Masterarbeit

Phänotypische und funktionelle Charakterisierung von CD4+ CD28- FoxP3+ T- Zellen in Patienten mit immun vermittelten Erkrankungen

eingereicht von

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zur Erlangung des akademischen Grades

Master of Science
(MSc)

an der

Karl Franzens Universität Graz

ausgeführt an der

Abteilung für Rheumatologie und Immunologie der Universitätsklinik für Innere Medizin, Medizinische Universität Graz

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Graz, Dezember 2014

Master thesis

Phenotypic and functional characterisation of CD4+ CD28- FoxP3+ T- cells in patients with immune-mediated diseases

submitted by

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to obtain the academic degree

Master of Science (MSc)

at the

Karl Franzens University of Graz

performed on the

Division of Rheumatology and Immunology at the University Clinic for Internal Medicine, Medical University of Graz

under the guidance of

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Graz, December 2014

Acknowledgement

At first, I would like to express my gratitude to my supervisor Priv.-Doz. Dr. med. univ. Christian Dejaco, PhD for the useful comments, remarks and engagement through the learning process of this master thesis.

Furthermore, I would like to thank my laboratory supervisor Johannes Fessler, MSc. BSc. for introducing me to the topic as well for his support in the laboratory.

I would like to thank my family, who have supported me throughout the entire process, and who stood by me during my whole (study) life.

Finally, I am obliged to my laboratory co-workers, who taught me so many things.

Zusammenfassung

Hintergrund

Die steigende Lebenserwartung ist ein zentrales Thema in der „Biologie des Alterns“ und verdeutlicht die Notwendigkeit, die Mechanismen der altersbedingten Veränderungen des Immunsystems, zu verstehen. Diese Veränderungen können zu einer höheren Anfälligkeit von Immundefekten wie Infektionen, Krebs oder Autoimmunerkrankungen wie der Rheumatoide Arthritis (RA) bei älteren Menschen führen.

Vorzeitige Immunoseneszenz ist ein markantes Merkmal der RA. Zusätzlich wurden quantitative und qualitative Mängel von regulatorischen T-Zellen (Tregs) bei RA Patienten beschrieben. In dieser Arbeit stellen wir die Hypothese auf, dass gealterte Tregs existieren und diese zur Krankheitsentstehung beitragen. Aus diesem Grund untersuchen wir die Prävalenz von gealterten Tregs bei Patienten mit RA sowie bei Gesunden. In Funktionsexperimenten testen wir wie gealterte Tregs die Regulation des Immunsystems beeinflussen.

Methoden

In einer prospektiven Studie von 35 RA Patienten in Vergleich zu 25 gesunden Kontrollen wurde die Prävalenz von CD4+CD28+FoxP3+ und CD4+CD28-FoxP3+ T-Zellen sowie die Expression der Oberflächenmarker CTLA-4 und PD-1 mittels Durchflusszytometrie (BD FACS Canto II) bestimmt. Des Weiteren wurde in Zellkulturexperimenten die *in vitro* Generierung von seneszenten regulatorischen T-Zellen (Tregs) durchgeführt. Mittels magnetischer Beads wurden $CD4^{+}CD25^{+}CD127^{low}$ Tregs isoliert und mit anti-CD3/CD28-Beads, Interleukin (IL) -2, mit oder ohne Tumor Nekrosis Faktor α [TNF-α (100ng/ml)] oder IL-15 (100ng/ml) für 14 Tage stimuliert. Nach 14 Tagen wurde die Expression von CD28 sowie die Produktion zahlreicher Zytokine (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α und Interferon-gamma (IFN-γ)) von verschiedenen Treg Subpopulationen gemessen. Schließlich wurde die supprimierende Aktivität in einem funktionellen CFSE- basierten Assays bestimmt.

Resultate

Eine erhöhte Anzahl von CD4+CD28-FoxP3+ T-Zellen im peripheren Blut von RA-Patienten konnte im Vergleich zu gesunden Kontrollen nachgewiesen werden (2% der gesamten CD4+ Population [±2,8] vs. 0,6% [±0,8]; p = 0,077). Die Analyse der Expression von Oberflächenrezeptoren der CD4+CD28-FoxP3+ und CD4+CD28+FoxP3+ Tregs hat gezeigt, dass CD28-FoxP3+ Zellen vermehrt das regulatorische Protein PD-1 exprimieren [17,45% (0-36,4) vs. 5,45% (1,8 bis 13,5), p = 0.034]; währenddessen die CTLA-4 Expression in beiden Populationen ähnlich war. Die *in vitro* Exposition von CD4+CD25+CD127- Tregs mit TNF-α verursachte eine Herunterregulierung von CD28 (Median MFI: 3.295 [Bereich: 1.293 bis 16.853] vs. 7.423 [3.986-132.529] p = 0.05) und wies somit einen CD28-FoxP3+ Phänotyp auf. Zusätzlich kam es zu einer Hochregulierung von CD25 (27.649 [15.085-43.991] vs. 14.779 [10.119-28.332] p = 0.025) und CD127 (996.5 [-35-1480] vs. 723.5 [-492-828] p = 0.028) bei den *in vitro* mit TNF-α behandelten Tregs im Gegensatz zu den Kontroll-Tregs.

Die Expression von FoxP3 war jedoch in allen Gruppen ähnlich. Bezüglich Zytokinproduktion zeigte die Behandlung mit IL-15 eine erhöhte Produktion von IL-4 (MFI: 637 [414-1247] vs. 461.5 [297-725] p = 0.028), IFN-γ (912.5 [644-1120] vs. 828 [566-1075] p = 0.028) sowie IL-17 (781.5 [515-1039] vs. 475.5 [356-792] p = 0,028). Die Stimulation mit TNF-α führte zu keinen Veränderungen im Zytokinprofil. Schließlich war die supprimierende Aktivität der TNF-α behandelten Tregs signifikant erhöht verglichen mit Kontroll- Tregs (Suppression 12.8% [48.3 -50.8-] vs. -26% [-58-25] p = 0.28).

Schlussfolgerung

In dieser Arbeit zeigen wir eine neuartige T-Zell Subpopulation, die sowohl seneszente sowie regulatorische Eigenschaft aufweist. Diese Subpopulation wurde bei RA Patienten, aber nicht bei gesunden Kontrollen gefunden und zeigte deutliche Änderungen hinsichtlich Phänotyp und Funktion. Darüber hinaus waren wir in der Lage CD28- Tregs *in vitro* durch TNF-α Stimulation zu erzeugen. Diese Daten deuten auf eine Beteiligung von seneszenten regulatorischen T-Zellen bei der Pathogenese der RA hin. Um die exakte Rolle dieses T-Zell Subsets zu klären sind weitere Studien durchzuführen.

Abstract

Background

Increasing life expectancy is crucial topic in the biology of aging and evokes the need to understand the mechanisms of age-related alterations in the immune system. These alterations may lead to higher susceptibility of immune failures such as infection, cancer or autoimmunity in elderly individuals like Rheumatoid arthritis (RA).

Premature immunosenescence is a hallmark feature of RA. In addition, quantitative and qualitative defects of regulatory T-cells (Tregs) were described in RA patients. In this work we hypothesize the existence of senescent Tregs which contribute to the disease pathogenesis. For this reason, we examine the prevalence of senescent Tregs in patients with RA and healthy individuals. In functional assays we test the mechanisms how senescent Tregs may influence immune regulation.

Methods

A prospective study on 35 patients with RA and 25 healthy controls was performed to determine prevalences of CD4+CD28+FoxP3+ and CD4+CD28-FoxP3+ T-cells as well as the expression of surface markers CTLA-4 and PD-1 via flow cytometry (BD FACS Canto II). Furthermore, *in vitro* generation of senescent Tregs was performed in cell culture experiments using magnetic bead isolated $CD4^{+}CD25^{+}CD127^{low}$ Tregs and stimulation with anti-CD3/CD28 beads, interleukin (IL)-2 with or without TNF-α (100ng/ml) or IL 15 (100ng/ml) for 14 days. After 14 days the expression of CD28 and the production of several cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ) of different Treg-subsets was measured. At last, the suppressive activity was determined in a functional CFSE-based assay.

