis or NF-κB activation (Tracey 2008). The importance of TNF-α is highlighted by the success of anti-TNF drugs (Taylor 2009). The relative roles of TNF-α and LT-α are not completely elucidated.

The first approved biologic agents for the treatment of RA were inhibitors of TNF-α, and the first three drugs available of this kind were Enbrel/Etanercept, Remicade/Infliximab and Humira/Adalimumab. The RA

patients in paper III were treated with one of these three different anti-TNF drugs. All three drugs bind to TNF-α preventing it from activating TNF-α receptors: Remicade/Infliximab is a mouse-human chimeric antibody, and Humira/Adalimumab is a chimeric antibody resembling Infliximab, but it contains only human components (Taylor 2009). Enbrel/Etanercept is a TNF receptor 2-IgG fusion protein, a decoy receptor for both TNF-α and LT-α, and contains only human components. Cytokines other than TNF-α have been introduced as therapeutic targets: Anakinra is a recombinant form of a naturally occurring IL-1 receptor antagonist, and Tocilizumab is a chimeric monoclonal antibody blocking IL-6 mediated signal transduction (Taylor 2009). Also, other types of therapies have emerged: Rituximab is a monoclonal antibody directed against CD20+ B cells. It induces transient depletion of (auto-) antibody production and co-stimulatory B cells (Papagoras 2010). Abatacept is a CTLA-4-IgG fusion protein and functions as a selective inhibitor of T-cell co-stimulation by blocking the interaction between CD28 and the antigen presenting cell (Papagoras 2010).

The Immune System and RA - *a brief and selective introduction*

The immune system consists of a myriad of different cell populations and soluble factors, each participating in maintaining an effective immune response to infectious agents. It is a common belief that dys-regulated T cells are involved in RA based on the well-known HLA association. Additionally, non-cellular agents such as autoantibodies, complement factors, and lectins are also involved in the pathogenesis of RA (Okroj 2007). Furthermore, microRNAs have recently been implicated in autoimmunity and diseases such as RA (Furer 2010; Tili 2008). MicroRNAs are small RNA molecules that regulate gene expression by binding to target mRNAs and prevent translation of the protein product by mechanisms involving antisense microRNA-mRNA interaction and subsequent cleavage of the RNAs (Furer 2010; Tili 2008).

T cells comprise subsets of lymphocytes developed in the thymus, where the T cell receptor repertoire is formed by positive and negative clonal selection. The T cell receptor of the CD4+ T cells reacts to peptide fragments presented by MHC class II on the surface of an antigen presenting cell. The CD4+ T cell population may be further subdivided into T helper 1 (Th1) and T helper 2 (Th2) cells with somewhat different biological activities: The Th1 cells activate macrophages, produce a number of pro-inflammatory cytokines, and stimulate B cells to produce antibodies mainly IgG and IgA; while Th2 cells produce anti-inflammatory cytokines and stimulate B cells to produce antibodies, especially switching to IgE (Murphy 2008). RA has traditionally been viewed as a Th1-driven disease with a relative predominance of the Th1 cytokine IFN-γ over IL-4, although neither Th1 nor Th2 cytokines are present in large amounts in SF (Raza 2005). Th17 cells are a new subset of CD4+ T helper cells that have been implicated in immune-mediated tissue injury such as in the synovium in RA (Steinman 2007). The CD4+ Th17 cells stimulate chondrocytes and osteoclasts, and activates fibroblasts to secrete chemokines (Lubberts 2004; Lundy 2007).

In order to avoid autoimmune reactions the immune system must be able to eliminate or inactivate auto-reactive cells. Central T cell tolerance is established when T cells specific for self antigens are eliminated in the thymus. Occasionally self-reactive T cells may escape deletion in the thymus and enter the periphery. Peripheral T

cell tolerance is acquired by mature lymphocytes in the peripheral tissues by cellular inactivation through weak signalling without co-stimulus (Murphy 2008). Activated effector T cells specific for self peptide:MHC complexes can cause local inflammation by activating macrophages or can damage tissue cells directly. Diseases in which these actions of T cells are likely to be important include RA (Cao 2006). A further level of peripheral tolerance is obtained through the function of regulatory T cells (see later).

Models for the role of T cells in arthritis

T cells clearly play a central role in joint inflammation in arthritis, but whether T cells are involved in initiation of the disease process, or are secondary to the inflammatory response originating within the joints, is unknown. It is also unknown whether the antigen is "an autoantigen (whether specific to the joints or ubiquitous), a highly conserved foreign protein cross-reacting with its human homolog, or a neo-antigen expressed as a result of posttranslational events" (Fournier 2005).

Witebsky's postulates revisited by Rose in 1993 may be used as the defining criteria for autoimmune diseases in human, and they include "direct evidence from transfer of pathogenic antibody or pathogenic T cells; indirect evidence based on reproduction of the autoimmune disease in experimental animals; and circumstantial evidence from clinical clues" (Rose 1993). For obvious reasons, direct evidence as human-to-human transfer of pathogenic T cells is not possible to come by. Therefore an updated version of the postulates require that "an autoimmune reaction is identified in the form of autoantibody or cell-mediated immune reaction; the corresponding antigen is known; and an analogous response causes a similar disease in experimental animals" (van Gaalen 2005). Several animal models of arthritis, such as collagen-induced arthritis or adjuvant arthritis in rodents, exists and are T-cell dependent (Firestein 2003; Fournier 2005). There is general agreement that T cells play a role in arthritis, leaving the corresponding antigen to be identified. Several potential candidates have been suggested including collagen type II, proteoglycans, heat shock proteins, and lately citrullinated proteins (Fournier 2005).

van Gaalen proposes a two-step model which emphasizes the involvement of citrullinated proteins and anti-CCP. The first step involves the presentation of citrullinated proteins to T cells by activated dendritic cells resulting in a T cell response. Citrullination is a result of the local inflammatory process and not subjected to regular B-cell tolerance development. The T cells stimulate B cells to produce high affinity anti-CCP which then enter the joint due to local inflammation or as a result of immune complex-facilitated vascular leakage. The second step involves migration of immune cells to the joint during inflammation or after a trauma, citrullination of synovial proteins by enzymes activated during cell death, and activation of complement by anti-CCP in complex with citrullinated antigens. More immune cells are attracted to the joint, more citrullinated antigens are produced, and an inflammatory circle contributing to the chronic disease is generated (van Gaalen 2005).

Firestein proposes a multi-stage pathogenic model: "a combination of stochastic events (activation of innate immunity), pre-determined events (genetic background), and adaptive immune responses directed against autologous antigens" (Firestein 2003). The early stage is proposed as activation of innate immunity, and any pathogen that gains entry to the joint could be responsible in a given individual. Proposed mechanisms are the

activation of innate immunity by agonists for toll-like receptors and/or engagement of Fc receptors by autoantibodies. Synovial dendritic cells activated by toll-like receptors migrate to the lymph nodes where they activate T cells. Adhesion molecules up-regulated on the endothelium allow circulating cells expressing the right receptors to accumulate in the inflamed joint. Then, in the appropriate genetic conditions such as the presence of the right HLA-DR alleles, there is a loss of tolerance related to the effects of HLA-DR background, or the T-cell repertoire might contribute to autoreactivity to newly exposed articular antigens. Direct T cell contact can also activate other cells in an antigen-independent manner (Firestein 2002; Firestein 2003).

nTreg and iTreg

Dysfunctions of Tregs fit the afore mentioned models as they are probably primed in the lymph nodes and later summoned to the joint by chemokines and adhesion molecules. Tregs are dedicated to the task of specializing in suppressing the activation, expansion, and function of other T cells and B cells (Fontenot 2003; Hori 2003; Shevach 2006; von Boehmer 2005). Hereditary as well as experimental elimination of Tregs lead to autoimmune manifestations (Afzali 2007; Allan 2008; Flores-Borja 2008; Hori 2003; Sakaguchi 2008), and quantitative or qualitative abnormalities in Tregs may contribute to inflammation in RA. There are a number of T cell subsets with immune modulating function (Cools 2007), however, the focus of the present thesis is the FoxP3 expressing T cell subsets, nTregs and iTregs, described below.

Current evidence suggests the existence of two subsets of FoxP3 expressing Tregs, the natural (nTreg) and the induced (iTreg) Treg cells (Horwitz 2008; Khattar 2009). nTregs develop in the thymus and then migrate to the periphery where they comprise 5-10 % of T cells. nTregs are characterised by the expression of cell surface markers CD4, CD25high, CD127low, and also CTLA-4 and glucocorticoid-induced tumour necrosis factor receptor (GITR). nTregs express FoxP3high, an intracellular transcription factor. The iTregs are generated in the periphery from CD4+CD25- cells after antigen recognition and in the right environment rich in TGF-β. iTregs start to express some of the same cell markers as nTregs (CD4, CD25low, and FoxP3low) (Horwitz 2008; Khattar 2009). CTLA-4 is one of the key molecules in Treg cells and is supposed to take part in the development of iTregs (Curotto de Lafaille 2009; Horwitz 2008) as well as in the suppressive mechanism of Tregs. Another difference between nTregs and iTregs is the differential sensitivity to modulation of the suppressive capacity upon interaction with IL-6 (Zheng 2008).

Both nTregs and iTregs need triggering of their T cell receptor (TCR) for suppressive function (Cools 2007). The TCR specificity of Tregs is currently unknown, but a model has been proposed predicting differences in specificity of the TCR between nTregs and iTregs (Fujimoto 2011; Horwitz 2008). As Tregs are CD4+, the antigen is expected to be presented by MHC II. It has been speculated that since activated T cells express MHC II, the thymus-generated nTregs are selected to recognise self-peptide including TCR fragments on activated T cells. In contrast, iTregs are generated from conventional activated T cells, and accordingly their TCR repertoire is expected to be directed against foreign antigen fragments presented by antigen presenting cells (Vandenbark 2008).

Isolation of highly purified Tregs is difficult and time consuming because there is no unique cell surface

marker, and because Tregs share phenotypic similarities with activated T cells (Aerts 2008; Bour-Jordan 2009; Milpied 2009). FoxP3 is the marker best correlated to Treg cells (Hori 2003; Khattri 2003; Ramsdell 2003), but it is a transcription factor located in the nucleus and it is therefore difficult to determine with flow cytometry. The staining of FoxP3+ cells can be highly variable and depends on the choice of antibody/buffer pair and the setting of the FoxP3 gate (Law 2009). Additionally, the estimation of FoxP3+ cells in flow cytometry and western blot has been further complicated by cross-reactivity with until now unidentified proteins of a FoxP3 antibody commonly used in flow cytometry (Woetmann, personal communication).

The mechanism of action

The mechanism of action is thought to be different for the two subsets – the nTregs suppress primarily via a cell contact-dependent mechanism, while the iTregs suppress through a cell contact-independent mechanism using cytokines such as IL-10 and TGF-β (Ramsdell 2003; Tang 2004). It is likely, that there is more than one mechanism by which Treg cells exert suppression, and that they do so in a context-dependent manner depending on several factors, for example the target cell, the local cytokine milieu, and the activation status of the antigen presenting cells.

One possible mechanism for cell contact-dependent suppression through CTLA-4 is the aggregation of Tregs on dendritic cells resulting in down-modulation of the CD80 and CD86 molecules on the dendritic cells, as described in mice (Oderup 2006; Onishi 2008). CD80 and CD86 provide a co-stimulatory signal through CD28 which is necessary for T cell activation and survival. Down-modulation of this signal would result in suppression of T cell activity. CTLA-4 and CD28 are similar molecules, and both bind to CD80 and CD86 but with opposite effect, the former inhibitory and the latter stimulatory (Magistrelli 1999). Another suppressive mechanism involving CTLA-4 is the induction of indoleamine 2,3-dioxygenase (IDO) in dendritic cells through the interaction between CTLA-4 and CD80/CD86. IDO is an enzyme involved in tryptophan catabolism in dendritic cells, and cells expressing IDO can suppress T cell responses through deprivation of the essential amino acid tryptophan and promote tolerance (Mellor 2004). A different proposed mechanism of immune suppression is the inactivation and conversion of extracellular ATP to AMP by the cell surface associated ectonucleotidase CD39, present on FoxP3+ Treg cells (Borsellino 2007). Extracellular ATP is released by damaged cells and functions as an activator of pro-inflammatory responses, and as a chemo-attractant for lymphocytes (Bours 2006). An additional suppressive mechanism involving cAMP is the formation of GAP junctions between Tregs and effector T cells allowing cAMP to diffuse from inside the Treg cell to inside the effector T cell (Bopp 2007) resulting in the inhibition of T-cell activation. Yet another proposed mechanism is the consumption by Treg cells of survival and growth promoting cytokines, such as IL-2, resulting in induction of apoptosis in effector T cells (Pandiyan 2007; Scheffold 2007). Tregs express high levels of the IL-2 receptor, CD25, but do not produce IL-2 themselves. This mechanism of action may be lost when large amount of cytokines are available, such as in the arthritic joint.

Another example of the importance of cytokines is the balance between the anti-inflammatory Treg cells and the pro-inflammatory Th17 cells. The cytokine TGF-β is required for both Treg and Th17 differentiation, as it

induces the expression of the transcription factors FoxP3 and RORγt required for differentiation of the Treg and Th17 cell lineages, respectively. FoxP3 can inhibit RORγt transcriptional activation resulting in Treg differentiation and not Th17, but in the presence of IL-6 the effect is abrogated leading to Th17 differentiation and not Tregs (Ivanov 2006; Zhou 2008). Th17 cells are also involved in activation of osteoclasts, causing bone resorption (Steinman 2007), and the role of IL-17, produced by Th17 cells, includes promotion of neutrophil accumulation and release of pro-inflammatory cytokines by a wide range of cell types (Brennan 2007; Xu 2010).

Also TNF-α is important in RA and has been shown to inhibit the suppressive function of Treg cells (Valencia 2006). Treg cells express TNFR2, and signalling through this receptor resulted in reduced FoxP3 expression and decrease in suppressive function. Supposedly anti-TNF treatment restored their suppressive function (Valencia 2006).

FoxP3

The marker best correlated to Tregs is the transcription factor FoxP3, which controls the expression of a number of key genes in the Treg cell itself and is required for the development of suppressive function of Treg cells (Hori 2003; Khattri 2003; Ramsdell 2003). However, there are reports that FoxP3 is transiently expressed in human activated non-regulatory T cells, although in significantly lower levels than those in suppressive nTregs (Allan 2007; Wang 2007). Supposedly, this transient expression of FoxP3 is a consequence of CD4+ T cell activation and the activated cells do not convert to Tregs (Allan 2007).
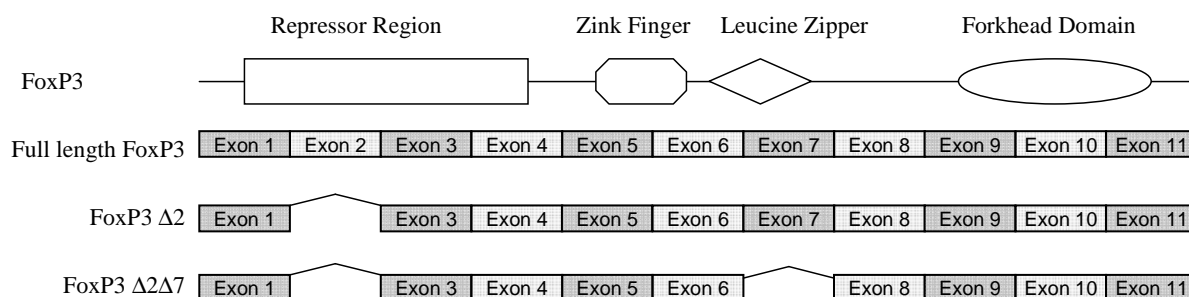
Figure 3. Schematic structure of the coding exons of human FoxP3 mRNA and the splice forms, and the predicted structural and functional domains. Modified from paper I (Ryder 2010).

Patients with genetic deficiencies in FoxP3 develop severe abnormalities in immune homeostasis, the IPEX syndrome (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked), causing e.g. excessive cytokine production, and chronic inflammation leading to death (van der Vliet 2007).