Results

Elevated numbers of CD4+CD28-FoxP3+ Tregs in peripheral blood from RA patients could be detected in comparison to healthy individuals (2% of total CD4+ Population [±2.8] vs. 0.6% [±0.8]; p = 0.077). Surface receptor expression analysis of CD4+CD28-FoxP3+ and CD4+CD28+FoxP3+ Tregs demonstrated that CD28-FoxP3+ cells expressed higher levels of the regulatory protein PD-1 [17.45% (0-36.4) vs. 5.45% (1.8-13.5), p=0.034], whereas CTLA-4 expression was similar in both subsets.

The exposure of CD4+CD25+CD127- Tregs to TNF-α in an *in vitro* assay caused a downregulation of CD28 (median MFI: 3.295 [range: 1.293-16.853] vs. 7.423 [3.986-132.529] p=0.05) and thus indicating a CD4+CD28-FoxP3+ phenotype. In addition, an upregulation of CD25 (27.649 [15.085-43.991] vs. 14.779,5 [10.119-28.332] p=0,025) and CD127 (996.5 [-35-1480] vs. 723.5 [-492-828] p=0,028) on TNF-α treated Tregs in contrast to unstimulated Tregs could be detected. The expression of FoxP3, however, was similar in all groups. Regarding cytokine production treatment with IL-15 caused an increased production of IL-4 (MFI: 637 [414-1247] vs. 461.5 [297-725] p=0.028), IFN-γ (912.5 [644-1120] vs. 828 [566-1075] p=0.028) as well as IL-17 (781.5 [515-1039] vs. 475.5 [356-792] p=0.028). Stimulation with TNF-α did not lead to changes in the cytokine profile of Tregs.

At last the suppressive activity of TNF-α treated Tregs was significantly increased compared to untreated Tregs (suppression 12.8% [-50.8- 48.3] vs. -26% [-58-25] p=0.28).

Conclusion

In this work, we discovered a novel T-cell subset which features both senescent as well as regulatory properties. This subset was detected in RA patients but not healthy individuals and indicated changes regarding phenotype and function. Moreover, we were able to generate CD28- Tregs *in vitro* by stimulation with TNF-α. These data, suggest an involvement of senescent regulatory T-cells in the pathogenesis of RA. To elucidate the exact role of this subset, however, has to be explored in future studies.

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Abbreviations

ACR American College of Rheumatology

APC antigen presenting cell

aTregs adaptive Tregs

Bcl-2 (b-cell lymphoma 2)

cAMP Cyclic adenosine monophosphate

CCR CC chemokine receptors

CD cluster of differentiation

CFSE Carboxyfluorescein succinimidyl ester

CMV cytomegalovirus

COX Cyclooxygenase

CRP C-reactive protein

CTLA4 cytotoxic T lymphocyte antigen 4

DC dendritic cell

DMARDS disease modifying antirheumatic drugs

EULAR European League against Rheumatism

Fas receptor/ FasL- Fas ligand

Fc- receptor fragment crystallizable region

FCS fetal calf serum

FOXP3 forkhead box protein P3

GATA3 erythroid transcription factor

GITR glucocorticoid- induced tumor necosis factor receptor

GM-CSF granulocyte-macrophage colony-stimulating factor

HLA- DRB 1 HLA class II histocompatibility antigen, DRB1-9 beta chain

HLA human leukocyte antigen

IDO Indolamin-2,3 –Dioxygenase

IFN interferon

IL interleukin

IL-6R Interleukin-6 receptor

IPEX Immundysfunction- Polyendokrinopathy syndrome

ITAM immunoreceptor tyrosine-based activation motif

iTregs inducer Tregs

KIR killer cell immunoglobulin-like receptor

LAG- 3 Lymphocyte-activation gene 3

LFA-1 lymphocyte function-associated antigen 1

MFI mean fluorescence intensity

MHC Major histocompatibility complex

MoAB monoclonal antibody

NK cell natural killer cell

NKG2D- natural – killer group 2, member D

NSAIDs Nonsteroidal anti-inflammatory drugs

OKT-3 monoclonal antibody against CD3

PBMC peripheral blood mononuclear cell

PBS phosphate buffered saline

PD-1 programmed cell death 1

PGE2 Prostaglandin E2

PTPN22 protein tyrosine phosphatase, non-receptor type 22

RA rheumatoid arthritis

RANK receptor activator of nuclear factor κB

RANKL receptor activator of nuclear factor κB Ligand

rHU IL-2 recombinant human IL-2

RPMI medium Roswell Park Memorial Institute medium

sDAI simple Disease Activity Index

SLE Systemic lupus erythematosus

STAT5 signal transducer and activator of transcription factor 5

TCR T cell receptor

TGF transforming growth factor

Th cell T-helper cell

TLR Toll like receptor

TNF tumor nekrosis factor

TSLP Thymic stromal lymphopoietin

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1 Background

The field of aging is of particular interest in the current society as well as medical research. Especially, the activity of the immune system is sorely afflicted by changes caused by ageing of the individual – a process called “immunoageing”. It is well known, that elderly people suffer more often from infections, neoplasms and autoimmune diseases. Moreover, immunoageing leads to a diminished response to vaccination, a reduction of natural killer cell function and progressive erosion of telomeres in lymphocytes going hand in hand with an accumulation of terminally differentiated (senescent) lymphocytes. Growing evidence suggests a pivotal role of immunoageing in younger individuals suffering from chronic inflammatory disorders of autoimmune origin, like rheumatoid arthritis (RA), also. [34]

To regulate immune responses and activation a lot of distinct regulatory immune cells are known. Among them, regulatory T-cells (Tregs) are of major interest in recent decades. In RA, there is an ongoing debate on the significance of Tregs in the disease pathogenesis. [36] My work has focused on a possible relationship between signs of immune ageing and Tregs in rheumatoid arthritis. I formed the hypothesis that RA patients have cells that combine regulatory as well as senescent characteristics and that these cells are altered compared to conventional Tregs.

1.1 Definition, pathogenesis and clinical background of Rheumatoid arthritis

RA is a chronic inflammatory disorder that is associated with synovial inflammation, autoantibody production, cartilage and bone destruction as well as other systemic features like cardiovascular, skeletal and psychological disorders. [1]

Epidemiology:

RA affects approximately 1.0% of adults in developed countries. [2] The disease predominately occurs in women (the female-to-male ratio being 3:1) and in Northern Europe and North America rheumatoid arthritis is more common than in developing

countries. [3] In many cases it causes joint destruction, inflammation, disability and early death. [4]

Potential risk factors are smoking, caffeine intake, alcohol intake, oral contraceptive use, vitamin D status as well as low socioeconomic status. [5,6]

Pathophysiology:

The pathogenesis of RA is not very well understood. In general, the interplay of genetic predisposition and environmental factors results in a complex system that recognizes some of the body´s own molecules as foreign and has destructive effects on the body´s own organs and tissues that can lead to the development of severe autoimmune diseases like RA. Both arms of the immune system, the innate as well as the adaptive immune system contribute to the initiation and perpetuation.

Genetic factors contribute to the pathogenesis of the disease. A key role is assigned to differences in the major histocompatibility (MHC) locus (human leukocyte antigen (HLA) DRB1 alleles) that seem to be the cause for disease susceptibility and severity. [2] It is the current understanding of the disease that a so far undefined antigen anomalously activates immune cells in individuals with a specific MHC background.

The effect of this aberrant antigen presentation is the activation of auto reactive T-cells. A clonal expansion of autoreactive T-cells results in direct destruction of host tissue and an exacerbated immune response. Activated autoreactive T-cells then stimulate B cells that produce autoantibodies. [6]

Lesions of synovial tissue are caused by macrophages and infiltrating T-cells and synovial membrane cells. Inflammatory cytokines and metalloproteinases are produced by stimulated macrophages and phenotypically and functionally altered synovial fibroblasts gain the ability to invade into bone and cartilage. [7]

Antigen presenting cells (APCs) like macrophages, dendritic cells as well as activated B cells are involved in the presentation of arthritis-associated antigens to T-cells.

CD4+ T-cells infiltrate into the synovial membrane and secrete interferon-gamma (IFN-γ) and interleukin 2 (IL-2). As mentioned before, RA patients share disease-associated HLA-DRB1 alleles that present arthritis related peptides to auto antigen-

specific T-cells which expand in the lymph nodes and joints and become stimulated. [5]

On the other hand B-cells are implied in the disease development via various mechanisms including antibody-dependent and antibody-independent functions.