The FoxP3 gene consists of 12 exons, but the first exon in the 5' end is not translated and therefore dubbed exon -1. There is consensus that FoxP3fl comprise of exon 1-11. Two low molecular splice forms of FoxP3 have been found, FoxP3Δ2 and FoxP3Δ2Δ7. As can be seen in figure 3, they both lack exon two, and the FoxP3Δ2Δ7 isoform also lacks exon seven (Allan 2005; Krejsgaard 2008; Smith 2006). Exon two is a part of the proline-rich repressor region at the N-terminal part of the protein, which has been shown to be important for transcriptional repression by FoxP3 (Bettelli 2005; Du 2008; Lopes 2006). Exon seven is part of the leucine zipper domain

necessary for FoxP3 homodimerization (Lopes 2006). The Forkhead/winged helix domain at the C-terminal of the FoxP3 protein mediates its nuclear localisation and ability to bind DNA (Schubert 2001).

The full length FoxP3 protein acts as functional repressor of RORα, NFAT, and NF-κB mediated gene expression (Bettelli 2005; Du 2008; Krejsgaard 2008). RORα has been implicated in the maintenance of bone tissue (Meyer 2000) and as a negative regulator of inflammatory response (Delerive 2001), and NF-κB and NFAT are among other things essential for cytokine gene expression and T cell functions (Bettelli 2005). The two isoforms differ functionally from FoxPfl, although the lack of exons two and seven did not prevent FoxP3-dependent inhibition of T-cell activation (Smith 2006). The FoxP3Δ2 splice variant differs functionally from full length by not being able to interact with RORα and inhibit RORα-mediated transcriptional activation (Du 2008). Both low molecular isoforms of FoxP3 have no inhibitory effect on NF-κB transcriptional activity, while the full length version is a strong inhibitor (Bettelli 2005; Krejsgaard 2008). There are, however, conflicting results regarding the splice variants as another group showed that all isoforms reduced both NFAT-mediated and NF-κB-mediated transcription, and that the lack of exon seven did not affect dimerization (Mailer 2009). On the other hand, previous reports have identified exon two as a central part of the transcriptional repressor domain in FoxP3 (Bettelli 2005; Du 2008; Lopes 2006).

The focus of this thesis is Tregs isolated from arthritis patients. For logistic reasons (e.g. lack of equipment, money, time) I have not performed any flow cytometry or functional studies of these Tregs. At the time these data were generated, no antibodies able to distinguish between the different isoforms of FoxP3 were available for flow cytometry, and it has only recently been possible to buy kits for isolation of a highly purified Treg population. Hence, I decided to isolate CD4+ T cells using magnetic beads, and froze the cells for RNA extraction. Functional as well as flow cytometry data are most reliable when performed on freshly isolated cells, and therefore a post-hoc analysis was not in question.

Methods

Validation of the QRT-PCR

The choice of reference gene for QRT-PCR

In all samples, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and CD4 were used as reference genes. It is standard to use housekeeping genes such as GAPDH as an internal reference gene to normalize the PCRs for the amount of RNA added to the initial cDNA reactions. A commercially available ready-to-use GAPDH TaqMan Assay Reagent was used. As the focus of this work was FoxP3-expressing CD4+ T cells isolated by CD4 positive selection, the CD4 gene was used as cell-specific control gene. It was not possible to do an actual control of the reference genes, as the material used originated from patients and was not subjected to controlled experimental variation. However, Mocellin demonstrated that the gene expression of CD4 by resting T cells did not change significantly after stimulation and it is therefore suitable to use as a T cell subset-specific reference gene (Mocellin 2003). Hence, the cycle threshold (Ct) values were normalized against the corresponding CD4 values. Additionally, there was a positive correlation between the Ct values of GAPDH and CD4 in blood CD4+ cells from arthritis patients, see figure 4, and the Pearson product moment correlation coefficient (R^2) was calculated in Excel to be 0.953. Because 95.3% of the variance in Ct(CD4) was attributable to the variance in Ct(GAPDH) it was assumed to be safe to replace GAPDH with CD4.
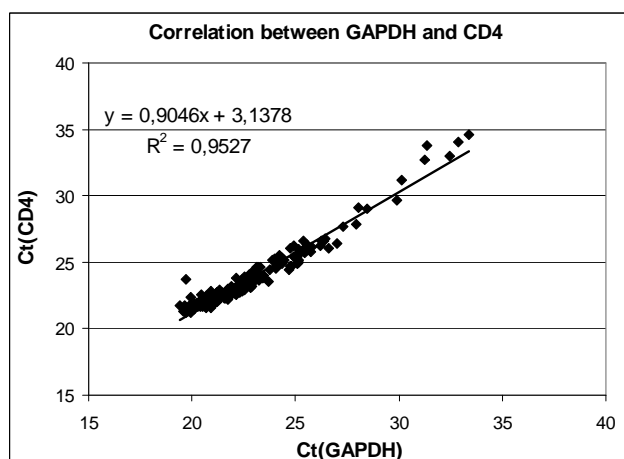
Figure 4. The correlation between GAPDH and CD4 Ct values. Data from 50 patients included in the study (paper I) is shown. The trendline equation was obtained using Microsoft Excel.

The principle

The quantitative real-time RT-PCR method theoretically doubles the amount of DNA with every cycle. The principle of the QRT-PCR reaction is outlined in figure 5. In short, the Taqman probe consists of a DNA sequence modified with a reporter dye in the 5' end and a quencher in the 3' end. When the quencher is in close proximity of the dye, it quenches the fluorescence signal by the phenomenon of fluorescence resonance energy transfer. During the PCR cycle the Taqman probe is cleaved, releasing the reporter dye from the effect of the quencher. The instrument quantitatively measures the fluorescence generated after each PCR cycle, and the expression of a gene is quantified in terms of cycle threshold (Ct). The Ct is the number of cycles at which the measured fluorescence exceeds the determined threshold. Hence, a large Ct for a given target gene translates to a small amount of starting

material and vice versa.
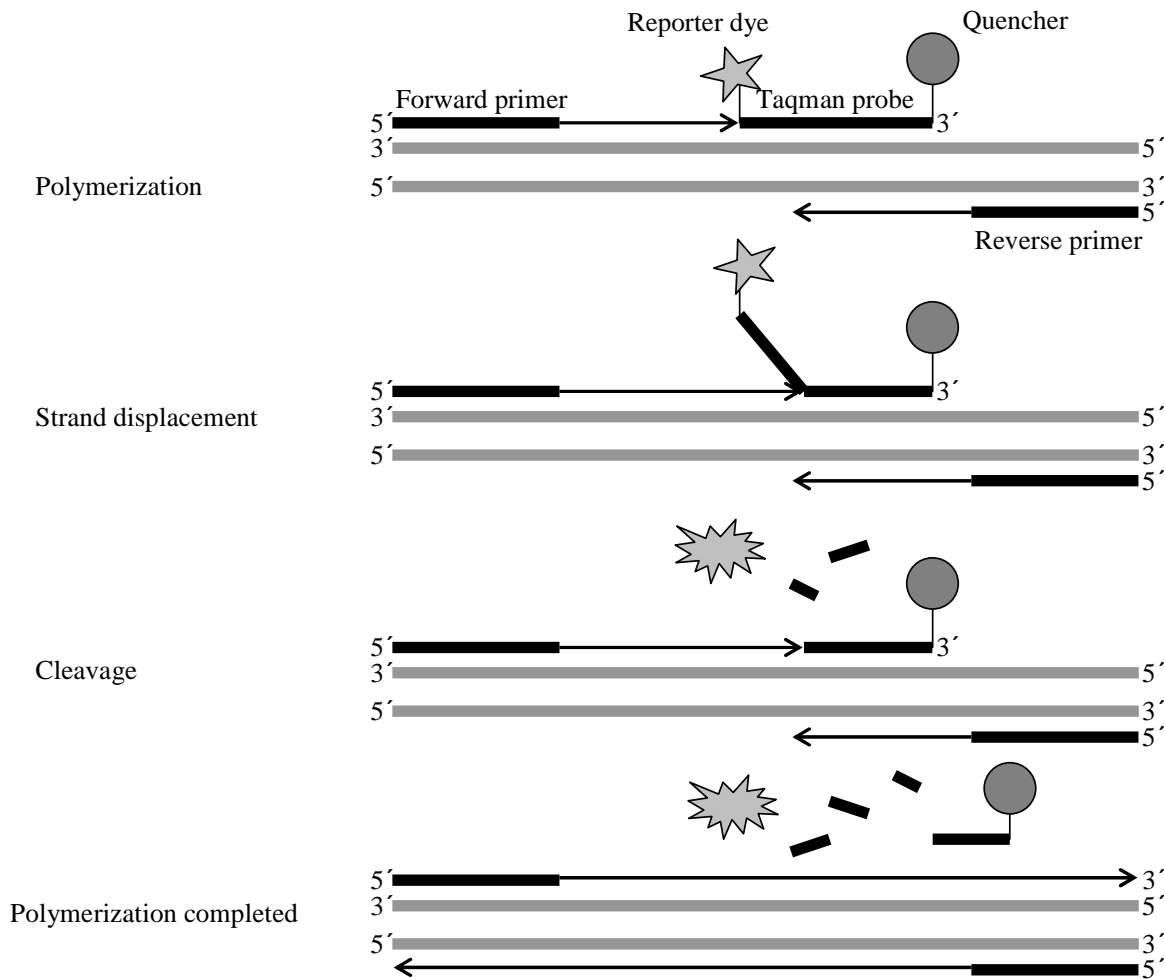
Figure 5. The principle of the QRT-PCR reaction. Reporter dye: 6-FAM; Quencher: TAMRA

The big question is how to analyze these RT-PCR data, and there are two methods one can use: the relative quantification and the absolute quantification. The absolute quantification method determines the starting copy number usually by relating the specific signal to a standard curve. The relative quantification method is used to analyze relative changes in gene expression by relating the specific signal in one sample to another, e.g. PB versus SF; before and after treatment. I chose relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak 2001), where $\Delta\Delta Ct = \Delta Ct$(target, patient) – ΔCt(target, healthy control group). The relative expression was calculated as the amount of target normalized to the internal reference CD4, and relative to the healthy controls used in the comparison in paper I, II, and III. Additionally, when comparing the individual expression levels in SF relative to the expression in paired samples of PB in paper II the expression in SF was calculated relative to the paired PB sample, and $\Delta\Delta Ct = \Delta Ct$(target, SF) – ΔCt(target, PB).

Assumptions, dilutions, slopes, and efficiencies

When applying the $2^{-\Delta\Delta Ct}$ method to QRT-PCR data it is assumed that the amplification efficiencies of the target

and reference transcripts are approximately equal. The efficiency is derived from the theoretical function for the amount of PCR product formed: $N = N_0 * E^n$, where N is the number of amplified copies, N_0 is the starting number of copies, n is the number of amplification cycles and E is the efficiency (Mygind 2002). The ideal efficiency is 2, and theoretically the amount of copies is doubled with every PCR cycle. The efficiency can be calculated from the slope of the line in the plot of log cDNA dilution (x-axis) versus Ct (y-axis). The equation below is derived from the function above:

$n = - (1/\log E)*\log N_0 + (\log N/\log E)$, and the slope equals $-(1/\log E)$ (Mygind 2002).
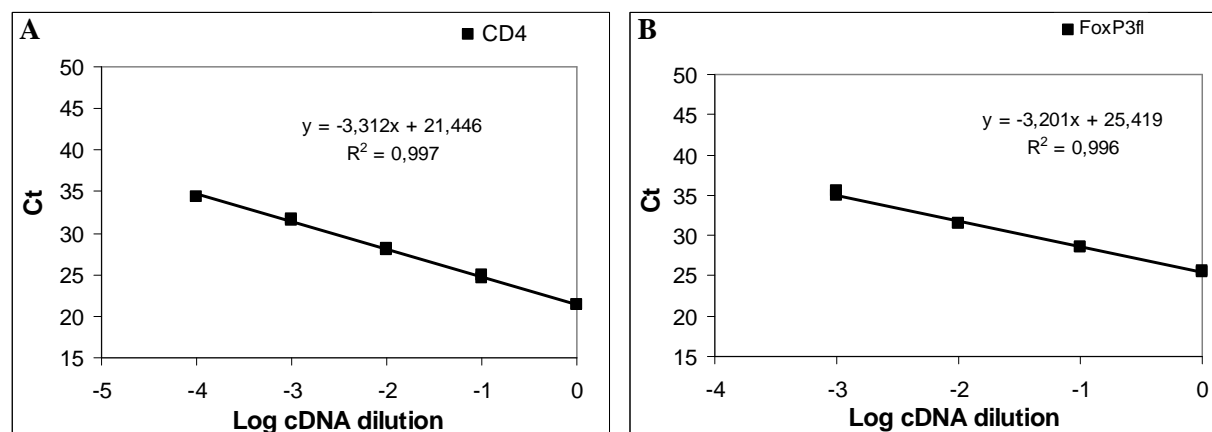
Figure 6. 10-fold dilutions series of cDNA using primer sets for A) CD4 and B) FoxP3fl. The slopes were determined from the trendline equations.

A ten fold serial dilutions of cDNA were amplified using all primer sets and plotted as described above. In figure 6 the plot for CD4 and FoxP3fl dilution series are shown, and the plots for the remaining five systems are shown in Appendix D. The slopes were determined from the trendline equations in the Excel plots, and the efficiency of each primer pairs were calculated as stated above and shown in table 2.

Table 2. The slopes and efficiencies of each probe and primer pairs in the QRT-PCR.

	GAPDH	CD4	CD25	CTLA-4	FoxP3fl	FoxP3Δ2	FoxP3Δ2Δ7
Slope	-3.33	-3.31	-3.44	-3.31	-3.20	-3.36	-3.57
Efficiency	2.00	2.01	1.95	2.01	2.05	1.98	1.91

The ideal efficiency is 2.00 and the corresponding slope is $-(1/\log 2) = 3.32$. There are no rules as to how similar the efficiencies have to be in order to use the $2^{-\Delta\Delta Ct}$ method. Schmittgen suggests that the efficiency of two genes are similar enough if they are within 10% of each other, that is efficiencies in the range of 1.8 to 2.2 with corresponding slopes of -3.92 to -2.92 (Schmittgen 2008). So what is the effect of difference in efficiency between target and reference gene? This can best be illustrated by an example. At cycle number 25 with efficiencies of 1.8 and 2.0, and using the equation above, there is difference in Ct of 3.7 corresponding to a 13-fold difference:

$N = N_0*E^n = N_0*1.8^{25} = N_0*2.41*10^6$ and $N = N_0*E^n = N_0*2.0^{25} = N_0*3.36*10^7$

During the experimental work it was discovered that the primer pair amplifying FoxP3fl were suboptimal. A new pair was designed and tested using the existing taqman probe, see figure 6 above. Data using both the new and

24

old primer pairs were compared, showing differing results only if the Ct values obtained using the old pair were higher than 32. As a consequence all samples with a Ct value higher than 32 in that particular system were reanalyzed with the new set.

TRIzol versus KingFisher

For practical reasons I used two different RNA isolation methods, the automatic KingFisher technology using magnetic rods and magnetic beads with oligo-dT probes catching mRNA, and the TRIzol reagent method isolating total RNA. In order to certify that the two methods of RNA isolation were comparable I performed two separate RNA extractions on the same samples using both methods followed by cDNA synthesis and QRT-PCR. The samples used were CD4+ T cells from PB and SF from an RA patient. Sample preparation and total RNA isolation using TRIzol Reagent were described in paper II, while real-time quantitative RT-PCR and mRNA isolation using the KingFisher technology were described in detail in paper I.
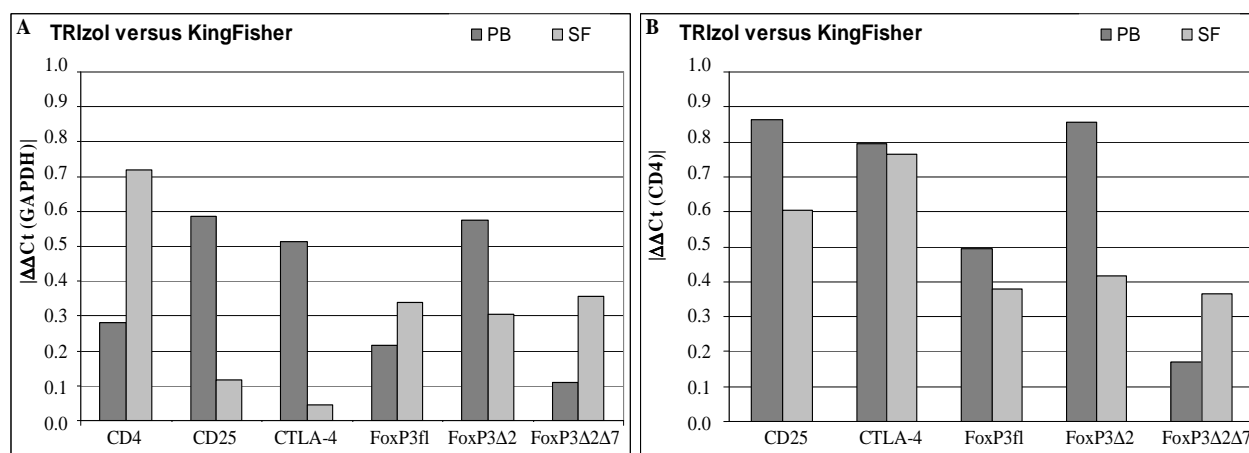
Figure 7. The numerical difference in $\Delta\Delta Ct$ for the two RNA isolation techniques used when A) GAPDH and B) CD4 was used as reference gene. $|\Delta\Delta Ct(GAPDH)| = |\Delta Ct_{TRIzol}(target\ gene) - \Delta Ct_{KingFisher}(target\ gene)|$ where $\Delta Ct(target\ gene) = Ct(target\ gene) - Ct(GAPDH)$. For the calculation of $|\Delta\Delta Ct(CD4)|$ the Ct(GAPDH) values were substituted with the Ct(CD4) values.

To ensure comparability and to correct the results for differing amounts of RNA I used GAPDH as reference and calculated the ΔCt value for CD4, CD25, CTLA-4, and the three FoxP3 splice forms as $\Delta Ct(target\ gene) = Ct(target\ gene) - Ct(GAPDH)$. The differences in ΔCt values between the two methods were less than one for all target genes (figure 7), and I therefore assume the two methods are comparable. I repeated the calculation for each target gene replacing the Ct values for GAPDH with CD4, and obtained similar results with differences in ΔCt values of less than one (figure 7).

Synovial fluid samples – volumes and cell numbers

In the paired samples study (paper II) all rheumatic patients with flare in the knee joint from whom a cellular SF could be obtained were included. Volumes of SF aspirated were between 22-285 ml with a median volume of 50 ml. The number of MNC isolated from each sample varied between 0.08-3.2 x 10^6 MNC/ml. The percentage

25

represented by MNC was different from patient to patient. Some patients had a large percentage of granulocyte/macrophages others a few, based on personal observations of the precipitate after Lymphoprep gradient centrifugation.

Clinical parameters

In paper I and III we tested the correlation between the presence and amount of FoxP3 mRNA and several clinical parameters. In this section I would like to describe these parameters with a few more words.

DAS28 score

The DAS28 score is a validated tool for quantitative assessment of RA (Prevoo 1995; van Gestel 1998; Wells 2009). It is a combined measure of information on swollen joints, tender joints, the acute phase response CRP and the patient assessment of general health. DAS28 provides a number on a scale from 0 to 10, indicating the current activity of arthritis in the patient. A DAS28 score of above 5.1 translates to a high disease activity whereas a DAS28 score below 3.2 indicates low disease activity. DAS28-defined remission is classified as a score lower than 2.6. Remission is the state of absence of disease activity in patients with a chronic illness, with the possibility of return of disease activity. The DAS28 score is used in both patient care and clinical research. The EULAR response criteria (Aletaha 2010) includes changes in disease activity, as well as current disease activity, and states that good responders are patients with an improvement of > 1.2 and a present score of ≤ 3.2. DAS28 scores are calculated as follows, where TJC = Tender joint count; SJC = Swollen joint count; GH = general health using the 100 mm visual analogue scale (VAS); and CRP mg/L.

DAS28 = $0.56*\sqrt{(TJC28)} + 0.28*\sqrt{(SJC28)} + 0.014*GH + 0.36*\ln(CRP+1) + 0.96$

CRP

C-reactive protein is one of the plasma proteins known as acute-phase proteins. They are proteins which may increase or decrease their plasma concentrations by 25% or more during inflammation. Conditions that commonly lead to marked changes in CRP include infection, trauma, surgery, burns, advanced cancer, and inflammatory conditions such as RA. CRP is produced in the liver (Brahn 1988), and the production is stimulated by the cytokines IL-6 and TNF-α. At Frederiksberg Hospital a normal CRP value is defined as <10 mg/L.

VAS

Self-reported general health assessment using the 100 mm visual analogue scale (VAS) where 0 = best and 100 = worst.

HAQ

The health assessment questionnaire (HAQ) is a questionnaire that evaluates self-perceived physical disability to perform different activities of daily living grouped into eight areas: dressing and grooming, rising, eating, walking,

hygiene, reaching, gripping, and other activities. The HAQ result in a number between 0-3 where 0 is normal and 3 is indicating severe physical disability.

ESR

The erythrocyte sedimentation rate (ESR) measures the rate at which red blood cells separate from blood serum over time, becoming sediment in the bottom of the test tube. The sedimentation rate increases with inflammation mainly due to elevated concentrations of fibrinogen (Brahn 1988), and at Frederiksberg Hospital a normal ESR value lies within 2-20 arbitrary units.

Anti-CCP

Some patients develop antibodies against modified (citrullinated) arginine residues (anti-CCP). Citrullination results in a small change in molecular mass and the loss of a positive charge in the modified proteins (van Gaalen 2005). The physiological role of citrullination remains to be elucidated. At Frederiksberg hospital a normal anti-CCP value is < 5 kU/L.

RF

Rheumatoid factor (RF) is an autoantibody against the Fc portion of IgG. RF is a combination of autoantibodies from the three major classes of immunoglobulins IgM, IgG, and IgA, with the IgM and IgG as the most abundant (Mannik 1988). RF forms immune complexes with the IgG and can be used prognostically as it correlates with functional and radiographic outcomes in RA. At Frederiksberg hospital a patient is defined positive at a RF concentration > 17 kiU/L.

Results & Discussion

We established a QRT-PCR method to measure the relative amount of Treg-related mRNAs

We designed assays for Treg relevant markers CD4, CD25 and CTLA-4. We also designed four FoxP3 assays each amplifying different transcripts (figure 8, paper I+II+III). The assays were designed with primers or probes spanning exon-exon junctions to avoid amplification of genomic DNA. Samples were run in duplicates starting with the reverse transcription step, and a sample specific control containing mRNA, but without random hexamer, RNase inhibitor or reverse transcriptase was included in the QRT-PCR analysis. These samples were negative and served as a control for the amplification of genomic DNA.
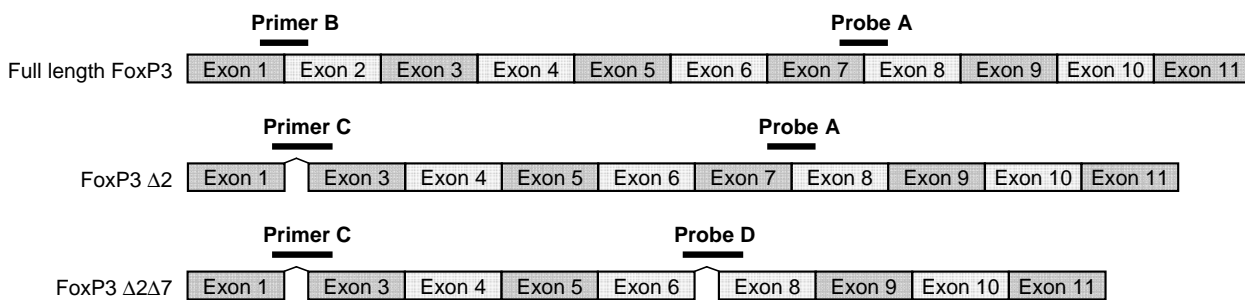
Figure 8. Schematic structure of the coding exons of human FoxP3 mRNA and the splice forms. From paper I (Ryder 2010).

The efficiencies were determined to be the same for all systems. The largest difference in efficiency was between the CD4 and FoxP3Δ2Δ7 (splice form lacking exon 7) primer pairs with efficiencies of 2.01 and 1.91, respectively. At cycle number 25 with these efficiencies and using the equation mentioned before, there is a 1.8 difference in Ct values corresponding to a 3.6-fold difference:

$N = N_0 * E^n = N_0 * 2.01^{25} = N_0 * 3.80*10^7$ and $N = N_0 * E^n = N_0 * 1.91^{25} = N_0 * 1.06*10^7$

Unlike the other FoxP3 assays, the one amplifying the splice form lacking exon 7 gave very large Ct values, which translates to very small amounts of that particular splice form. We can therefore conclude that the assay measuring splice forms lacking exon 2 is mainly measuring FoxP3Δ2, and that:

FoxP3Δ2 + FoxP3Δ2Δ7 ≅ FoxP3Δ2.

It is also worth mentioning that when the concentration of template is close to one molecule pr well, the distribution of template molecules per well is expected to follow the Poisson distribution (the Poisson law of small numbers). For example, if the concentration is one copy of cDNA per well, you would expect no signal in 37% of the wells.

Increased expression of FoxP3 mRNA in blood CD4+ T cells from RA patients compared to HC

In our studies (paper I+II+III) both full length and the Δ2 splice form of FoxP3 were present in PB cells in patients as well as in HC; and the Δ2 splice form was more abundant than the full length version in almost all our patients and in every HC. In paper I we found an increased expression of FoxP3fl and CD25 mRNA in the patients compared to the healthy control group, but no significant difference in FoxP3Δ2 or CTLA-4 mRNA levels between

the two groups. In our second study (paper II) our data on PB CD4+ T cells from RA patients differed slightly from that of our first study (paper I). In the second study, we found a significant increase in expression of both FoxP3fl and Δ2 in RA patients compared to HC, and a non-significant increase in CD25 mRNA, while the CTLA-4 mRNA levels were significantly lower. However, the overall tendencies in both studies were the same: increased mRNA levels of CD25 and both full length and Δ2 splice forms and decreased levels of CTLA-4 mRNA (figure 9).
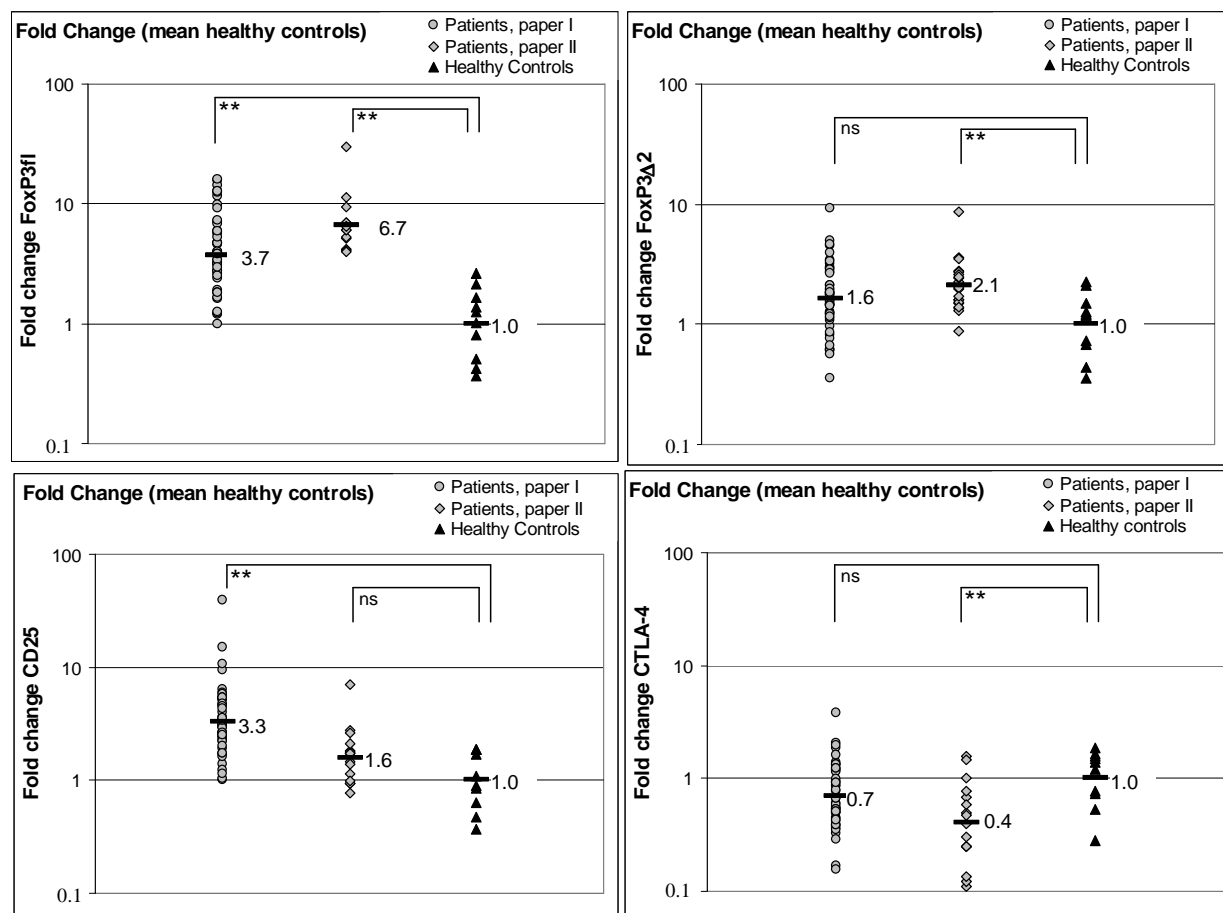
Figure 9. Comparison of the individual expression levels (mRNA) in peripheral blood CD4+ T cells of A) FoxP3fl, B) FoxP3Δ2, C) CD25, and D) CTLA-4 in the RA patients in paper I + II and 10 healthy controls relative to the average expression of the healthy controls. A fold change above 1 represents an increase, and a fold change below 1 represents a decrease. The mean value is shown with a bar. *p<0.05, **p<0.01, ns: not significant.

We interpreted these data in support of the hypothesis that Tregs are increased in RA patients, but suggested, however, that these Tregs had a compromised expression of CTLA-4. CTLA-4 is thought to play an important part in the suppressive function of nTregs (Flores-Borja 2008; Tang 2004), and the relative deficiency of CTLA-4 expression could also indicate the presence of iTregs rather than nTregs. These findings were in accordance with previously published data suggesting increased numbers of Tregs in RA patients (Cao 2004; Mottonen 2005; Raghavan 2009), as well as non-functional Tregs prior to anti-TNF treatment (Ehrenstein 2004; Flores-Borja 2008; Nadkarni 2007). An alternative interpretation could be an increased expression of FoxP3 and CD25 in single cells and not an increase in numbers, but it is more likely a combination of the two. We are looking at the average CD4+ cell, and cannot distinguish between a few cells expressing large amounts or many cells expressing small amounts.

Also, it is important to remember that the amount of mRNA is not directly proportional to the amount of protein produced. A large increase in expression of FoxP3fl mRNA does not necessarily correspond to a large increase in FoxP3fl at the protein level.