B-cells present antigens but also produce antibodies, cytokines and autoantibodies that occur in patients with RA. [8]

These autoantibodies form larger immune complexes and lead to the production of pro-inflammatory cytokines such as TNF-α that cause Fc (Fragment, crystallizable) - receptor and complement activation. Through the activation of B and T-cells more chemokines and cytokines are produced; leading to a feedback loop for additional interactions between T-cells, B-cells and macrophages. [9, 8]

Clinical presentation

RA is a chronic inflammatory disease that affects joints of the hands and feet. It has a chronic course and leads to a decreased quality of life, joint replacement surgery and mortality. [5, 4] The first symptoms of RA are often stiffness and pain, and in many cases damage of joints, bones and tendons result. Patients with long-standing disease suffer from rheumatoid nodules or RA vasculitis and have a higher risk for cardiovascular events and cancer. [10]

Untreated, RA would result in progressive destruction of affected joints. As a consequence, patients would be limited in their course of the day and their quality of life would attenuate.

Nevertheless, it is the highest task to diagnose and treat the disorder as early as possible.

Diagnosis/ classification

The diagnosis of RA combines typical symptoms and laboratory findings.

In the following table (**table 1**) the new classification of ACR (American College of Rheumatology) and EULAR (European League Against Rheumatism) of 2010 for the diagnosis of RA are listed.

The new classification includes antibody status, acute-phase response, joint involvement and symptom duration. For a definite diagnosis of RA 6 points or more are needed. (Scott, Wolfe, & Huizinga, 2010) [5]

Table 1 Four different categories for the diagnosis of RA that are defined by the American College of Rheumatology. For the diagnosis of RA 6 points or more are needed.

1. Joint involvement (0–5)
• Two to ten medium-to-large joints (1)
• One to three small joints (large joints not counted) (2)
• Four to ten small joints (large joints not counted) (3)
• More than ten joints (at least one small joint) (5)
2. Serology (0–3)
• Negative RF *and* negative ACPA (0)
• Low positive RF *or* low positive ACPA (2)
• High positive RF *or* high positive ACPA (3)
3. Acute-phase reactants (0–1)
• Normal CRP *and* normal ESR (0)
• Abnormal CRP *or* abnormal ESR (1)
4. Duration of symptoms (0–1)
• Less than 6 weeks (0)
• 6 weeks or more (1)

Treatment/ therapy:

The aim of RA therapy is to influence the course of the disease, suppress inflammation and alleviate occurring symptoms. Over the last 15 years many new biologic and non-biologic disease-modifying antirheumatic drugs (DMARDs) have been used in RA to reduce disease activity to very low levels. [11]

At the moment different biologic therapies are available, partly targeting pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. [12] A more recent agent for the treatment that selectively interferes with the CD80/CD86 - CD28 costimulatory signal cascade is Abatacept. [13]

To facilitate T-cell activation CD80 or CD86, that are both expressed on the surface on antigen-presenting cells (APCs), bind to CD28 on the T-cell. Afterwards, the important naturally occurring inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) is activated on the T-cell surface and binds to CD80 or CD86 with a greater affinity than CD28 does, thus competing with CD28 for the binding to CD80 or CD86. [14]

The drug abatacept combines the extracellular domain of human CTLA-4 as well as a modified part of the Fc domain of human IgG1 [15]. Like CTLA-4, abatacept then competes with CD28 for CD80 and CD86 binding and can therefore be used for a selective modulation of the T-cell activation. [16]

Non-steroidal anti-inflammatory drugs (NSAIDs) interfere with small pieces of the inflammatory cascade (COXs) and disease-modifying anti-rheumatic drugs (DMARDs) with the underlying immune- inflammatory events. [10]

1.2 The immune system & autoimmunity

The immune system is a broad network of cells and soluble factors that can eliminate bacteria, viruses and parasites and can be divided into two parts: the innate immune system including dendritic cells (DCs), natural killer cells (NK), macrophages and granulocytes as well as complement factors and the adaptive immune system including T and B-cells.

The innate response is very fast and broad whereas the adaptive immune system is slower and specific, but long-lasting. Most intruders are recognized by the innate immune system. The immune cells of the innate part are also responsible for the activation of adaptive immune cells that can recognize specific antigens. After the recognition of the antigen, adaptive immune cells multiply themselves and become highly specific. Adaptive immune cells eliminate infections and can become memory cells. [17]

Failures in the fine balanced immune system can lead to autoimmune diseases like RA. T-cells play a central role in the development of autoimmunity and therefore, in this work, we focus on T-cells in the context of RA.

1.3 The impact of T-cells in the pathogenesis of RA

The common sense of RA pathogenesis delineates a self-perpetuating immune reaction in individuals with high susceptibility to the disease triggered by environmental factors. Consequently, immune cells get aberrantly activated and cause synovial inflammation and autoantibody production resulting in destruction of bone and cartilage.

In the pathogenesis of RA a pivotal role is attributed to T-cells. [18] As mentioned above there is a strong linkage between certain genes that are involved in T-cell activation (e.g. HLA-DR4, PTPN22, CTLA-4) and increased susceptibility to RA. For example, mutations in the PTPN22 gene may lead to a selectional shift in thymic development and favoring the generation of high-avidity autoreactive T-cells in the periphery. [19]

The clinical efficiency of Abatacept, a fusion protein that inhibits T-cell costimulation, moreover supports the importance of T-cells in the disease development. [20]

Further, T-cell numbers in rheumatoid joints are abnormally elevated and in animal models it has been described that T-cells drive the pathogenesis of RA. [5]

Antigen-activated CD4+ T-cells can be classified into several T-cell subpopulations based on the cytokines that they produce or via their transcription factor profile. In the past, this subdivision was limited to T-helper (Th) 1 cells and Th2 cells. T-cell mediated inflammation was proposed by a high Th1 to Th2 ratio and a low antibody-mediated response. [21] Th1 cells are known to produce IFN-γ and are regulated through the transcription factor Tbet whereas Th2 cells are regulated by the transcription factor GATA3 and produce IL-4, IL-5 as well as IL-13. While Th1 cells are important for the protection against intracellular pathogens, Th2 cells are involved in the regulation of humoral immunity and are necessary for the protection against extracellular pathogens. [22]

Today, we know numerous subclasses of T-cells (such as Th1, Th2, Th9, Th17, Tregs) and slowly we begin to understand the plasticity of the T-cell in general.

It has been observed that during the pathogenesis of RA a shift in the predominance of different naïve T-cell subsets occurs. [21]

Currently, the spotlight is on the interplay of IL-17 producing cells (Th17 cells) and regulatory T-cells (Tregs). Former Th1 cells that produce interferon-γ have long been attributed for T-cell mediated autoimmunity but recent data suggest that Th17 cells are important for the progression of certain autoimmune diseases. In the knee joint and in synovial biopsies from RA patients IL-17 positive cells could be detected. Neutralizing antibodies against IL-17 ameliorated the disease both in RA mouse models and in RA patients. [23] In addition, it has been observed that the overexpression of IL-17 in mice induces RA-like features

The expansion of Th17 cells depends on a variety of cytokines and transcription factors that work together to increase the Th17 numbers and also to suppress the induction of other lineages of T-helper cells. IL-6 as well as Transforming growth factor β (TGF-β) have been described to play a central role in generating *de novo* Th17 cells. [24, 25, 26]

Furthermore, studies have shown that Th17 cells can be induced by IL-23, IL-6 and IL-1β independent of TGF-β [27]

Th17, therefore, are regarded as the counterpart to Tregs, a T-cell subset that is essential in the down-regulation of the immune response. The relevance of Tregs in RA will be discussed later in this work.