Recently, a novel splice variant of FoxP3 lacking *only* exon 7 was identified in *ex vivo* stimulated CD4+CD25+ T cells and CD8+ T cells (Kaur 2010). Kaur reported an increase in expression of FoxP3Δ7 by up to 136-fold in *ex vivo* stimulated CD4+CD25+ T cells. They also detected the FoxP3Δ7 splice form in freshly isolated CD4+CD25+ T cells comprising <1% of total FoxP3 (Kaur 2010). The FoxP3Δ2Δ7 isoform has been found to be over-expressed in Sézary Syndrome, an aggressive variant of cutaneous T cell lymphoma (Krejsgaard 2008). A disadvantage of the QRT-PCR technique is that it is not possible to quantify the double deletion splice form such as FoxP3Δ2Δ7 directly. In our dataset the splice form lacking exon seven, with or without exon two, was not measurable in the ten healthy controls and only expressed in small amounts in the RA patients, suggesting that CD4+ T cells from arthritis patients predominantly express FoxP3fl and FoxP3Δ2. Also, sequencing of FoxP3 from 23 cloned bacterial colonies (paper I) revealed that none of them was missing exon seven. In our dataset the Δ7 isoform, with or without exon two, was not very common.

A physiological role of these different splice forms has yet to be found. Alternative splicing allows a gene to produce more than one isoform of mRNA, often resulting in functionally distinct proteins. It is a well-known, albeit not well-characterised, phenomenon in the immune system and in activated T cells (Grigoryev 2009; Ip 2007; Lynch 2004). One known example of alternative splicing in T cells are the markers CD45RA and CD45RO, used to distinguish between memory CD45RO T cells and virgin CD45RA T cells, where the CD45RO splice form is lacking three exons (Hermiston 2003; Holmes 2008; Oberdoerffer 2008). Another example is the alternative splice form of the cytokine IL-7 lacking exon 5 (Vudattu 2009). The FoxP3 splice forms are functionally distinct, and especially exon two has been identified as a central part of the transcriptional repressor domain able to interact with RORα (Du 2008). A change in the balance between the different isoforms within the Treg cell might affect the ability of FoxP3 to interact with different transcription factors and molecules and mediate a change in gene expression and cell function. Thus it may be hypothesised that the Tregs found in RA represent a stage of frustrated Tregs incapable of suppressing the inflammatory process. Alternative splice forms in general might represent therapeutic targets and/or candidates for diagnostic use.

What is the difference between the PB CD4+ T cells presented in paper I and II?

In the paired samples study (paper II), irrespective of sub-diagnosis and treatment, all rheumatic patients with flare in the knee joint from whom a cellular SF could be obtained, were included. These patients were included on short notice on the day the knee was aspirated, contrasting the patients in the first study (paper I) who participated in a planned and scheduled course of treatment. The data on the patients in the first study (paper I) were baseline data prior to treatment with anti-TNF drugs, which are used for treatment based on a most often wide spread disease activity. In contrast, the patients in the paired samples study (paper II) were included based on a flare in a single joint, i.e. a more local phenomenon.

An overall increase in expression of FoxP3 in CD4+ T cells in SF compared to PB in RA patients

Analysis of the expression of FoxP3 mRNA in paired samples of CD4+ T cells from PB and SF (paper II) confirmed the overall increase in expression of FoxP3 splice forms in arthritis patients compared to healthy PB CD4+ T cells. Especially, FoxP3fl was noticeably increased with a fold change of 21.3 in SF and 6.7 in PB CD4+ T cells from arthritis patients when compared to HC. Also the $\Delta 2$ splice form was increased in the patients compared to HC although not as dramatically with a fold change of 6.0 in SF and 2.1 in PB CD4+ T cells. Furthermore CD25 was increased with a fold change of 2.8 in SF and 1.6 in PB (not significant) compared to HC, while the expression of CTLA-4 was decreased in PB from RA patients with a fold change of 0.4 (corresponding to -2.5) compared to HC. The expression of CTLA-4 in SF was equal to that in PB from HC.

The full length version of FoxP3 is a well-characterised marker of Treg cells and plays an important role in the induction of fully functional Treg cells (Hori 2003; Yagi 2004), while the importance and function of the two low molecular splice forms are not fully elucidated. Thus, our data on the selective increase in expression of FoxP3fl in SF support previous reports of an accumulation of Tregs in inflamed joints of arthritis patients (Cao 2006; Jiao 2007; Mottonen 2005; Van Amelsfort 2004). Additionally, the high increase in FoxP3 expression without a concomitant increase in CTLA-4 expression in blood and SF support the hypothesis of abnormal Tregs with a compromised function in RA (Flores-Borja 2008), both in the inflamed joint but also systemically. It could be explained as an increase in iTreg cells, as this cell population uses a contact-independent mechanism of suppression which does not involve CTLA-4.

When comparing the individual expression levels of the different splice forms of FoxP3 in samples of SF CD4+ T cells relative to the expression in paired samples of PB CD4+ T cells (paper II), the patients displayed a differentiated expression pattern based on the arthritis sub-type. The three splice forms were increased in SF compared to PB in RAneg and PsA, but not in RApos. The RApos patients had a high FoxP3 expression in both PB and SF CD4+ T cells. The PB-SF ratio of FoxP3 mRNA splice forms appeared to be ranked according to diagnosis: RApos < RAneg ≤ PsA. Although the ranking was not as clear for the expression of the low molecular splice forms as it was for FoxP3fl, a definite trend was observed. The marked increase in FoxP3fl expression in SF CD4+ T cells in all patients irrespective of specific sub-diagnosis compared to HC indicated that it is a common feature of arthritis and not linked to the pathology of the arthritis in question. The observation that there was no increase in FoxP3 expression in synovial CD4+ T cells compared to blood in RApos patients was attributable to a high expression of FoxP3 in blood CD4+ T cells, indicating the involvement of blood due to the systemic nature of the disease. Also the presence of several cytokines in blood and SF, and especially a higher concentration of IL-8 found in SF from RApos compared to RAneg and PsA, may reflect the differences in disease mechanisms. IL-8 is a chemokine involved in monocyte migration (Hayashida 2001), and elevated levels in SF favour the hypothesis of a selective recruitment of immune cells to the joint as part of the inflammatory process.

Anti-TNF drugs and the expression pattern of FoxP3 mRNA

In our material, anti-TNF treatment had no general effect on the expression of the different splice forms of FoxP3, CD25, and CTLA-4 mRNA (paper III). The three anti-TNF drugs used were the decoy receptor Etanercept, and the antibody-based therapies Adalimumab and Infliximab. There are different reports on the effect of anti-TNF drugs, many of which are looking solely on those patients who did benefit from the treatment. In line with this approach we performed a *post hoc* analysis of the RA patients who benefitted from the anti-TNF treatment.
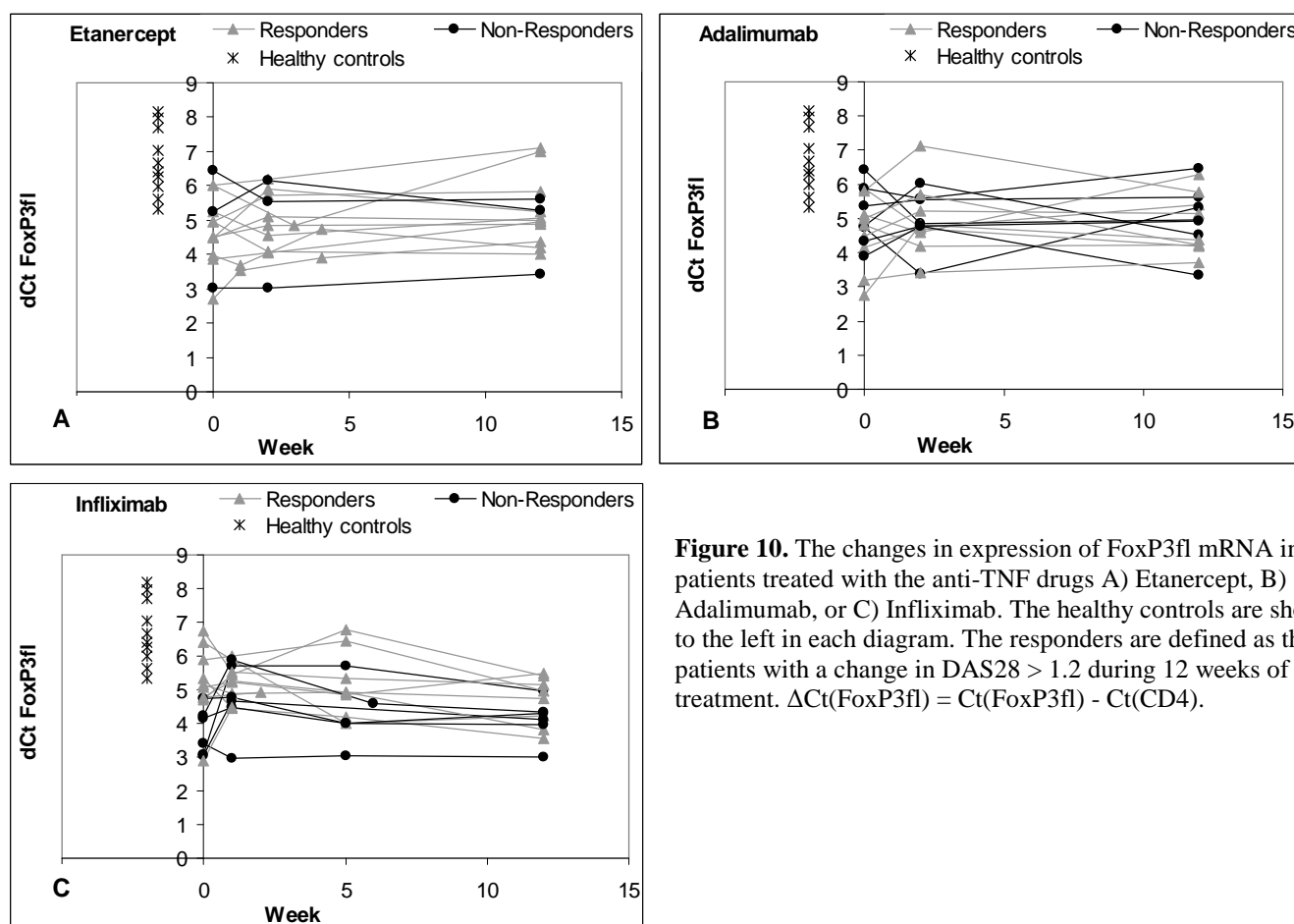
Figure 10. The changes in expression of FoxP3fl mRNA in the patients treated with the anti-TNF drugs A) Etanercept, B) Adalimumab, or C) Infliximab. The healthy controls are shown to the left in each diagram. The responders are defined as those patients with a change in DAS28 > 1.2 during 12 weeks of treatment. ΔCt(FoxP3fl) = Ct(FoxP3fl) - Ct(CD4).

We observed a significant decrease in expression of FoxP3fl after 12 weeks of beneficial Etanercept treatment (figure 10A), where the expression was partially normalized and reduced from 4.6 times more to 3.2 times more than the average HC. This reduction in expression of FoxP3fl, along with a concomitant reduction in CD25 expression from 2.9 to 2.0 times more than in the average HC, indicate a decrease in Treg cells towards that of healthy controls. Importantly, the expression of CTLA-4 was not significantly altered in these patients and remained reduced at to almost half the amount as in HC (fold change 0.6). This lead to a change in the ratio between FoxP3fl and CTLA-4 towards that of HC, even though the ratio was still different as the HC has 7.7 times more CTLA-4 than FoxP3fl mRNA and the Etanercept responders have 1.5.

In contrast, successful treatment with the antibody-based therapies Adalimumab and Infliximab did not

influence the expression of FoxP3fl (figure 10B+C), or the other mRNAs investigated. In the Infliximab group the opposite trend, an increase in expression of FoxP3fl, was seen. This trend towards an increase in expression of FoxP3fl in the Infliximab group was in accordance with findings by other groups (Ehrenstein 2004; Nadkarni 2007; Valencia 2006), and Nadkarni suggested that nTreg cells remained defective even after Infliximab treatment, and that the reported restoration of function could be due to induction of iTregs (Nadkarni 2007). Our data (paper I+II) support a previous report of an impaired CTLA-4 expression in RA patients compared to healthy controls (Flores-Borja 2008). In our biological samples, the anti-TNF treatment (decoy receptor or antibody-based therapy) did not return the expression of CTLA-4 to normal (paper III), which is also in agreement with previously published data (Flores-Borja 2008). Flores-Borja speculated that a reduced expression and functional abnormalities in Treg-associated CTLA-4 could explain Treg defects in RA patients, and that anti-TNF therapy induces a population of iTregs using a TGF-β dependant suppressive mechanism as opposed to contact-dependant mechanism involving CTLA-4 (Flores-Borja 2008). Our data show decreased expression of CTLA-4 in RA patients supporting this theory.

Our data suggest that the TNF decoy receptor Etanercept and the antibody-based therapies Adalimumab and Infliximab have different effect on the expression of FoxP3 in responsive patients (paper III). We speculate that it is due to the differences in mechanisms between the two types of drugs. Etanercept is a decoy receptor which binds and eliminates both TNF-α and LT-α, while Adalimumab and Infliximab are neutralizing antibodies only targeting TNF-α. The two proteins TNF-α and LT-α share significant amino acid sequence homology and they bind to the same receptors TNFR1 and TNFR2. TNF-α is produced by a number of different cell types including monocytes, macrophages and T cells, while LT-α is produced by activated T cells (Tracey 2008). Further we speculate that LT-α regulates FoxP3 expression in a subset of RA patients, and that individual patients may experience different treatment effects depending on whether the TNF receptor ligand is mainly of monocyte/macrophage origin (TNF-α) or T cell origin (LT-α). Overall, however, our findings support the view that anti-TNF treatment is mostly symptomatic and does not cure the underlying abnormality of the immune system.

Definition of responders to the anti-TNF drugs

We used the changes in DAS28 scores to identify the patients who benefitted from the anti-TNF treatment (paper III). We used part of the EULAR criteria for good responders: an improvement >1.2 in DAS28 score, based on the assumption that a change in DAS28 of more than 1.2 is a good response within 12 weeks regardless of the present score. For the patients included in our study (paper III), the current DAS28 at week 12 (x-axis) versus the change in DAS28 from week 0 to 12 (y-axis) were plotted (figure 11). In the Etanercept group (N=14), a total of 10 patients achieved a change in DAS28 score of more than 1.2 out of which three patients achieved remission and another three were scored with low disease activity. In the Adalimumab group (N=17), nine patients had a change of more than 1.2, and out of this group three patients achieved remission while one patient was scored with low disease activity. Finally, in the Infliximab group (N=17), 10 patients had a change in DAS28 score of more than 1.2, while no patients achieved remission, but three were reduced to low disease activity.
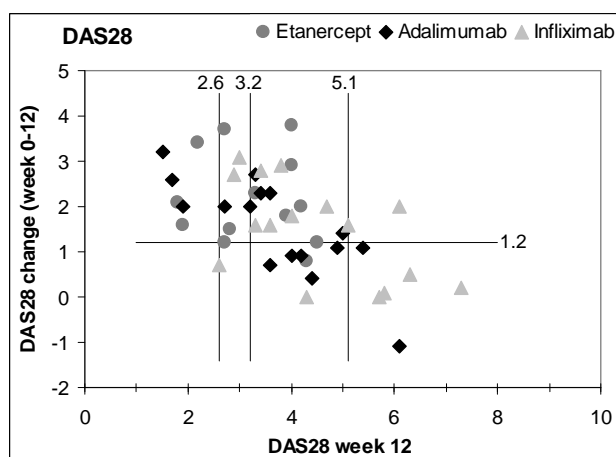
Figure 11. DAS28 scores at week 12 and the change in DAS28 from week 0-12. The lines indicate EULAR defined remission at 2.6; low disease activity 3.2; and high disease activity 5.1, as well as the change in DAS28 scores at 1.2.

Responders versus non-responders

We examined whether there was a different expression pattern of FoxP3 splice forms in the patients with a clinical response to anti-TNF compared to the non-responders (paper III). We were not able to identify baseline predictors of treatment response, i.e. identify patients who would benefit the most from anti-cytokine drugs based on its effect on the expression of FoxP3 mRNA isoforms present in CD4+ T cells in peripheral blood during therapy. Arthritis results from a multidimensional abnormality of the immune system, and effect of anti-TNF drugs seems to be based on other pathways than Tregs and FoxP3.

Correlation between FoxP3 expression and clinical parameters

One of the first questions we addressed was whether the expression of FoxP3 mRNA splice forms correlated to disease duration as a measure of the chronicity of the disease. There was no correlation, and disease duration had no influence on the expression of FoxP3 in RA patients (paper I). Factors having an impact on the FoxP3 mRNA expression were CRP, ESR and MTX. We speculate that a positive correlation between the amount of FoxP3 mRNA and the biochemical markers of inflammation reflects a frustrated compensatory Treg response. The role of MTX is difficult to ascertain. The dosage of MTX reflects the disease activity; while at the same time may by itself reduce inflammatory markers. In our material, the FoxP3 expression varied widely in the patients without MTX, while the correlation was seen in those treated with this drug. Thus, MTX might be speculated to inhibit FoxP3 expression in a dose-dependent manner, which would point towards a beneficial effect of MTX on the root of arthritis in contrast to the more superficial effect of anti-TNFs. However, confirmatory evidence of the possible MTX effect on FoxP3 needs to be presented before further considerations are made in this respect.

Our data are in agreement with Ehrenstein who reported a correlation between CRP and the percentage of CD4+CD25high T cells (not CD4+CD25low) (Ehrenstein 2004). Both expression data (paper I) and flow cytometry data (Ehrenstein 2004) indicate a relationship between inflammation and Tregs, where inflammation is measured as CRP and Tregs as FoxP3 mRNA or CD4+CD25high on the protein level.

Since we observed a significant improvement in DAS28 and VAS scores within the first two weeks of anti-TNF treatment (paper III), we addressed the question whether the individual changes in expression of FoxP3

mRNA within the first two weeks of treatment correlated to different clinical parameters. We thus investigated the correlation between the changes in expression of FoxP3 (week 2-0) and the changes in DAS28 (week 12-0), doses of MTX and/or Prednisolone at baseline, female/male, RF status, and anti-CCP at baseline. There were no correlations between changes in FoxP3 expression and the DAS28 changes, doses of Prednisolone, or gender in either group. The doses of MTX at baseline correlated to the change in FoxP3fl in the Infliximab group, indicating an effect of MTX on the response. RF status and anti-CCP correlated to some of the changes in expression in the Etanercept group, but as the number of seronegative patients was only three, we disregarded these results. In conclusion, we could not definitively relate the changes in expression within the first two weeks to clinical data at baseline.

We then addressed the question whether the changes in expression of FoxP3 mRNA during 12 weeks of treatment correlated to changes in DAS28 scores or changes in CRP in the Etanercept responders. We chose to look at the Etanercept responders only as they exhibited significant changes in expression during 12 weeks of treatment, while the responders to the antibody-based therapies did not. We found negative correlations between the changes in DAS28 and FoxP3fl ($r = -0.25$) and FoxP3Δ2 ($r = -0.82$) suggesting that a clinical improvement correlates to a reduction in FoxP3 mRNA expression. Similarly, we found significant negative correlation between the changes in DAS28 and CTLA-4 ($r = -0.80$) and also between changes in CRP and FoxP3Δ2 ($r = -0.67$) all in agreement with the data from paper I indicating a relationship between inflammation and Tregs.

Supplementary experiments using DNA Microarray technology

Introduction

DNA microarray technology has been developed for gene expression profiling of thousands of transcripts, and many genes in various biological systems have been demonstrated to be differentially regulated. This technology has mainly been used in two ways:

- As a tool for classification where disease signatures can be helpful in patient stratification (Aigner 2003; Heller 1997). This application requires, however, a rather large number of samples.
- Screening for candidate genes or pathways involved in a disease process.

In both cases the results may primarily be considered hypothesis generating. Thus, comparing genes differentially expressed in synovial CD4+ T cells with CD4+ T cells from blood may elucidate inflammatory mechanisms and possible targets, or simply confirm or refute theories. These analyses may disclose a molecular signature of the difference between circulating CD4+ T cells and CD4+ T cells at the site of inflammation. In this study the GeneChip® technology was chosen for transcript profiling due to the robustness and ease of use of this microarray technology. The principle of the DNA microarray analysis is outlined in figure 12. The details of the technique are beyond the scope of this thesis. For a detailed description of the technology please see: www.affymetrix.com.
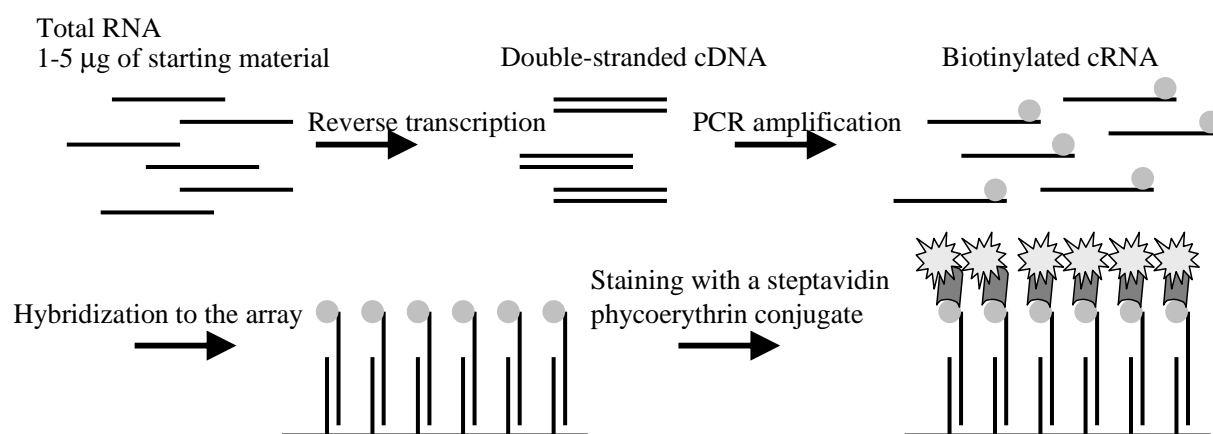
Figure 12. The principle of the DNA Microarray analysis. Total RNA is isolated from the sample of interest, reverse-transcribed into double-stranded cDNA, and further amplified by PCR resulting in biotinylated cRNA. The biotinylated product is hybridized to the array followed by staining with a streptavidin phycoerythrin conjugate. The microarray is then scanned in an instrument, where a laser excites the dye and a detector measures its emission.

Methods

DNA Microarray analysis was performed on CD4+ T cells isolated from blood and SF from three RAneg patients and three PsA patients. For logistic reasons it was not possible to include a group of RApos patients in the array study, as paired RNA samples from three RApos patients were not available, where the quality and amount of RNA met the high quality and large amount required for array analysis. The DNA Microarray technique required RNA preparations of 2-5 µg total RNA with a very high purity. Additionally, quantitative real-time RT-PCR was performed on the paired samples from two patients in each group.

Study population

Patients were recruited consecutively from the outpatients' clinic, Frederiksberg Hospital, Denmark. Inclusion criteria were: Flare of inflammatory arthritis in a knee joint; and indication for intra-articular injection of steroid. Exclusion criteria were: Osteoarthritis; contra-indications to joint aspiration; and the joint in question had been treated intra-articularly within the preceding month. Paired samples of blood and SF were collected from six patients; three diagnosed with rheumatoid arthritis (Arnett 1988) and three patients fulfilling the criteria for psoriatic arthritis (Veale 1994). All six patients were seronegative. See table 3 for patient characteristics.

Table 3. Patient characteristics.

Patient ID	Diagnosis	Female/Male	Age	Disease duration	prednisolone	MTX	QRT-PCR
PsA 14	PsA	F	63	5	No	No	Yes
PsA 50	PsA	F	57	37	No	No	No
PsA 59	PsA	F	46	2	No	Yes	Yes
RAneg 21	RAneg	F	45	9	No	Yes	Yes
RAneg 28	RAneg	M	62	1	Yes (oral)	No	No
RAneg 58	RAneg	F	52	10	No	No	Yes

F: female; M: male; Age and disease duration in years; MTX: methotrexate; QRT-PCR: quantitative real-time RT-PCR applied to sample.

cRNA and hybridisation

Preparations of cRNA and hybridisation were performed according to the protocols from Affymetrix. Briefly, total RNA was prepared from cells using Trizol followed by RNA cleanup using a Qiagen kit as described in paper II. Synthesis of double-stranded cDNA was performed using an oligo dT primer with the T7 RNA polymerase recognition sequence appended. Subsequently, biotinylated nucleotides were incorporated by in vitro transcription using T7 RNA polymerase, and the resulting cRNA transcripts were fragmented before hybridisation. The Human Genome U133 Plus 2.0 Arrays were used, and the chips were scanned using a laser scanner according to instructions from Affymetrix.

Treatment of data

Hybridisation results were captured and further processed using the Microarray Suite MAS 5.0 (Affymetrix) software. All hybridisation results were scaled to 100 using all probe sets, and the data were analysed using Data Mining Tools 3.1 (DMT) from Affymetrix and DNA chip analyzer 1.3 (dCHIP) (Li 2001). Initially samples from blood (PB) were compared to samples from SF. Comparison was made between samples from same individuals, using the PB samples as baseline. This resulted in six data sets, one from each patient involved. In the subsequent analysis differentially regulated genes were isolated.

In the Affymetrix programme DMT differentially regulated genes were scored as either increased (I) with a signal log ratio ≥ 1 or decreased (D) with a signal log ratio ≤ -1. A signal log ratio of +/- 1 corresponded to a two-fold up- or down-regulation. Furthermore, all D transcripts had to be scored as present in the PB sample, and all I transcripts had to be scored as present in the SF sample. In the dCHIP programme a gene was scored as differentially regulated if it had an expression value ratio SF/PB > 2.0 or PB/SF > 2.0; and a difference of more than 100 in signals between PB and SF samples.

Long lists of genes were created for each patient in each programme, but only genes scored as differentially regulated in both DMT and dCHIP in all three patient sets were analysed further. To be included in the list of differentially regulated genes common to all patients in a diagnosis group, the direction of the change also had to be the same in all the patients of the group.

RAneg, 3 patients   PsA, 3 patients
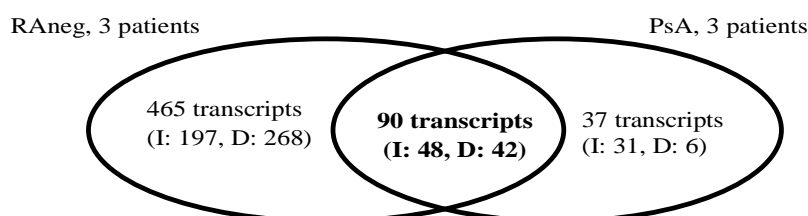
Figure 13. The number of differentially regulated genes common or specific for both patient groups using DMT *and* dCHIP.

465 transcripts (I: 197, D: 268) **90 transcripts (I: 48, D: 42)** 37 transcripts (I: 31, D: 6)

In the RAneg patients, 555 genes were differentially regulated, and in the PsA patients it was 127 genes. 90 genes were differentially regulated in both patient groups, and the direction of the change was the same, so that a gene up-regulated in one patient group was also up-regulated in the other patient group, see figure 13. The resulting numbers of differentially regulated genes using both programmes are listed in table 4.

Table 4. The number of differentially regulated gene transcripts in each patient using DMT, dCHIP or the intersection of the results from both. Gene lists shown in bold was the basis of the Gene Ontology pathway analysis, and gene lists marked with * was the basis of the Ingenuity Pathway analysis. Ptt: patients.

Patient ID	Data analysis	Number of transcripts	Increased	Decreased
RAneg 21	DMT	4352	2053	2299
	dCHIP	2164	1109	1055
	both	1979	1031	948
RAneg 28	DMT	5560	2050	3510
	dCHIP	3358	977	2381
	both	2940	914	2026
RAneg 58	DMT	5194	2222	2972
	dCHIP	2808	1213	1595
	both	2482	1132	1350
RAneg (3 ptt)	DMT	1257	543	714
	dCHIP	601	256	344
	both	**555***	**245**	**310**
PsA 14	DMT	3849	1919	1930
	dCHIP	1476	1103	373
	both	1375	1016	359
PsA 50	DMT	2011	1044	967
	dCHIP	1195	658	537
	both	931	503	428
PsA 59	DMT	5565	2856	2709
	dCHIP	2572	1593	979
	both	2380	1447	933
PsA (3 ptt)	DMT	326	162	164
	dCHIP	145	90	55
	Both	**127***	**79**	**48**

Results & Discussion

In four out of six patients, both the DNA Microarray analysis and the QRT-PCR were performed on the same samples. This gave me the opportunity to carry out a mutual validation of the techniques. Additionally to this analysis, the gene expression of a selected group of genes of special relevance will be discussed, and finally, a pathway analysis based on some of the gene lists shown in table 4 has been performed.

Comparing DNA Microarray and quantitative real-time PCR

In this section I the two techniques DNA Microarray and quantitative real-time PCR were compared. The two techniques were applied to eight paired samples from four patients, but also the additional results from the four paired samples included in the DNA Microarray, but not run in quantitative real-time PCR, will be discussed.

GAPDH and CD4

In the DNA Microarray analysis GAPDH and CD4 were scored as present in all 12 samples. GAPDH was scored as increased using PB samples as baseline, with a mean fold change of 1.8 for PsA and 2.0 for RAneg. This indicates that GAPDH is not a very good candidate for a housekeeping gene in these analyses, supporting the actual choice of CD4 as the internal reference when analyzing the QRT-PCR data. However, CD4 is not a perfect candidate either. On the DNA Microarrays, CD4 was not differentially regulated in three patients, while it was found decreased in one PsA patient (fold change: -1.5) and increased in two RAneg patients (fold change: 1.4 and 1.9). Related to CD4, the four patients which were analysed with QRT-PCR, two PsA patients were scored as decreased (fold change: -1.5) and no change in the DNA Microarray analysis, and two RAneg patients were scored as increased (fold change: 1.4) and no change in the DNA Microarray analysis.

It should be noted that, when looking at QRT-PCR results, samples that differ by a factor of two in the starting concentration of cDNA will theoretically be one cycle apart. Due to the semi-quantitative nature of as well QRT-PCR as the hybridisation readout from array studies, the QRT-PCR method requires a difference of 1 or more Ct to be considered reliable, and a fold change in the array readout of +/- 1.5 does not hamper the results. It is therefore concluded that a CD4 fold change within the range of +/- 1.5 does not influence the results, and thus support the use of CD4 as internal reference in the QRT-PCR analysis.

Comparing DNA Microarray analysis with QRT-PCR, it is not possible directly to compare the Ct values from the PCR system to the hybridisation signals from the array. Therefore, comparison of the fold changes in the paired samples was performed using PB samples as baseline.

CD25, CTLA-4 and FoxP3

With respect to CD25 and CTLA-4, the two methods are roughly in agreement. Table 5 lists the fold changes in the four patients in the PCR system and on the arrays. Also the change defined by Affymetrix is shown. A direct fold change comparison of the two methods with regard to the expression of FoxP3 splice forms is not possible, as the probes on the genechip do not distinguish between the splice forms.

Table 5. Fold changes using PB samples as baseline.

Patient	CD25 PCR	CD25 Array	Change Array	CTLA-4 PCR	CTLA-4 Array	Change Array
PsA 14	9.9	9.1	I	3.3	4.3	I
PsA 59	1.0	-1.5	NC	1.4	-1.2	NC
RAneg 21	1.1	1.6	I	1.1	1.9	I
RAneg 58	1.3	2.1	I	0.83 (-1.2)	1.1	NC

PCR: using QRT-PCR; Array: using DNA Microarray; I: Increased, NC: no change.