1.4 The importance of cytokines in the pathogenesis of RA

In general, cytokines play a major role in the pathogenesis of RA because they influence the function of T-cells. A variety of T-cell related cytokines were described to impact the pathogenesis of RA. [28]

Proinflammatory cytokines like tumor necrosis factor α (TNF-α) and IL-6 serum levels are elevated in the elderly and seem to be the key players in the pathogenesis of RA. These two proinflammatory cytokines have different functions in the immune system and are involved in autoimmune responses in particular in tissue destruction in RA patients. [17]

Through IL-6, T-cells get activated and promote antibody production. IL-6 modulates autoimmunity not only by impacting T-cells, it enhances tissue destruction by

activating macrophages and osteoclasts which are responsible for cell matrix degradation and bone resorption. [28]

As mentioned in the first chapter different biological agents that target these two cytokines were established. The blockade of TNF-α belongs to the standard treatment therapy for RA and the targeting of the IL-6 receptor via antibody predict therapeutic benefit. [10]

The cytokine IL-15 is able to promote the synthesis of important proinflammatory cytokines such as IL-1, TNF-α, a phenomenon that has a pivotal role in different inflammatory conditions. The enhanced synthesis of IL-15 was found in HTLV-1-associated conditions (adult T-cell leukaemia, myelopathy) and in autoimmune and inflammatory diseases, including RA, sarcoidosis, inflammatory bowel disease and multiple sclerosis, systemic lupus erythematosus. [29]

1.5 T-cell development, homeostasis and mechanisms of aging

Age seems to be of tremendous interest dealing with the disorder RA. T-cells undergo different developmental stages in their life and thus T-cell development seems to be linked to the pathogenesis of RA, as well.

The first step of T-cell development takes place in the bone marrow. Lymphoid progenitor cells, afterwards, migrate to the thymus where these cells mature before they leave in the periphery. [23] Progenitor cells don´t have any specific adult T-cell markers until they interact with the thymic stroma. At that time the precursor cells will express some surface molecules but are still negative for CD4 and CD8, which are the most important molecules to distinguish T-helper cells from cytotoxic T-cells in later T-cell life. Moreover, the rearrangement of the ß-chain genes for the TCR starts at this stage. Some cells cannot successfully generate ß-chains and will be eliminated while the remaining multiply. [17] Proliferated cells then start to express both T-cell markers, CD4 and CD8. At last, the rearrangement of the genes for the α-chain of the TCR starts.

Cells that express the full T-cell receptor complex will migrate to the corticomedullary junction where the antigen presentation takes place. T-cells recognize the antigen via major histocompability complexes (MHC) - molecules. Two classes of MHC molecules exist: MHC class I and MHC class II. MHC class I molecules recognize proteins that are synthesised in the cytosol and are transported to the cell surface (e.g. viral proteins) whereas MHC class II molecules bind proteins that come from intracellular vesicles (e.g. macrophage vesicles or phagocytized cells). T-cells that express CD8 bind preferentially to MHC class I molecules and T-cells that express CD4 bind MHC class II molecules.

Over the course of time precursor, T-cells undergo a negative and positive selection. T-cells that cannot bind any class of MHC molecules will be eliminated. The rest undergoes a second selection (negative selection); T-cells that recognise MHC molecules with self-peptides will be disposed. This selection is crucial to remove potential autoimmune cells. Then the T-cells are released as naïve CD45RA+ cells in the periphery. After the contact with the antigen, central and effector memory T-cells gain the expression of the surface marker CD45RO and lose the expression of CD45RA. Consequently, either CD45RA or CD45RO is used to generally differentiate the naïve from memory populations. [17]

T-cells and aging

In the elderly a deterioration of the immune system is a common phenomenon. Aging results in the failure of an effective immune response that makes elderly individuals more susceptible for infections and cancers. Aging is also associated with autoimmune diseases such as rheumatoid arthritis. [1] Patients with RA are immunocompromised and premature aging of the immune system is involved in the pathogenesis of RA. [30] Additionally, the chronic immune stimulation in RA may contribute itself to the aging of the immune system and is responsible for disease progression. [31]

One important phenomenon in the immunobiology of aging is a decrease of new T-cells. Since age-related changes in the thymic function affect sites of T-cell differentiation and maturation, a negative output of T-cells would be expected.

Thymic involution has been suggested to promote the age- related decline of T-cells. [32]

The atrophy of the thymus starts with puberty and goes along with changes in structure, internal cytokine milieu and a decline in T-cell production. Thymic involution starts early although the progressive decline in the T-cell generation may reach its peak late in life. [21]

As mentioned above with increasing age only an irrelevant level of functional thymic tissue in humans remains that is in position to produce T-cells [33]; in consequence, a homeostatic proliferation of peripheral T-cells is necessary for compensation and maintenance of naïve T-cell numbers. [34] The renewal of peripheral T-cells may not be infinite. After the seventh decade of life telomere lengths reach the so- called "Hayflick- limit", a turning point, where T-cells do not proliferate anymore. [35]

The cells then undergo specific phenotypical and functional changes. CD28, an important co-stimulatory molecule is downregulated and the cells acquire cytotoxic potential that may then result in immune dysfunction and higher suspicion for immune-mediated diseases. [36]

Furthermore, TCR diversity is declined in elderly persons [46] because of extensive antigen-driven homeostatic proliferation of T-cells. T-cells that have a high affinity TCR for self-antigens or antigens that origin from chronic virus infections show a survival advantage over other T-cells. [37]

In mouse studies aging was accompanied with a defect in the differentiation of CD4+ T-cells into Th1 and Th2 cells although the differentiation into Th17 cells remained unaffected, interestingly the number of Th17 cells was increased as well as the amount of IL-17 produced by each Th17 cell. [38] However, it is still unclear if this finding can be extrapolated to RA-associated inflammation and joint pathology in elderly humans.

1.6 Senescent T-cells and their role in the pathogenesis of RA

Immunosenescence causes an inadequate accumulation of terminally differentiated effector-memory T-cells both at sites of inflammation and peripheral blood. [36, 39, 40, 41] In RA, the process of immunosenescence is believed to be caused by chronic immune activation [31] but it is still unclear if premature immunosenescence is the cause or the consequence of the disease. Interestingly, healthy individuals that share genetic risk factors show an inadequate thymus atrophy and signs of early T-cell aging. [42]

Some signs of immunosenescence in RA are: (1) reduced thymic T-cell production and (2) decreased TCR repertoire of naïve and memory T-cells even in young RA patients. [7]

Features of immunosenescence:

1. Immunosenescence is associated with variances in the cell membrane that causes altered cellular signalling [43]
2. Increased T-cell stimulation due to reappearing infections lead to an exhaustion of the T-cell reserve [44]
3. Peripheral T-cell proliferation leads to a decline of telomere length, epigenetic changes as well as an increase of differentiated effector-memory T-cells [45]
4. The T-cell receptor diversity decreases over time [46]

The increasing antigenic load that occurs with chronic immune activation causes changes in the receptor expression and thus in function of memory T-cells. The loss of CD28 (an important co- stimulatory molecule) as well as the contracted TCR repertoire on these end-differentiated memory T- cells is striking. [47] Bryl et al. showed that the loss of the co-stimulatory molecule CD28 is induced by TNF-α. [48] In patients that were treated with TNF-α blocking agents the CD28 expression by CD4+ cells could be restored. [49]

CD4+ and CD8+ T-cells undergo an age-related loss of CD28 expression, but it has been observed that CD8+ T-cells do so more rapidly. [50, 30] Studies have shown that a repeated exposure of T-cells to the same antigen suppresses CD28

expression and telomerase activity. [51, 31] *In vitro* and *in vivo* these CD28- T-cells have shorter telomeres than CD28+ T-cells. [52]

CD28- T-cells are not only observed in the periphery rather they also occur at inflammatory sites depending on the absence or presence of tissue specific homing receptors. Concretely, CD28- T–cells express a variety of Th1 type chemokine receptors and cytokines that are important for the cells to home to sites of inflammation and to the lymph nodes. [47, 37]

CD28- T-cells in ankylosing spondylitis (AS) for example mostly express chemokine receptors of the pro-inflammatory type 1. [53] In RA patients, the subsets of CD4+ CD28- T-cells combine the characteristics of short-lived effector memory cells and long-lived central memory cells with the expression of CCR-5 (C-motif-chemokine-receptor 5) and CCR-7 and are therefore in position to find a niche in the inflamed synovium as well as in the lymph nodes. [54]

CD28 is a type I transmembrane protein and belongs to the Ig superfamily [55] and is crucial for T-cell activation through ligation of CD28 with CD80 (B7-1) or CD86 (B7-2) on the target cells. [12]

Furthermore, CD28- T-cells are in position to produce large amounts of perforin and granzyme B as well as IFN-γ that allows them to lyse target cells. [56] Additionally, CD28- T-cells feature longevity and persistence because of defects in the apoptotic pathway with an up-regulation of the pro- apoptotic proteins bcl-2 (B-cell lymphoma 2) and FLIP (Fas-associated death domain like IL-2-converting enzyme-like inhibitory protein) that results in inhibition of the Fas (Fas-receptor) - meditated apoptosis. [57]