There are three probes related to FoxP3 present on the array; one probe (221334_S_AT) covers sequences in exons 8, 9, 10, and 11, which means it recognises all splice forms. In the PCR system, three of the four patients have increased expression of the different splice forms using PB samples as baseline, while one patient showed a decrease in expression. Comparable results were obtained with the array system as the direction of the change was the same on the arrays. One of the two other probes (221333_AT) covers sequences in introns right after exon 11 and right before exon 12, and as expected it gives no signals on the arrays. The third probe is situated in exon 12 after the stop codon and is therefore not relevant for this analysis. Based on this, it was concluded that the two methods are in general agreement, and the array data was therefore used for further studies.

Impurities/contamination

Monocytes and macrophages also express limited amounts of CD4 on the cell surface, and since CD4-positive selection was performed, the possibility of impurities from these cell types were analysed. At first two rather specific markers of the monocyte/macrophage lineage were analysed: CD14 and CD68. CD14 was scored as present on all 12 arrays, while CD68 was scored as present on 9 of the 12 arrays. From this it was concluded that both monocytes and macrophages were present in the CD4+ cell populations. CD14 gave rise to rather high signals on the arrays in both PB and SF samples. However, this does not imply that the relative abundance of monocytes was also high, since monocytes are both CD14high and CD4low when scored in flow cytometry. This means that a few monocytes can give rise to a strong CD14 mRNA signal. Secondly, contamination from B cells and CD8+ T cells were analysed. CD19 and CD8 were scored as absent on all 12 chips, and it was therefore assumed that there were no contaminating B cells or CD8+ T cells in the CD4-positive selected cell preparations.

Selected genes

Th17 cells

One type of cells, the Th17 cells, has recently attracted great attention in inflammatory conditions, specifically in immune-mediated tissue injury such as in the synovium in RA (Steinman 2007). The expression of IL-17 (IL-17A and IL-17F) appears to be restricted mainly to a small group of activated CD4+ T cells, Th17 cells. CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by the transcription factor RORγt (Maggi 2010). The orphan nuclear receptor RORγt is the key transcription factor that orchestrates the differentiation of this cell lineage, as RORγt induces transcription of the genes encoding IL-17 and the related cytokine IL-17F in naïve CD4+ T helper cells (Ivanov 2006). IL17A and IL17F, as well as RORγt, were scored as absent on the arrays, indicating the absence of Th17 cells in these samples. CD161 was scored as present in all samples with rather high

signals, indicating, perhaps, the presence of naïve CD4+ T cells underway to differentiation to Th17 cells as Th17 cells originate from CD161+ naïve CD4+ T cells (Cosmi 2008).

GARP

Recently, GARP (Glycoprotein A Repetitions Predominantly) synonymous with LRRC32 (Leucine Rich Repeat Containing 32) was identified as a Treg-specific protein induced upon activation of the T cell receptor (TCR) (Probst-Kepper 2010). GARP is supposedly involved in the control of FoxP3 by a positive feedback loop and is supposed to be a key receptor involved in the maintenance of the phenotype and function of activated Treg cells (Probst-Kepper 2010). GARP was scored as absent on all 12 arrays and thus not supporting the presence of active Tregs, unless the Tregs in these patients are not functioning. Alternatively, the absence of GARP could be interpreted as the presence of iTregs as these cells do not up-regulate GARP (Probst-Kepper 2010).

CD39

Recently, CD39, an ectonucleotidase responsible for enzymatic cleavage of ATP to AMP, has been reported on the surface of human Tregs (Borsellino 2007; Mandapathil 2009). ATP is released during tissues injury and activates the immune system. A proposed anti-inflammatory mechanism for Treg cells is the catalytic inactivation and conversion of extracellular ATP to AMP by surface associated CD39 (Borsellino 2007). In all but one of the six PB samples CD39 was scored as absent, or present with a low signal. CD39 was scored as present in all six SF samples with medium high signals. This resulted in CD39 being scored as increased in SF in five of six patients with fold changes between 2.7 and 5.5. In one PsA patient there was no change in expression of CD39 between PB and SF, reflecting a higher signal in the blood sample. These data support the increased presence of Treg cells in SF.

IL-7R

Treg cells are among other things characterised in flow cytometry by low expression of IL-7 receptor (IL-7R; a heterodimer consisting of CD127 and CD132) and high expression of CD25 (IL-2 receptor) on the cell surface (Seddiki 2006). IL-7R was present in all samples and gave rise to high signals on the arrays, and it was decreased in all SF samples except one PsA SF sample. These data indicate the presence of a mix of Treg cells and resting CD4+ T cells as the latter express high levels of IL-7R and very low levels of CD25 on the cell surface. IL-7 itself seems to be involved in the homeostasis of T cells in humans with a selective increase in non-Treg CD4+ T cells (Rosenberg 2006), but no IL-7 protein was found in the PB or SF samples, supporting the suggestion that the T cells present in the joint were recruited to the site of inflammation.

IL-2

IL-2 is a T cell growth factor produced by conventional T cells upon exposure to antigens, and it is critical for the development and peripheral expansion of CD4+CD25+ regulatory T cells (Nelson 2004; Scheffold 2005). IL-2 was scored as absent on all 12 arrays, indicating the presence of a large number of already committed Treg cells as well as naïve T cells among these CD4+ T cells isolated, as only activated T cells, and not Tregs, produce IL-2 (Scheffold 2005). Additionally, the cytokine IL-2 was not measurable in any of the PB or SF samples, which is in

agreement with previous results by other groups (Steiner 1999; Tukaj 2010). These data indicate that CD4+ T cells, including Tregs, present in SF are recruited to the site of inflammation rather than proliferate in the joint, cf. later.

Ki-67 protein

Ki-67 protein (MKI67; antigen identified by monoclonal antibody Ki-67) is a proliferation marker present only during active phases of the cell cycle, and absent in resting cells (Scholzen 2000). In this data set the Ki-67 protein was scored as absent or present with very low signals on the arrays supporting the hypothesis of Tregs being recruited to the joint rather than proliferate at the site. Additionally, it indicates the absence of activated T cells, as activated T cells proliferate.

CCR4

The issue of recruitment versus local proliferation has been addressed by others (Flytlie 2009; Iellem 2001; Jiao 2007; Oo 2010), and Jiao 2007 found that Treg cells in SF from RA patients expressed high levels of especially CCR4, a chemokine receptor possibly involved in controlling the trafficking of Treg cells into inflammatory tissues (Wei 2006). CCR4 is also reported as expressed on circulating CD4+ memory T cells, including skin-homing T cells (Flytlie 2009; Iellem 2001), and is therefore of interest regarding PsA. Moreover, CCR4 is up-regulated upon TCR-mediated activation and is more likely a marker for activated T cells (Iellem 2001). In this limited data set, differing results were found with increased expression in two PsA patients and one RAneg patient while the remaining three patients had no change in expression between PB and SF samples. The signals on the arrays varied between medium high signals in the three SF samples with increased expression to being scored as absent in two other SF samples. The two patients (one RAneg and one PsA) with CCR4 scored absent both had disease duration of 1- 2 years. Flytlie 2009 proposed that CCR4 expressing CD4+ T cells could be involved in spreading psoriasis to the joints as CCR4 was shown to be necessary for the migration of T cells to the skin, and both CCR4+ cells and the CCR4 ligand MDC/CCL22 were increased locally in the inflamed synovial membrane. However, my data does not necessarily support this hypothesis as the newly diagnosed PsA patient did not express CCR4 in SF.

Pathway study

Due to the large amounts of data generated in an array study, a pathway analysis is an obvious choice of approach to systematise the differentially expressed genes. The gene lists shown in bold in table 4 were analysed further using the Gene Ontology (GO) classification browser in the NetAffx™ Analysis Center (www.affymetrix.com) and the Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com).

Using the **GO annotations,** several pathways were represented among the differentially regulated genes. They are listed in table 6. Some genes are represented in more than one pathway, for example it was noticed that vitamin D receptor is present in the two pathways "nuclear receptors" and "ovarian infertility genes". The pathways were grouped into three categories: "Immune response and immune cell trafficking", "Cell cycle and metabolism", and "Miscellaneous".

In the category "Immune response and immune cell trafficking" several pathways are involved in cell migration, which may relate to the accumulation of immune cells in the SF: G-protein coupled receptors are associated with many cell surface receptors including chemokine receptors which may be especially relevant to homing of regulatory T cells to the synovium (Flytlie 2009). Calcium is part of signal transduction pathways such as G protein-coupled receptors, and the pathway "Calcium regulation in cardiac cells" includes genes involved in ion transport, protein binding, and receptor signalling, and does not indicate the presence of cardiac cells in the synovium. Integrins are cell adhesion molecules also involved in cell migration, and Wnt proteins are involved in calcium signalling through G proteins and gene activation in T cells. The TGF-β signalling pathway is involved in many cellular processes including cell differentiation, cell growth, and apoptosis. The differentiation of Treg cells requires TGF-β but is dependent on the transcription factor FoxP3. The inflammatory response pathway is relevant for this dataset, as arthritis is considered an inflammatory disorder. The category "Cell cycle and metabolism" comprises of several pathways involved in cell cycle, cell metabolism and growth, and includes DNA replication, mRNA processing, and glycolysis. The category "Miscellaneous" constitute the rest.

Table 6. Gene Ontology pathways	RAneg		PsA	
	Increased (Total 245)	Decreased (Total 310)	Increased (Total 79)	Decreased (Total 48)
Immune response and immune cell trafficking				
G-protein coupled receptor protein signalling pathway	21	5	4	4
Integrin-mediated cell adhesion	1	2	0	2
Calcium regulation in cardiac cells	7	4	4	1
Inflammatory Response Pathway	7	2	4	0
TGF-Beta Signalling Pathway	1	4	2	1
Wnt signalling	0	2	1	1
Cell cycle and metabolism				
Cell cycle	3	2	1	0
G1-to-S cell cycle	1	3	1	0
Glycolysis and Gluconeogenesis	4	0	0	0
Krebs-TCA Cycle	0	1	0	0
Pentose Phosphate Pathway	0	1	0	1
mRNA processing	0	5	0	0
Ribosomal Proteins	0	4	0	1
DNA replication	0	0	1	0
Nuclear Receptors	2	0	1	0
Miscellaneous				
Apoptosis	1	3	0	0
Circadian Exercise	3	0	1	0
Complement Activation Classical	3	0	0	0
Fatty Acid Degradation	3	1	0	0
Hypertrophy model	1	0	1	0
Ovarian Infertility Genes	2	2	1	0
Prostaglandin synthesis regulation	2	0	0	0
Smooth muscle contraction	3	1	1	1
Statin Pathway	0	0	1	0
Striated muscle contraction	1	0	0	0

Analysis based on gene list shown in bold in table 4

Using the **IPA software,** it was investigated if there was an association between the differentially regulated

genes and a specific pathway, and whether this was due to chance. Based on gene lists marked with * in table 4, IPA generated a list of canonical pathways associated with the dataset, as well as a p-value and a ratio for each pathway. Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. The ratio was calculated as: the number of molecules in a given pathway that is differentially regulated divided by the total number of molecules that makes up the given pathway.

However, the canonical pathways linked by IPA to the dataset were primarily pathways involving HLA molecules (MHC II) which are expressed on monocytes, but also on activated T cells and B cells. MHC II is highly expressed on monocytes giving rise to a strong fluorescence signal in flow cytometry compared to a low fluorescence signal from activated T cells. Together with the absence of IL-2 expression in the analysed CD4+ cells this indicates that the MHC II signals originate from monocytes. Hence, the pathways linked to the dataset by IPA reflect the minority of monocytes in the samples and not the CD4+ T cells present.

Through another feature of IPA it was possible to identify the biological functions that were most significant to the molecules differentially regulated in the SF samples compared to the PB samples. The molecules associated with biological functions in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned was due to chance alone. The molecular and cellular functions are listed in table 7.

Table 7.
Ingenuity Pathway Analysis - Molecular and cellular functions

RAneg

Name	p-value	# Molecules
Cell-To-Cell Signalling and Interaction	$4.57*10^{-7}$ - $2.10*10^{-2}$	45
Cellular Growth and Proliferation	$7.55*10^{-7}$ - $2.44*10^{-2}$	86
Cellular Movement	$2.25*10^{-5}$ - $2.45*10^{-2}$	50
Cellular Development	$5.25*10^{-5}$ - $2.51*10^{-2}$	47
Cell Cycle	$3.18*10^{-4}$ - $2.16*10^{-2}$	16

PsA

Name	p-value	# Molecules
Cellular Movement	$2.72*10^{-7}$ - $3.31*10^{-2}$	23
Cellular Growth and Proliferation	$1.41*10^{-6}$ - $3.31*10^{-2}$	32
Cell-To-Cell Signalling and Interaction	$4.88*10^{-5}$ - $3.31*10^{-2}$	17
Cell Cycle	$6.96*10^{-5}$ - $3.31*10^{-2}$	7
Cell Death	$1.94*10^{-4}$ - $3.31*10^{-2}$	28

Analysis based on gene list marked with * in table 4

In conclusion the array data point mostly towards the presence of cell-traffic related molecules as well as to antigen presentation due to monocyte contamination. This indicates that the cells present in the SF are cells attracted to the site of inflammation.

Closing remarks

Several questions arose regarding FoxP3 in RA: What kind of Tregs are increased, nTregs or iTregs? What are the implications of an increase in numbers of iTregs rather than nTregs or vice versa? CTLA-4 is thought to play an important part in the suppressive function of nTregs, and the lack of a concomitant increase in CTLA-4 expression suggests the presence of dysfunctional nTregs in arthritis patients. However, based on our data we cannot exclude the possibility of an increase of in vivo generated iTregs rather than dysfunctional nTregs. The importance of CTLA-4 is highlighted by the fact that Abatacept, which is a CLTA-4-IgG fusion protein, is effective in RA, and one could speculate that Abatacept mimics the actions of functional nTregs.

The iTregs are generated in the periphery after TCR activation and in the presence of TGF-beta and, unlike nTregs, they are reported to be resistant to the influence of IL-6. The cytokine IL-6 can convert nTregs to Th17 cells, while the iTregs are resistant to this cytokine and may retain their suppressive function at inflammatory sites, such as SF, rich in IL-6. It is quite possible that nTregs and iTregs have different roles in immune responses and react differently in an inflammatory milieu. Fujimoto suggested that "the TCR repertoire between nTreg and iTregs are different and that nTregs may be important for the maintenance of self-tolerance via recognizing self-antigens, whereas iTregs generated from conventional T cells may be involved in tolerance to non-self antigens" (Fujimoto 2011). Based on their study in mice which implied a different effect of IL-6 on nTregs and iTregs, Fujimoto further suggested that "IL-6 may thus enhance foreign antigen-specific immune response by acting differently on nTregs and iTregs" (Fujimoto 2011). Using this model in RA, the high levels of IL-6 reported in SF from RA patients by us and others would tip the balance towards iTregs and enhance a specific immune response against foreign antigens when a response against self antigens is needed.

Another relevant question came up while interpreting the QRT-PCR data: Are we looking at activated T cells rather than Tregs? Based only on the QRT-PCR data we cannot say for certain because Tregs and activated T cells all express high levels of CD25 and also CTLA-4, but based on the DNA Microarray data the answer is no. The activation/proliferation markers IL-2 and Ki-67 were scored as absent or present with low signals on the arrays. Additionally, IL-7R was present with rather high signals on the arrays indicating the presence of resting CD4+ T cells, as they express high levels of IL-7R and very low levels of CD25 on the cell surface. FoxP3 is transiently expressed in activated T cells, but according to our array data there were no activated T cells in these samples, and increased FoxP3 expression therefore indicated increased Treg counts. From this we conclude that we are looking at a mix of resting CD4+ T cells and Treg cells, but that we cannot distinguish between nTregs and iTregs.

An alternative splice form of CTLA-4 has been reported, lacking the trans-membrane region encoded by exon two resulting in a soluble form of the protein. Our probe and primers are located in exon two, and we can only detect the full length CTLA-4 mRNA resulting in the membrane form. The membrane version of CTLA-4 is up-regulated upon T cell activation while the soluble form is down-regulated by T cell activation. This information further supports the data on the lack of activated T cells in our samples as membrane CTLA-4 is not up-regulated while FoxP3 is.