Terminally effector-memory T-cells lose specific co-stimulatory molecules like CD28 and CD27 but acquire new stimulatory receptors such as killer cell immunoglobulin-like receptors (KIRs), natural-killer group 2, member D (NKG2D) and lymphocyte function-associated antigen 1 (LFA-1) as well as toll like receptors (TLRs) that are important recognition receptors that are not normally expressed on T-cells of the adaptive immune system. See **figure 1**. [58, 59]

In conclusion, an activation of CD4+CD28- T cells not only depends on professional APCs anymore rather than on stress molecules (ligands for KIRs and TLRs- bacterial and viral products) that cause excessive immune activation. [56]

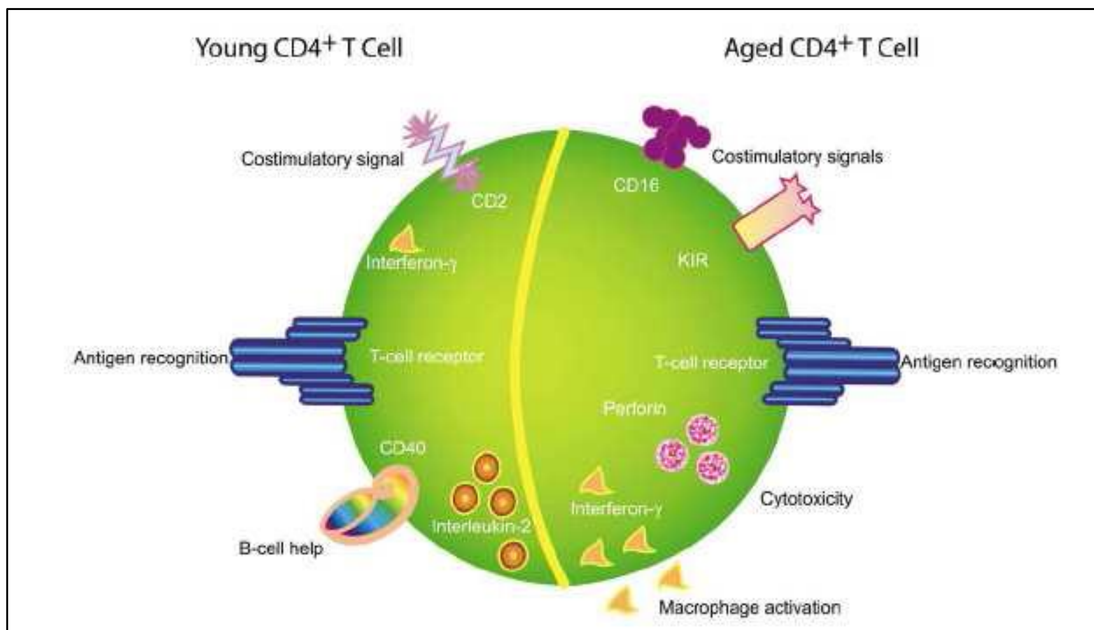

Figure 1 Surface molecules of young CD4+ T-cells in comparison to aged CD4+ T-cells. Characteristics are the loss of the co stimulatory molecule CD28 and the expression of alternative molecules such as KIRs and TLRs. Furthermore, aged CD4+ T-cells release perforin and granzyme B as well as large amounts of IFN-γ. [60]

An elevated number of CD28- T-cells causes a higher risk of erosions and extraarticular manifestation in patients with RA. Furthermore, these patients faced a higher risk for cardiovascular events and responded worse to the drug abatacept. [61, 62]

Besides, it has been found out that CD28- T-cells are in position to lyse endothelial cells by perforin and thus promote prearteriosclerotic vascular damage in RA. [63]

<u>Cytokines and its relevance in the process of aging</u>

The process of aging includes a low- grade inflammation of the adipose tissue and in animal models it has been observed that TNF-α and IL- 6 expression was increased in the adipocytes from aged mice compared to young mice. [64]

On the one hand, aging and chronic inflammatory syndromes (such as RA) are closely linked with a high frequency of CD4+CD28- T-cells and on the other hand elevated levels of TNF-α influence CD28 expression. Bryl et al. demonstrated that the incubation of T-cell lines with TNF-α reduced the surface expression of CD28.

This effect was reversible although a continuous cultivation with TNF-α resulted in the appearance of a CD28- subset. [48]

Another important cytokine is IL-15. Studies reported that IL-15 regulates the generation and growth of CD8+CD28- T-cells and possibly plays a role in the increased occurrence of CD8+CD28- T-cells in the elderly. [65]

Unlike TNF-α, IL-15 does not trigger the downregulation of CD28 expression on CD4+ T-cells, however as mentioned before, IL-15 does also play a role in CD4+ memory T-cell proliferation (both under physiological conditions and after in vitro stimulation). [66, 67, 68]

Furthermore, IL-15 increases the cytolytic properties of CD4+CD28- T-cells and improve the antigen-specific response. [68] IL-15 plays a crucial role in the immune response to early infections and chronic inflammation through multiplying the effects of pro-inflammatory cytokines on IFN-γ secretion as well as by enhancing the antigen-specific responses of CD4+CD28- T-cells. [68, 69]

1.7 Regulatory T-cells, a distinct subset down regulating the immune system

A number of different T-cell subsets with regulatory function have been shown including natural Tregs (nTreg), adaptive or induced Tregs (iTreg), type 1 regulatory T cells (Tr1), T helper 3 cells (Th3), double-negative (dn) T cells, γδ T cells and iNKT cells. In RA, a pivotal role in the regulation of immune activation is attributed to Tregs. [70]

Distinct Tregs can originate from the thymus as well as from the periphery but are indistinguishable because of their expression of typical Tregs markers. CD25+CD4+ regulatory T-cells that are produced in the thymus are also known as natural Tregs (nTregs) and are specialized, antigen-primed cells that suppress excessive and abnormal immune responses that are injurious to the host. nTregs are able to

control other immune cells (effector lymphocytes), particularly helper T (Th) cells subsets like Th1, Th2, Th17 and follicular Th (Tfh) cells. [71]

For the development of nTregs several costimulatory signals are important; for instance CD28, IL-2 receptor α (CD25), CD40 ligand (CD40L, CD154), glucocorticoid- induced tumor necrosis factor receptor (GITR) and signal transducer and activator of transcription factor 5 (STAT5). [16] Tregs that originate from the periphery are known as inducer (iTregs) or adaptive (aTregs). Naïve T-cells are capable of inducing Tregs by antigenic stimulation and through dedicated factors that are not ideal for the development of effector T-cells. [72] Tregs are generated under high levels of IL-2, IL-10 and TGF-ß and aberrant antigen presentation by APCs. [24]

1.7.1 Phenotypical characterisation

Currently, the forkhead transcription factor FoxP3 is the most specific Treg marker and plays an important role in the function and development of Treg cells. [73] The expression of FoxP3 is accompanied with the expression of CD25 and CTLA-4 and other important Treg- associated markers. [74] FoxP3 seems to be an activator and/or repressor for genes that are important for T-cell activation like IL-2, CD25, TNF-α and CTLA-4 and forms complexes with other transcription factors as well as histone and chromatin modulators. [75] Mutations in the human FoxP3 gene cause the severe autoimmune disease IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) that triggers non-functional Treg cells, allergy and inflammatory bowel disease. [76]

Unfortunately, data indicate that human FoxP3 is up-regulated in activated T cells without suppressive function as well and thus FoxP3 is not always sufficient for the identification of Tregs. [73] Alternatively, other surface molecules are used to characterize Tregs as effectively as possible: CD4+CD25+CD127-. On the on hand CD127 (IL-17Rα) is used to facilitate the determination of the purification grade of Tregs [77]. Tregs with a high FoxP3 expression showed lower expression of CD127 [78]. Nevertheless, in human T-cells was shown that interleukin 7 down-regulates CD127. [79] On the other hand Tregs highly express CD25 (IL-2 receptor) levels on

their surface and high doses of IL- 2 are crucial for the survival and maintenance of Tregs. [80] CD25 is expressed on the majority of Tregs (around 70-80%). [9] Certainly, the expression of CD25 is also enhanced following stimulation [81]

At last, Helios, a member of the transcription factor family Ikaros was shown to be an alternative marker for human regulatory T-cells recently. [82] Unfortunately, Helios is also up-regulated in activated non-regulatory T-cells. [83] Besides, another study of Cantor et al. recently described a regulatory T-cell subset that expressed Helios, but not FoxP3. [84]

1.7.2 Mechanisms of suppression

Regulatory T-cells (Tregs), both nTregs and iTregs, have the ability to suppress a variety of pathological and physiological immune responses including anti-tumour, anti-microbial responses as well as transplantation immunity. One key issue in current lymphocyte biology is to understand how regulatory T-cells function and how they suppress other lymphocytes *in vivo* and *in vitro* at the molecular level (**see Figure 2**). [85]

Tregs are in position to inhibit the maturation of antigen- presenting cells (APCs) and block the expression of MHC molecules as well as co- stimulatory molecules like CD80 and CD86. Furthermore, Tregs have cytolytic effects on target T-cells and APCs through the release of perforin and granzymes. In addition, Tregs secrete inhibitory cytokines like transforming growth factor- (TGF), IL-10 and IL-35 that suppresses the activation and proliferation of T-cells. The deprivation of γ_c family cytokines leads to the induction of the expression of certain pro-apoptotic proteins by conventional T-cells and enhances their apoptotic rate. Important factors for the suppression of dendritic cells are: CTLA4, lymphocyte activation gene 3 (LAG3) and, thymic stromal lymphopoietin (TSLP). [86]

In the following chapter the most important Treg effector functions are described:

Suppression by soluble factors

Through the secretion of soluble factors (inhibitory cytokines) Tregs are able to suppress target cells. IL-10, TGF-ß and IL-35 are the most important inhibitory cytokines. Interleukin-10 causes a down-regulation of different costimulatory and adhesion molecules and MHC IIs on APCs. [87]

Moreover, IL-10 is responsible for long-lasting anergy in CD8+ and CD4+ T-cells and inhibits cytokine-synthesis by monocytes and T-cells.) [88]

TGF-ß inhibits T-cell proliferation and blocks the differentiation of naïve T-cells into Th1 and Th2. [89]

Tregs highly produce IL-35 which inhibits the proliferation of T-cells. Culturing Tregs with IL-35 result in proliferation [90] and high expression of IL-10. [91]

Contact dependent suppression

Next, contact-dependent suppression includes the interaction of CTLA-4 (CD152) or TGF-β on Tregs with cognate receptors on the target cell such as B7 (CD80/86) and TGF-β RII on conventional cells. [92]

CTLA-4 is closely related with the surface antigen CD28 and is proposed to be a powerful antagonist to CD28 and has a tremendous role as a negative regulator on T-cell activation. [93] Moreover, treatment with abatacept functions the same way to modulate immune activation. [94]

It is well known that the costimulatory molecule CD28 is required for the intrathymic generation of nTregs. Mice deficient for CD28 or its ligands show dramatically reduced number of nTregs and develop an accelerated autoimmunity. [95]

Suppression by cytolysis

Tregs are able to kill their target cells by death receptor signalling through Fas- FasL interaction [96] or through the release of granzymes and perforin. Several different types of granzymes exist in mouse and human, but granzyme A and B are mostly occurring. [97]

Tregs control various cell types including CD4+ and CD8+ T-cells, monocytes, DC, B cells and NK-cells. [98]

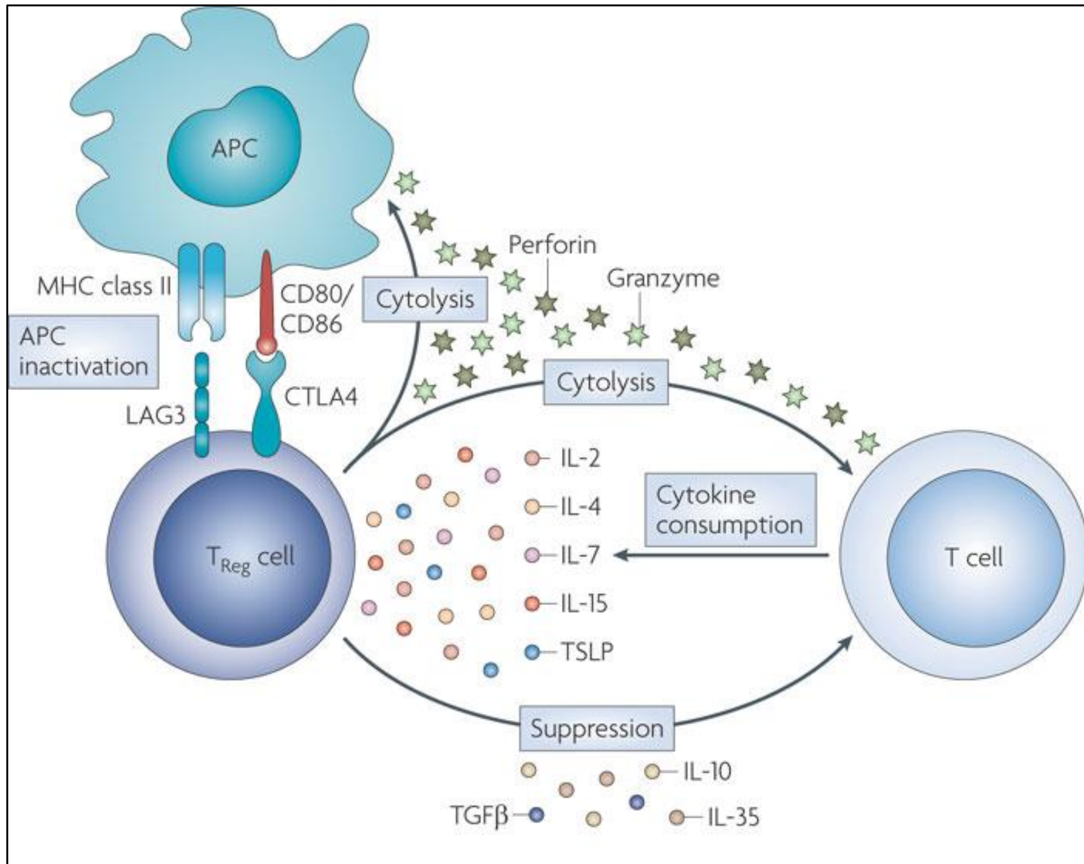

Figure 2 Tregs use several mechanisms to suppress the activation as well as proliferation of conventional T cells. [86]

Suppression of dendritic cells (DCs)

CTLA-4 affects not only non-regulatory T-cells but also has an important regulatory function in Tregs *in vivo*. Surface CTLA-4 is able to induce IDO production (Indolamin-2, 3 -Dioxygenase) by dendritic cells that results in induction of apoptosis of Th1 and Th2. [99]

Another important factor for DC inhibition is LAG-3 a CD4 homologue that interferes with the ITAM (immunoreceptor tyrosine-based activation motif) signalling in DCs and inhibits DC maturation and immunostimulation. [100]

Suppression by metabolic disruption

Another way of suppression is metabolic disruption. Tregs are able to disturb the metabolism of effector T-cells in multiple ways. cAMP (cyclic adenosine monophosphate) for example stops IL-2 production and therefore the proliferation by T-cells. [101] The release of the substance prostaglandin E2 (PGE2) upregulates the cAMP level and thus has suppressive capacity. [102]

Furthermore, Tregs deprive T-cells of thiols such as cysteine. DCs provide a cysteine rich environment for T cells although Tregs represent strong competitive consumers of these substances. [103]

1.8 Importance of regulatory T-cells for the pathogenesis of RA

The pathogenesis of RA is maybe influenced by an imbalance of Tregs and effector cells. [53]

Nevertheless, contradictory data regarding prevalences and function of Tregs in RA have been published in the last years, although growing evidence suggests an altered efficacy of Tregs in RA patients. [104]

It has been observed that Tregs that origin from RA patients had a reduced capacity to erase the production of pro-inflammatory cytokines by conventional T-cells [106] Further, synovial fluid non-regulatory T-cells from RA patients exhibited a resistance against Treg mediated suppression. [107] This defect of non-regulatory T-cells, however, could be partially restored by TNF- treatment. A possible explanation for this reduced effectivity of Tregs in RA is a relatively low CTLA-4 expression. The reason for CTLA-4 downregulation remains unclear but there is a susceptibility that a CTLA-4 gene polymorphism in RA may play a role. [105]

Interestingly, an artificial induction of CTLA-4 in Tregs from RA patients led to a restored suppressive function *in vitro*. [108]

In addition, recent studies observed that Tregs are able to differentiate into pro-inflammatory Th17 cells also. [109] These Th17 cells then showed the ability to stimulate osteoclastogenesis and expressed higher levels of transcription factor Sox4, chemokine receptor 6 (CCR6), chemokine ligand 20 (CCL20), IL-23 receptor

(IL-23R) and the osteoclastogenic factor RANKL [110,111] and therefore may favor the pro-inflammatory milieu and osteoporosis in RA.