We addressed the question of the increased presence of Tregs in SF, enrichment or proliferation, and based

on our data we favour the concept of enrichment. According to the array data the proliferation marker Ki-67 is not present, indicating the absence of proliferating Tregs, and leaving two options: selective recruitment (nTreg) or induction (iTreg). The presence of cell-traffic related molecules, according to the array data, could be in support of selective recruitment of nTregs but could also be due to general trafficking of immune cells attracted to the site of inflammation, and the absence of GARP on the array may indicate the presence of iTregs as these cells do not up-regulate GARP.

Possibly, there is a difference between activated and non-activated T cells regarding the expression of FoxP3 mRNA splice forms. In our data set, looking at non-activated T cells, we saw a trend towards a difference in the ratio between the full length and Δ2 splice of forms of FoxP3 between RA patients and HC. Previously a Δ2Δ7 splice form was identified as over-expressed in T cells from patients suffering from Sézary Syndrome, and also *ex vivo* stimulated CD4+CD25+ T cells had an increased expression of a Δ7 splice form of FoxP3. These results differ from ours as we only detected very small amounts of FoxP3 splice forms lacking exon seven in RA patients and HC. According to the array data these T cells were not activated, and that could explain the lack of either Δ7 splice form.

Anti-TNF drugs do have an effect in RA as demonstrated with changes in DAS28 and VAS scores. The effect on FoxP3 expression in our dataset was not unequivocal as we observed a decrease in the Etanercept group and a trend towards an increase in the Infliximab group when looking only at patients who benefitted from the treatment. It is difficult to be conclusive about the effect of anti-TNF therapies on Tregs, possibly because they are not the primary target and only influenced by changes in the entire inflammatory milieu.

The ongoing disease despite a relative abundance of Tregs in the blood and even more so locally in the joint is a paradox. Accumulation of Tregs close to the site of inflammation may represent a futile attempt to suppress the auto-reactive inflammation. The Tregs may be inherently dysfunctional or rendered dysfunctional due to the local inflammatory milieu.

Future perspectives

Flow cytometry or Western blot to verify the protein expression is an obvious choice for further studies, especially if it has become possible to buy splice form specific antibodies for flow cytometry and/or Western blot. Furthermore, functional studies elucidating possible suppressive mechanisms targeting the pathogenic processes involved in RA could be used to identify targets for future therapy. Also it would be important to identify the predicted differences in specificity of the TCR repertoire between nTregs and iTregs. The recently developed technologies of MHC multimer reagents, for example the Dextramer[TM] technology, enable detection of antigen-specific CD8+ T cells. When this technology includes detection of antigen-specific CD4+ T cells we can address this question and a whole new range of possibilities emerges.

Therapy using Treg cells is the next step. There are several approaches to consider when contemplating Treg cell therapy: activation and expansion of antigen-specific nTregs *in vivo*, the use of *in vitro* expanded nTregs transferred to the patient, or the conversion of naïve T cells to antigen-specific iTregs *in vivo* or *in vitro*. Future studies to identify specific surface markers that can differentiate between Treg cells and effector cells are essential for future manipulation of Tregs. Treg cells proliferate poorly *in vitro* and the expansion of possible contaminating effector T cells could be a factor. Also, it is important to elucidate the mechanisms of action of Tregs in order to be able to control their function in a clinical setting. Immunotherapy with Treg cells has been used for experimental treatment of acute graft-versus-host disease (Brunstein 2011). The study by Brunstein and colleagues was a "first-in-human" clinical trial and demonstrated that it is possible and safe, and that it decreased the risk of acute graft-versus-host disease in the recipients of these *ex vivo* expanded umbilical cord blood Tregs. One could speculate that the mechanism of action in the case of these *ex vivo* expanded Tregs is the work of CTLA-4 co-stimulation and the use of Abatacept would be equally successful. The effect of Treg cell therapy in RA is debatable, and if, as our data and others suggest, Tregs are dysfunctional in RA, the challenges regarding future Treg therapy in RA will be to expand the right Treg population and not a dysfunctional one. Another strategy could be to use *ex vivo* expanded umbilical cord blood Tregs as described by Brunstein. However allograft is not feasible when considering the pros and cons and would probably only be used in extremely severe cases. Whether Treg cell therapy will have an effect in RA patients still remains unanswered.

Reference List

1. Aerts NE, Dombrecht EJ, Ebo DG, Bridts CH, Stevens WJ, and De Clerck LS. (2008) *Activated T cells complicate the identification of regulatory T cells in rheumatoid arthritis.* Cellular Immunology. 251(2); 109-115.

2. Afzali B, Lombardi G, Lechler RI, and Lord GM. (2007) *The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease.* Clinical and Experimental Immunology. 148(1); 32-46.

3. Aigner T and Dudhia J. (2003) *Genomics of osteoarthritis.* Current Opinion in Rheumatology. 15(5); 634-640.

4. Albers JM, Kuper HH, van Riel PL, Prevoo ML, van 't Hof MA, van Gestel AM, and Severens JL. (1999) *Socio-economic consequences of rheumatoid arthritis in the first years of the disease.* Rheumatology.(Oxford). 38(5); 423-430.

5. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, III, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Menard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovsky J, Wolfe F, and Hawker G. (2010) *2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative.* Arthritis and Rheumatism. 62(9); 2569-2581.

6. Allan SE, Broady R, Gregori S, Himmel ME, Locke N, Roncarolo MG, Bacchetta R, and Levings MK. (2008) *CD4(+) T-regulatory cells: toward therapy for human diseases.* Immunological Reviews. 223 391-421.

7. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG, and Levings MK. (2007) *Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production.* International Immunology. 19(4); 345-354.

8. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai MY, Orban PC, Ziegler SF, Roncarolo MG, and Levings MK. (2005) *The role of 2 FOXP3 isoforms in the generation of human CD4(+) Tregs.* Journal of Clinical Investigation. 115(11); 3276-3284.

9. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, and Hunder GG. (1988) *The American-Rheumatism-Association 1987 Revised Criteria for the Classification of Rheumatoid-Arthritis.* Arthritis and Rheumatism. 31(3); 315-324.

10. Bettelli E, Dastrange M, and Oukka M. (2005) *Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells.* Proceedings of the National Academy of Sciences of the United States of America. 102(14); 5138-5143.

11. Bliddal H and Torp-Pedersen S. (2000) *Use of small amounts of ultrasound guided air for injections.* Annals of the Rheumatic Diseases. 59(11); 926-927.

12. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staege MS, Stassen M, Jonuleit H, and Schmitt E. (2007) *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression.* Journal of Experimental Medicine. 204(6); 1303-1310.

13. Borsellino G, Kleinewietfeld M, Di MD, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D,

Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Rotzschke O, and Falk K. (2007) *Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression.* Blood. 110(4); 1225-1232.

14. Bour-Jordan H and Bluestone JA. (2009) *Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells.* Immunological Reviews. 229 41-66.

15. Bours MJ, Swennen EL, Di VF, Cronstein BN, and Dagnelie PC. (2006) *Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation.* Pharmacology and Therapeutics. 112(2); 358-404.

16. Brahn E and Scoville CD. (1988) *Biochemical markers of disease activity.* Baillieres Clinical Rheumatology. 2(1); 153-183.

17. Brennan F and Beech J. (2007) *Update on cytokines in rheumatoid arthritis.* Current Opinion in Rheumatology. 19(3); 296-301.

18. Brennan FM and McInnes IB. (2008) *Evidence that cytokines play a role in rheumatoid arthritis.* J.Clin.Invest. 118(11); 3537-3545.

19. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, Defor T, Levine BL, June CH, Rubinstein P, McGlave PB, Blazar BR, and Wagner JE. (2011) *Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics.* Blood. 117(3); 1061-1070.

20. Cao D, Borjesson O, Larsson P, Rudin A, Gunnarsson I, Klareskog L, Malmstrom V, and Trollmo C. (2006) *FOXP3 identifies regulatory CD25(brignt)CD4(+) T cells in rheumatic joints.* Scandinavian Journal of Immunology. 63(6); 444-452.

21. Cao DJ, van Vollenhoven R, Klareskog L, Trollmo C, and Malmstrom V. (2004) *CD25(bright)CD4(+) regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease.* Arthritis Research & Therapy. 6(4); R335-R346.

22. Cools N, Ponsaerts P, Van T, V, and Berneman ZN. (2007) *Regulatory T cells and human disease.* Clin.Dev.Immunol. 2007 89195-

23. Cosmi L, De PR, Santarlasci V, Maggi L, Capone M, Frosali F, Rodolico G, Querci V, Abbate G, Angeli R, Berrino L, Fambrini M, Caproni M, Tonelli F, Lazzeri E, Parronchi P, Liotta F, Maggi E, Romagnani S, and Annunziato F. (2008) *Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor.* Journal of Experimental Medicine. 205(8); 1903-1916.

24. Costenbader KH, Feskanich D, Mandl LA, and Karlson EW. (2006) *Smoking intensity, duration, and cessation, and the risk of rheumatoid arthritis in women.* American Journal of Medicine. 119(6); 503-509.

25. Curotto de Lafaille MA and Lafaille JJ. (2009) *Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor?* Immunity. 30(5); 626-635.

26. da Mota LM, dos Santos Neto LL, and de Carvalho JF. (2009) *Autoantibodies and other serological markers in rheumatoid arthritis: predictors of disease activity?* Clinical Rheumatology. 28(10); 1127-1134.

27. Delerive P, Monte D, Dubois G, Trottein F, Fruchart-Najib J, Mariani J, Fruchart JC, and Staels B. (2001) *The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response.* EMBO Rep. 2(1); 42-48.

28. Doan T and Massarotti E. (2005) *Rheumatoid arthritis: An overview of new and emerging therapies.* Journal of Clinical Pharmacology. 45(7); 751-762.

29. Du J, Huang C, Zhou B, and Ziegler SF. (2008) *Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3.* Journal of Immunology. 180(7); 4785-4792.

30. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, and Mauri C. (2004) *Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF alpha therapy.* Journal of Experimental Medicine. 200(3); 277-285.

31. Feldmann M, Brennan FM, and Maini RN. (1996) *Role of cytokines in rheumatoid arthritis.* Annual Review of Immunology. 14 397-440.

32. Firestein GS. (2003) *Evolving concepts of rheumatoid arthritis.* Nature. 423(6937); 356-361.

33. Firestein GS and Zvaifler NJ. (2002) *How important are T cells in chronic rheumatoid synovitis? II. T cell-independent mechanisms from beginning to end.* Arthritis and Rheumatism. 46(2); 298-308.

34. Flores-Borja F, Jury EC, Mauri C, and Ehrenstein MR. (2008) *Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis.* Proc.Natl.Acad.Sci.U.S A. 105(49); 19396-19401.

35. Flytlie HA, Hvid M, Lindgreen E, Kofod-Olsen E, Petersen EL, Jorgensen A, Deleuran M, Vestergaard C, and Deleuran B. (2009) *Expression of MDC/CCL22 and its receptor CCR4 in rheumatoid arthritis, psoriatic arthritis and osteoarthritis.* Cytokine.

36. Fontenot JD, Gavin MA, and Rudensky AY. (2003) *Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells.* Nature Immunology. 4(4); 330-336.

37. Fournier C. (2005) *Where do T cells stand in rheumatoid arthritis?* Joint Bone Spine. 72(6); 527-532.

38. Fox DA. (2000) *Cytokine blockade as a new strategy to treat rheumatoid arthritis: inhibition of tumor necrosis factor.* Archives of Internal Medicine. 160(4); 437-444.

39. Fujimoto M, Nakano M, Terabe F, Kawahata H, Ohkawara T, Han Y, Ripley B, Serada S, Nishikawa T, Kimura A, Nomura S, Kishimoto T, and Naka T. (2011) *The influence of excessive IL-6 production in vivo on the development and function of Foxp3+ regulatory T cells.* Journal of Immunology. 186(1); 32-40.

40. Furer V, Greenberg JD, Attur M, Abramson SB, and Pillinger MH. (2010) *The role of microRNA in rheumatoid arthritis and other autoimmune diseases.* Clinical Immunology. 136(1); 1-15.

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

42. Grigoryev YA, Kurian SM, Nakorchevskiy AA, Burke JP, Campbell D, Head SR, Deng J, Kantor AB, Yates JR, III, and Salomon DR. (2009) *Genome-wide analysis of immune activation in human T and B cells reveals distinct classes of alternatively spliced genes.* PLoS.One. 4(11); e7906-

43. Hayashida K, Nanki T, Girschick H, Yavuz S, Ochi T, and Lipsky PE. (2001) *Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8.* Arthritis Research. 3(2); 118-126.

44. Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, Woolley DE, and Davis RW. (1997) *Discovery and analysis of inflammatory disease-related genes using cDNA microarrays.* Proceedings of

the National Academy of Sciences of the United States of America. 94(6); 2150-2155.

45. Hermiston ML, Xu Z, and Weiss A. (2003) *CD45: a critical regulator of signaling thresholds in immune cells.* Annual Review of Immunology. 21 107-137.

46. Holmes N. (2008) *Immunology. A splicing switch for T cells.* Science. 321(5889); 646-647.

47. Hori S, Nomura T, and Sakaguchi S. (2003) *Control of regulatory T cell development by the transcription factor Foxp3.* Science. 299(5609); 1057-1061.

48. Horwitz DA, Zheng SG, and Gray JD. (2008) *Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other.* Trends Immunol. 29(9); 429-435.

49. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Simigaglia F, and D'Ambrosio D. (2001) *Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells.* Journal of Experimental Medicine. 194(6); 847-853.

50. Ip JY, Tong A, Pan Q, Topp JD, Blencowe BJ, and Lynch KW. (2007) *Global analysis of alternative splicing during T-cell activation.* Rna-A Publication of the Rna Society. 13(4); 563-572.

51. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, and Littman DR. (2006) *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell. 126(6); 1121-1133.

52. Jiao Z, Wang W, Jia R, Li J, You H, Chen L, and Wang Y. (2007) *Accumulation of FoxP3-expressing CD4(+)CD25(+) T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis.* Scandinavian Journal of Rheumatology. 36(6); 428-433.

53. Kaur G, Goodall JC, Jarvis LB, and Hill Gaston JS. (2010) *Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells--identification of Foxp3Delta7 in human regulatory T cells.* Molecular Immunology. 48(1-3); 321-332.

54. Khattar M, Chen W, and Stepkowski SM. (2009) *Expanding and converting regulatory T cells: a horizon for immunotherapy.* Arch.Immunol.Ther.Exp.(Warsz.). 57(3); 199-204.

55. Khattri R, Cox T, Yasayko SA, and Ramsdell F. (2003) *An essential role for Scurfin in CD4(+)CD25(+) T regulatory cells.* Nature Immunology. 4(4); 337-342.

56. Kobayashi S, Momohara S, Kamatani N, and Okamoto H. (2008) *Molecular aspects of rheumatoid arthritis: role of environmental factors.* FEBS J. 275(18); 4456-4462.

57. Krejsgaard T, Gjerdrum LM, Ralfkiaer E, Lauenborg B, Eriksen KW, Mathiesen AM, Bovin LF, Gniadecki R, Geisler C, Ryder LP, Zhang Q, Wasik MA, Odum N, and Woetmann A. (2008) *Malignant Tregs express low molecular splice forms of FOXP3 in Sezary syndrome.* Leukemia.

58. Law JP, Hirschkorn DF, Owen RE, Biswas HH, Norris PJ, and Lanteri MC. (2009) *The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+CD25+Foxp3+ regulatory T cells.* Cytometry A. 75(12); 1040-1050.

59. Li C and Wong WH. (2001) *Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection.* Proc.Natl.Acad.Sci.U.S.A. 98(1); 31-36.

60. Livak KJ and Schmittgen TD. (2001) *Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method.* Methods. 25(4); 402-408.

61. Lopes JE, Torgerson TR, Schubert LA, Anover SD, Ocheltree EL, Ochs HD, and Ziegler SF. (2006) *Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor.* Journal of Immunology. 177(5); 3133-3142.