1.9 Regulatory T-cells and aging

In humans different cellular subsets of CD4+ FoxP3+Tregs have been observed; on the one hand a ´naïve- like´ phenotype that is characterized by the expression of CD25+CD45RA+ and on the other a ´memory- like´ phenotype that is CD25hiCD45RO+. [112]

"Naïve-like" Tregs are generated in the thymus of young individuals and are expanded in the periphery where they get in contact with antigens and develop into a "memory-like" phenotype. Homeostatic proliferation of "naïve-like" and "memory-like" Tregs and the conversion of non- regulatory Tregs into Tregs are necessary for Treg homeostasis. Within aging the function of the thymic is progressively lost and homeostatic proliferation as well as conversion of non- regulatory Tregs into Tregs are crucial for the compensation of the declining thymic function and to maintain the Treg pool. In "naïve-like" Tregs the telomere length and T-cell receptor diversity is higher in comparison to memory-like Tregs and a recurrent stimulation of Tregs suggest a terminal differentiation and an altered phenotype and function. [45]

The loss of TCR diversity of Tregs is a common phenomenon in the elderly and an increased immunosuppression in response to specific self or viral antigens could be mediated by Tregs. Permanent response to antigens is an incidence of malignancies and infections in the elderly and the reduced diversity of the TCR could be responsible for reduced protection from autoimmunity. [47]

In human studies it has been found out that Tregs from young and elderly individuals are in position to inhibit the proliferation of responder cells although the production of the important anti-inflammatory cytokine IL-10 was reduced in cells of older individuals. Besides, the expression of CD25, FoxP3 as well as Il-7Rα on Tregs or chemokine receptor expression was unchanged. [113]

Additionally, advanced age seems to have an impact on Treg function in animals. It has been shown that the transfer of CD25+ Tregs from old mice into young ones

resulted in a reduced suppression of delayed type hypersensivity responses in comparison to the injection of young Treg cells. [114] Another study showed that Tregs from aged mice are less efficient in the inhibition of the proinflammatory activity of Th17 cells in comparison to Tregs from young animals. [115]

To study the impact of CD28 in Tregs on the pathogenesis of RA a conditional knock-out mouse for CD28 in Tregs was used as a model. The mice developed a severe autoimmunity because of Treg dysfunction and uncontrolled immune activation. [95]

These data suggest a pivotal role of CD28 signaling in the well-functioning of Tregs.

In this work we hypothesize the existence of senescent CD28- Tregs in RA and want to characterize this subset phenotypically and functional.

1.10 Summary

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that is characterized by synovial inflammation, autoantibody production as well as cartilage and bone destruction [1]

T-cells are involved in the pathogenesis of RA [18] and patients exhibit abnormalities in the T-cell pool that goes along with a contraction in the T-cell receptor diversity as well as shortened telomeres. [46] With advancing age the thymic T-cell production declines. [33]

A variety of mechanisms, mainly antigen-driven clonal expansion, cause replicative stress on T-cells and induces cellular senescence. [31] A characteristic of immunosenescence is a profound change in the functional profile of T-cells that leads to an accumulation of CD4+ T-cells that have lost the important surface molecule CD28 [48] and have in contrast gained killer immunoglobulin-like receptors and cytolytic capability that goes along with the production of large amounts of interferon-γ. [56]

In RA, the occurrence of terminally differentiated (senescent) T-cells is a typical characteristic and these cells seem to be involved in the pathogenesis of RA. The increasing occurrence of CD28- T-cells is linked with a worse clinical outcome and Tregs from these patients are not in position to downregulate the immune system. [49]

Besides, the pathogenesis of RA is may be influenced by an imbalance of Tregs. [36] In patients with RA it has been observed that Tregs have a reduced capacity to reduce the production of pro-inflammatory cytokines by conventional T-cells. Furthermore, synovial fluid non-regulatory T-cells that came from RA patients were resistant against Treg mediated suppression. [107]

This work investigates the occurrence, phenotypical as well as functional characteristics of senescent CD28- Tregs in RA patients.

2 Materials and methods

2.1 Patient´s characteristics

In a prospective, cross-sectional study on 35 patients with RA [mean age 58 (±SD 9.5), 71.4% female, SDAI 8.15 (±1.2)] and 25 healthy controls [mean age 56.4 (±6.7), 60% female] the number of CD4+CD28-FoxP3+ Tregs in peripheral blood was determined via flow cytometry.

This study was permitted by the local institution review board and a written declaration of consent was obtained from the patients.

In the following **table 2** the patient’s characteristics are listed:

Table 2 Patients characteristics

	HC (n=25)	RA (n=35)
Age [years]*	56.4 (±6.7)	58 (±SD 9.5)
Female, n (%)	60	71.4
Disease activity scores		
sDAI#	n.d.	8.15 (±1.2)
DAS28#	n.d.	2.75 (±1.2)
Laboratory data		
CRP (mg/l)#	n.d.	5.38 (±6.6)
BSG (mm/1sth)#	n.d	15 (±13)

* mean (±standard deviation); # median (range)

2.2 Antibodies and flow cytometry

2.2.1 Phenotypical description of CD28- FoxP3+ Tregs

Phenotypic description of Tregs in patients and healthy controls was performed via flow cytometry. In brief, 100µl whole blood were stained with the following surface markers (all Becton Dickinson, San Diego, USA): CD3 (label: Allophycocyanine-Cyanine 7 APC-Cy7), CD4 (V500), CD8 (V450) CD28 (Peridinin chlorophyll protein-PerCP-Cy5.5), CD25 (Phycoerythrin- Cyanine 7 PE-Cy7), CD127 (Phycoerythrin

PE), CD25- Isotyp (Pe-Cy7), CD127- Isotyp (PE) CTLA-4 (CD152) and PD-1 (CD279) (Becton Dickinson, San Diego, USA). **(see table 3)** Appropriate isotype controls for CD25, CTLA-4 and PD-1 were used (Becton Dickinson, San Diego, USA).

Table 3 Whole- blood staining of Tregs. Antibodies for surface and intracellular staining (all Becton Dickinson, San Diego, USA).

Antibodies	Colour	Order number	Company	volume [µl]
CD3	APC-Cy7	341110	BD	3
CD4	V500	560768	BD	3
CD8	V450	560347	BD	3
CD28	PerCP-Cy5.5	560685	BD	3
CD25	PE-Cy7	335824	BD	5
CD127	PE	557938	BD	5
(CD 25- Isotyp)	Pe-Cy 7	557872	BD	5
(CD127- Isotyp)	PE	551436	BD	20
CTLA-4	PE	555853	BD	20
PD-1	FITC	557860	BD	2
(FoxP3-Isotyp)	Alexa Fluor647	557732	BD	5
FoxP3	Alexa Fluor647	560045	BD	20

After 30 min of incubation at room temperature the cells were fixed and permeabilized with 1ml of FoxP3 Fixation/Permeabilization solution for 30 min at 4°C. (FoxP3 / Transcription Factor Staining Buffer Set, eBioscience) After that the cells were washed two times with 1x Perm buffer (5min 1500 U) and the pellet was then resuspended in 100µl Perm Buffer.