62. Lubberts E, Koenders MI, and van den Berg WB. (2004) *The role of T cell interleukin-17 in conducting destructive arthritis: lessons from animal models.* Arthritis Research & Therapy. 7(1); 29-37.

63. Lundy SK, Sarkar S, Tesmer LA, and Fox DA. (2007) *Cells of the synovium in rheumatoid arthritis. T lymphocytes.* Arthritis Res.Ther. 9(1); 202-

64. Lynch KW. (2004) *Consequences of regulated pre-mRNA splicing in the immune system.* Nature Reviews Immunology. 4(12); 931-940.

65. Maggi L, Santarlasci V, Capone M, Peired A, Frosali F, Crome SQ, Querci V, Fambrini M, Liotta F, Levings MK, Maggi E, Cosmi L, Romagnani S, and Annunziato F. (2010) *CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC.* European Journal of Immunology. 40(8); 2174-2181.

66. Magistrelli G, Jeannin P, Herbault N, oit De CA, Gauchat JF, Bonnefoy JY, and Delneste Y. (1999) *A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells.* European Journal of Immunology. 29(11); 3596-3602.

67. Mailer RK, Falk K, and Rotzschke O. (2009) *Absence of leucine zipper in the natural FOXP3Delta2Delta7 isoform does not affect dimerization but abrogates suppressive capacity.* PLoS One. 4(7); e6104-

68. Mandapathil M, Lang S, Gorelik E, and Whiteside TL. (2009) *Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression.* Journal of Immunological Methods. 346(1-2); 55-63.

69. Mannik M, Nardella FA, and Sasso EH. (1988) *Rheumatoid factors in immune complexes of patients with rheumatoid arthritis.* Springer Seminars in Immunopathology. 10(2-3); 215-230.

70. Mellor AL and Munn DH. (2004) *IDO expression by dendritic cells: tolerance and tryptophan catabolism.* Nat.Rev.Immunol. 4(10); 762-774.

71. Meyer T, Kneissel M, Mariani J, and Fournier B. (2000) *In vitro and in vivo evidence for orphan nuclear receptor RORalpha function in bone metabolism.* Proc.Natl.Acad.Sci.U.S.A. 97(16); 9197-9202.

72. Milpied P, Renand A, Bruneau J, Mendes-da-Cruz DA, Jacquelin S, Asnafi V, Rubio MT, MacIntyre E, Lepelletier Y, and Hermine O. (2009) *Neuropilin-1 is not a marker of human Foxp3+ Treg.* European Journal of Immunology. 39(6); 1466-1471.

73. Mocellin S, Provenzano M, Rossi CR, Pilati P, Nitti D, and Lise M. (2003) *Use of quantitative real-time PCR to determine immune cell density and cytokine gene profile in the tumor microenvironment.* Journal of Immunological Methods. 280(1-2); 1-11.

74. Mottonen M, Heikkinen J, Mustonen L, Isomaki P, Luukkainen R, and Lassila O. (2005) *CD4(+) CD25(+) T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis.* Clinical and Experimental Immunology. 140(2); 360-367.

75. Murphy K, Travers P, and Walport M. (2008) *Janeway's Immunobiology.* 7th edition

76. Mygind T, Birkelund S, Birkebaek NH, Ostergaard L, Jensen JS, and Christiansen G. (2002) *Determination of PCR efficiency in chelex-100 purified clinical samples and comparison of real-time quantitative PCR and conventional PCR for detection of Chlamydia pneumoniae.* BMC.Microbiol. 2 17-

77. Nadkarni S, Mauri C, and Ehrenstein MR. (2007) *Anti-TNF-{alpha} therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-{beta}.* Journal of Experimental Medicine. 204(1); 33-39.

78. Naz SM and Symmons DP. (2007) *Mortality in established rheumatoid arthritis.* Best.Pract.Res.Clin.Rheumatol. 21(5); 871-883.

79. Nelson BH. (2004) *IL-2, regulatory T cells, and tolerance.* Journal of Immunology. 172(7); 3983-3988.

80. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, Saigo K, Morinobu A, Koshiba M, Kuntz KM, Kamae I, and Kumagai S. (2007) *Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis.* Annals of Internal Medicine. 146(11); 797-808.

81. Oberdoerffer S, Moita LF, Neems D, Freitas RP, Hacohen N, and Rao A. (2008) *Regulation of CD45 alternative splicing by heterogeneous ribonucleoprotein, hnRNPLL.* Science. 321(5889); 686-691.

82. Oderup C, Cederbom L, Makowska A, Cilio CM, and Ivars F. (2006) *Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression.* Immunology. 118(2); 240-249.

83. Okroj M, Heinegard D, Holmdahl R, and Blom AM. (2007) *Rheumatoid arthritis and the complement system.* Annals of Medicine. 39(7); 517-530.

84. Onishi Y, Fehervari Z, Yamaguchi T, and Sakaguchi S. (2008) *Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation.* Proc.Natl.Acad.Sci.U.S.A. 105(29); 10113-10118.

85. Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, Shetty S, Harki J, Shaw JC, Eksteen B, Hubscher SG, Walker LS, and Adams DH. (2010) *Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver.* Journal of Immunology. 184(6); 2886-2898.

86. Padyukov L, Silva C, Stolt P, Alfredsson L, and Klareskog L. (2004) *A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis.* Arthritis and Rheumatism. 50(10); 3085-3092.

87. Pandiyan P, Zheng L, Ishihara S, Reed J, and Lenardo MJ. (2007) *CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells.* Nat.Immunol. 8(12); 1353-1362.

88. Papagoras C, Voulgari PV, and Drosos AA. (2010) *Strategies after the failure of the first anti-tumor necrosis factor alpha agent in rheumatoid arthritis.* Autoimmun.Rev. 9(8); 574-582.

89. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, and van Riel PL. (1995) *Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis.* Arthritis and Rheumatism. 38(1); 44-48.

90. Probst-Kepper M, Balling R, and Buer J. (2010) *FOXP3: required but not sufficient. the role of GARP (LRRC32) as a safeguard of the regulatory phenotype.* Curr.Mol.Med. 10(6); 533-539.

91. Quinn MA, Gough AK, Green MJ, Devlin J, Hensor EM, Greenstein A, Fraser A, and Emery P. (2005) *Anti-CCP antibodies measured at disease onset help identify seronegative rheumatoid arthritis and predict radiological and functional outcome.* Rheumatology (Oxford). 45(4); 478-480.

92. Qvistgaard E, Rogind H, Torp-Pedersen S, Terslev L, Danneskiold-Samsoe B, and Bliddal H. (2001) *Quantitative ultrasonography in rheumatoid arthritis: evaluation of inflammation by Doppler technique.* Annals of the Rheumatic Diseases. 60(7); 690-693.

93. Raghavan S, Cao D, Widhe M, Roth K, Herrath J, Engstrom M, Roncador G, Banham AH, Trollmo C, Catrina AI, and Malmstrom V. (2009) *FOXP3 expression in blood, synovial fluid and synovial tissue during inflammatory arthritis and intra-articular corticosteroid treatment.* Annals of the Rheumatic Diseases. 68(12); 1908-1915.

94. Ramsdell F. (2003) *Foxp3 and natural regulatory T cells: key to a cell lineage?* Immunity. 19(2); 165-168.

95. Raza K, Falciani F, Curnow SJ, Ross EJ, Lee CY, Akbar AN, Lord JM, Gordon C, Buckley CD, and Salmon M. (2005) *Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin.* Arthritis Research & Therapy. 7(4); R784-R795.

96. Rose NR and Bona C. (1993) *Defining criteria for autoimmune diseases (Witebsky's postulates revisited).* Immunology Today. 14(9); 426-430.

97. Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, Stetler-Stevenson M, Morton KE, Mavroukakis SA, Morre M, Buffet R, Mackall CL, and Gress RE. (2006) *IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells.* Journal of Immunotherapy. 29(3); 313-319.

98. Rubbert-Roth A and Finckh A. (2009) *Treatment options in patients with rheumatoid arthritis failing initial TNF inhibitor therapy: a critical review.* Arthritis Res.Ther. 11 Suppl 1 S1-

99. Rudolph EH and Woods JM. (2005) *Chemokine expression and regulation of angiogenesis in rheumatoid arthritis.* Curr.Pharm.Des. 11(5); 613-631.

100. Ryder LR, Woetmann A, Madsen H, Odum N, Ryder LP, Bliddal H, Danneskiold-Samsoe B, Ribel-Madsen S, and Bartels E. (2010) *Expression of full-length and splice forms of FoxP3 in rheumatoid arthritis.* Scandinavian Journal of Rheumatology.

101. Sakaguchi S, Yamaguchi T, Nomura T, and Ono M. (2008) *Regulatory T cells and immune tolerance.* Cell. 133(5); 775-787.

102. Scheffold A, Huhn J, and Hofer T. (2005) *Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango.* European Journal of Immunology. 35(5); 1336-1341.

103. Scheffold A, Murphy KM, and Hofer T. (2007) *Competition for cytokines: T(reg) cells take all.* Nat.Immunol. 8(12); 1285-1287.

104. Schmittgen TD and Livak KJ. (2008) *Analyzing real-time PCR data by the comparative C(T) method.* Nat.Protoc. 3(6); 1101-1108.

105. Scholzen T and Gerdes J. (2000) *The Ki-67 protein: from the known and the unknown.* J.Cell Physiol. 182(3); 311-322.

106. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, and Ziegler SF. (2001) *Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation.* Journal of Biological Chemistry. 276(40); 37672-37679.

107. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, and Fazekas de St GB. (2006) *Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells.* Journal of Experimental Medicine. 203(7); 1693-1700.

108. Shevach EM. (2006) *From vanilla to 28 flavors: multiple varieties of T regulatory cells.* Immunity. 25(2); 195-201.

109. Silman AJ, MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, and Ollier WE. (1993) *Twin concordance rates for rheumatoid arthritis: results from a nationwide study.* British Journal of Rheumatology. 32(10); 903-907.

110. Smith EL, Finney HM, Nesbitt AM, Ramsdell F, and Robinson MK. (2006) *Splice variants of human FOXP3 are functional inhibitors of human CD4(+) T-cell activation.* Immunology. 119(2); 203-211.

111. Sokka T, Abelson B, and Pincus T. (2008) *Mortality in rheumatoid arthritis: 2008 update.* Clinical and Experimental Rheumatology. 26(5 Suppl 51); S35-S61.

112. Steiner G, Tohidast-Akrad M, Witzmann G, Vesely M, Studnicka-Benke A, Gal A, Kunaver M, Zenz P, and Smolen JS. (1999) *Cytokine production by synovial T cells in rheumatoid arthritis.* Rheumatology. 38(3); 202-213.

113. Steinman L. (2007) *A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage.* Nature Medicine. 13(2); 139-145.

114. Tang QZ, Boden EK, Henriksen KJ, Bour-Jordan H, Bi MY, and Bluestone JA. (2004) *Distinct robes of CTLA-4 and TGF-beta in CD4(+)CD25(+) regulatory T cell function.* European Journal of Immunology. 34(11); 2996-3005.

115. Taylor PC and Feldmann M. (2009) *Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis.* Nat.Rev.Rheumatol. 5(10); 578-582.

116. Terslev L, Torp-Pedersen S, Qvistgaard E, Danneskiold-Samsoe B, and Bliddal H. (2003) *Estimation of inflammation by Doppler ultrasound: Quantitative changes after intra-articular treatment in rheumatoid arthritis* . Ann Rheum Dis. 62 1049-1053.

117. Tili E, Michaille JJ, Costinean S, and Croce CM. (2008) *MicroRNAs, the immune system and rheumatic disease.* Nat.Clin.Pract.Rheumatol. 4(10); 534-541.

118. Tracey D, Klareskog L, Sasso EH, Salfeld JG, and Tak PP. (2008) *Tumor necrosis factor antagonist mechanisms of action: a comprehensive review.* Pharmacology and Therapeutics. 117(2); 244-279.

119. Tukaj S, Kotlarz A, Jozwik A, Smolenska Z, Bryl E, Witkowski JM, and Lipinska B. (2010) *Cytokines of the Th1 and Th2 type in sera of rheumatoid arthritis patients; correlations with anti-Hsp40 immune response and diagnostic markers.* Acta Biochimica Polonica. 57(3); 327-332.

120. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, and Lipsky PE. (2006) *TNF downmodulates the function of human CD4(+)CD25(hi) T-regulatory cells.* Blood. 108(1); 253-261.

121. Van Amelsfort JMR, Jacobs KMG, Bijlsma JWJ, Lafeber FPJG, and Taams LS. (2004) *CD4+CD25+ regulatory T cells in rheumatoid arthritis - Differences in the presence, phenotype, and function between peripheral blood and synovial fluid.* Arthritis and Rheumatism. 50(9); 2775-2785.

122. van der Vliet H and Nieuwenhuis EE. (2007) *IPEX as a result of mutations in FOXP3.* Clin.Dev.Immunol. 2007 89017-

123. van Gaalen F, Ioan-Facsinay A, Huizinga TW, and Toes RE. (2005) *The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis.* Journal of Immunology. 175(9); 5575-5580.

124. van Gestel AM, Haagsma CJ, and van Riel PL. (1998) *Validation of rheumatoid arthritis improvement*

criteria that include simplified joint counts. Arthritis and Rheumatism. 41(10); 1845-1850.

125. Vandenbark AA, Culbertson NE, Bartholomew RM, Huan J, Agotsch M, LaTocha D, Yadav V, Mass M, Whitham R, Lovera J, Milano J, Theofan G, Chou YK, Offner H, and Bourdette DN. (2008) *Therapeutic vaccination with a trivalent T-cell receptor (TCR) peptide vaccine restores deficient FoxP3 expression and TCR recognition in subjects with multiple sclerosis.* Immunology. 123(1); 66-78.

126. Veale D, Rogers S, and FitzGerald O. (1994) *Classification of clinical subsets in psoriatic arthritis.* British Journal of Rheumatology. 33(2); 133-138.

127. Veale DJ, Ritchlin C, and FitzGerald O. (2005) *Immunopathology of psoriasis and psoriatic arthritis.* Annals of the Rheumatic Diseases. 64 Suppl 2 ii26-ii29.

128. von Boehmer H. (2005) *Mechanisms of suppression by suppressor T cells.* Nat.Immunol. 6(4); 338-344.

129. Vudattu NK, Magalhaes I, Hoehn H, Pan D, and Maeurer MJ. (2009) *Expression analysis and functional activity of interleukin-7 splice variants.* Genes Immun. 10(2); 132-140.

130. Wang J, Ioan-Facsinay A, van d, V, Huizinga TW, and Toes RE. (2007) *Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells.* European Journal of Immunology. 37(1); 129-138.

131. Wei S, Kryczek I, and Zou WP. (2006) *Regulatory T-cell compartmentalization and trafficking.* Blood. 108(2); 426-431.

132. Wells G, Becker JC, Teng J, Dougados M, Schiff M, Smolen J, Aletaha D, and van Riel PL. (2009) *Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate.* Annals of the Rheumatic Diseases. 68(6); 954-960.

133. Xu S and Cao X. (2010) *Interleukin-17 and its expanding biological functions.* Cell Mol.Immunol. 7(3); 164-174.

134. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, and Sakaguchi S. (2004) *Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells.* International Immunology. 16(11); 1643-1656.

135. Yu P and Fu YX. (2006) *Tumor-infiltrating T lymphocytes: friends or foes?* Lab Invest. 86(3); 231-245.

136. Zheng SG, Wang J, and Horwitz DA. (2008) *Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6.* Journal of Immunology. 180(11); 7112-7116.

137. Zhou L, Lopes JE, Chong MMW, Ivanov II, Min R, Victora GD, Shen YL, Du JG, Rubtsov YP, Rudensky AY, Ziegler SF, and Littman DR. (2008) *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing ROR gamma t function.* Nature. 453(7192); 236-U14.

Appendix A

Supplementary figures

10-fold dilutions series of cDNA using primer sets for A) GAPDH, B) CD25, C) CTLA-4, D) FoxP3Δ2 and E) FoxP3Δ2Δ7. The slopes were determined from the trendline equations.