For intracellular staining 20µl of FoxP3 (Alexa Fluor) and 5µl of FoxP3 isotype control (Alexa Fluor) (Becton Dickinson, San Diego, USA) was added **(see table 3)**. After another 30min of incubation at room temperature the cells were washed once and resolved in 150µl Flow cytometry staining buffer (eBioscience). Stained cells were analyzed by a FACS Canto II (Becton Dickinson). For the measurement 300,000 events are counted at each acquisition. Data are analyzed with DIVA software. (Becton Dickinson)

2.2.2 Cytokine production

In vitro aged Tregs (see following chapter) were treated with Brefeldin A (10ng/ml) (Sigma Aldrich) and Golgi Stop (1µl/ml) (BD Bioscience) for 4 hours before cytokine staining. Afterwards the cells were harvested, resupended in 100µl Perm Buffer and stained for CD3 (label: APC-Cy7), CD4 (V500), CD8 (V450), CD28 (PerCP-Cy5.5), CD25 (PE-Cy7) and CD25-Isotyp (Pe-Cy7). (all Becton Dickinson)

After permeabilization and fixation, the cytokines were stained with IL-2 (FITC), IL-4 (PE), IL-6 (PE), IL-10 (FITC), IL-17 (PE), TNF-α (PE), IFN-γ (FITC). (all Becton Dickinson, San Diego, USA) and measured on FACS Canto II.

2.2.3 Cell preparation

For the functional assays the PBMCs were isolated as described in the following chapter. In the first step, peripheral venous blood was drawn and the peripheral mononuclear cells (PBMCs) were gained via Histopaque density gradient centrifugation. 15ml of Histopaque were given in a 50ml tube and the 1:1 blood-PBS (phosphate buffer saline) mixture were layered on Histopaque to an end-volume of 50ml. After the centrifugation (1870 U for 25 min, without break) the buffy coat that contains the white blood cells were taken off and washed twice (1500 U for 5min) in PBS. After that the total cell number and probe-purity was determined by a Beckmann Coulter.

2.3 Functional assays

2.3.1 Separation of T-cell subsets

For functional assays Tregs and non-regulatory Tregs were isolated with CD4+CD25+CD127$^{dim/-}$ Regulatory T Cell Isolation Kit II human (AutoMACS, Miltenyi biotec, Amsterdam, The Netherlands). After isolation of PBMCs (see chapter cell preparation) the cell number was determined.

In the first step, non-CD4+ and CD127 high cells were depleted with a cocktail of biotinylated antibodies and Anti-Biotin MicroBeads. Afterwards, the flow-through fraction of the of $CD4^{+}CD127^{dim/–}$ T cells was labeled with CD25 MicroBeads for the positive selection of $CD4^{+}CD25^{+}CD127^{dim/–}$ regulatory T cells.

2.3.2 Cell culture

Isolated $CD4^+CD28^+CD25^+CD127^{dim}$ Tregs (see chapter separation of T-cell subsets 2.3.1) were cultured according to a previously described conditions (Yang et al., 2012). In brief, $1x10^5$ cells/ml were suspended in a 96-well plate with RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM L- glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were then stimulated with anti-CD3/CD28 MoAb (monoclonal antibody)-coated microbeads (life sciences) at a 4:1 bead-to-cell ratio and 200 U/ml human recombinant (rHU) IL-2 (SIGMA), with or without 100 ng/ml TNF-α (SIGMA) interleukin-15 (IL-15; 100ng/ml) (added every 2-3 days).

After six days of cultivation, cells were harvested, washed and suspended in medium containing 20U/ml IL-2 for 2 days. After this “resting phase”, the expanded Tregs were stimulated for another six days with anti-CD3/CD28 MoAb- coated microbeads at a bead-to-cell ratio of 2:1 and 200 U/ml IL-2 (added every 2-3 days).

2.3.3 Determination of suppressive activity

The suppressive activity of cultured Tregs was characterized with a CFSE based assay. The CFSE stock (10mM DMSO, Invitrogen) that was stored at -20°C will be thawed and diluted in PBS to obtain the desired working solution. Afterwards, senescent autologous Tregs were resuspended in PBS to a total cell number of 5-10 × 10^6 cells/ml and incubated with CFSE (final concentration: 1µM) for 7 min at 37°C. After incubation, the cells were washed and resuspended with culture medium (RPMI1640 containing 10%FBS) for three times. CFSE-labeled autologous $CD4^+CD25^-CD127^+$ cells were co-incubated with Tregs at a 1:1 ratio. To induce proliferation, the cells were then stimulated with anti- CD3/CD28 beads at a 1:1 ratio for three days. Thereafter, cells were harvested and analyzed by flow cytometry.

2.4 Statistical analysis

All statistical analyses were performed using the SPSS program, version 20.0 (Chicago, IL, USA). Results were described as median and range (non-parametric data) or mean and standard deviation (parametric data) as appropriate and the Mann-Whitney-U and two-sided Student´s t-test, respectively for comparisons was performed. In case >2 groups were analyzed, the Kruskal-Wallis tests or the ANOVA were used. Paired data were compared with the t-test or the Wilcoxon test, respectively. Correlation between variables were evaluated by the Spearman´s rank correlation coefficient.

3 Results

3.1 $CD4^{+}CD28^{-}FoxP3^{+}$ T-cells are elevated in RA patients

Two [±2.8] percent of CD4+ T-cells were senescent Tregs in RA patients as indicated by the CD28-FoxP3+ phenotype, whereas this subset was almost absent in healthy individuals [0.6% (±0.8), p=0.077]. Interestingly, the number of ordinary non-senescent CD4+CD28+FoxP3+ regulatory T cells was similar in both groups [28.6% (±18.5) vs. 32.7% (±18), p=0.480]. (see **figure 3)**

Figure 3 Increased prevalences of $CD4^{+}CD28^{-}FoxP3^{+}$ T-cells in RA patients. Figure show median percentage of **(A)** CD4+CD28+FoxP3+ T-cells and **(B)** CD4+CD28-FoxP3+ T-cell

3.2 PD-1 expression is enhanced in CD4+CD28-FoxP3+ T-cells

To analyze the expression of surface proteins CTLA-4 (CD152) and PD-1 (CD279) that are linked to Treg activity we performed flow cytometry analysis.

Phenotypic analysis of these receptors revealed that CD28-FoxP3+ T cells express significantly more surface PD-1 in comparison to their CD28+ counterparts [17.45% (0-36.4) vs. 5.45% (1.8-13.5), p=0.034] **(see figure 4A)**. CTLA-4 expression was not different between the different subsets **(see figure 4B)**.

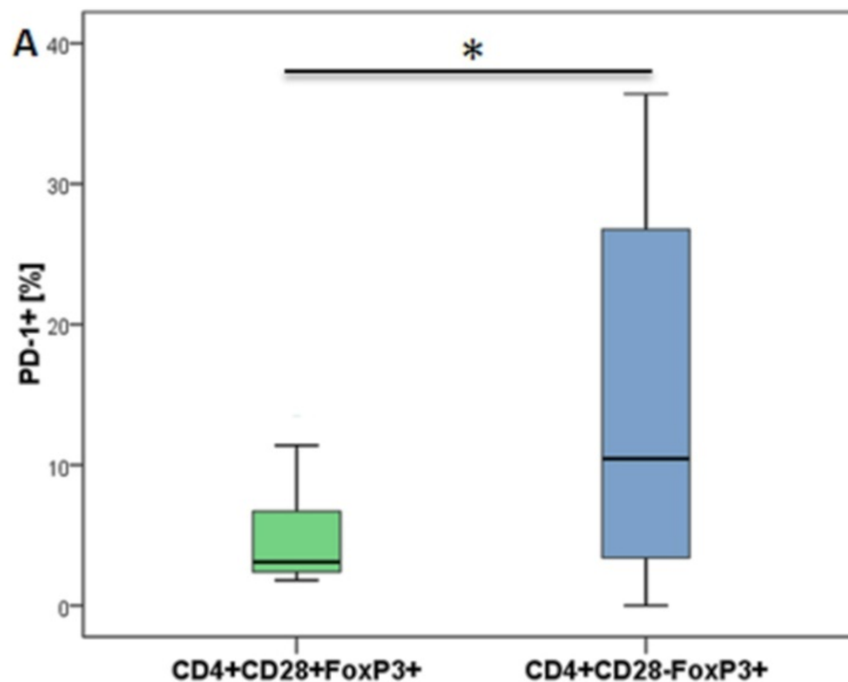

Figure 4a Differential expression of surface proteins PD-1. Expression of surface molecules of CD4+CD28+FoxP3+ (green) as well as CD4+CD28-FoxP3+cells (blue) **(A)** Box plots show percentages of PD-1+ cells. *…p<0.05

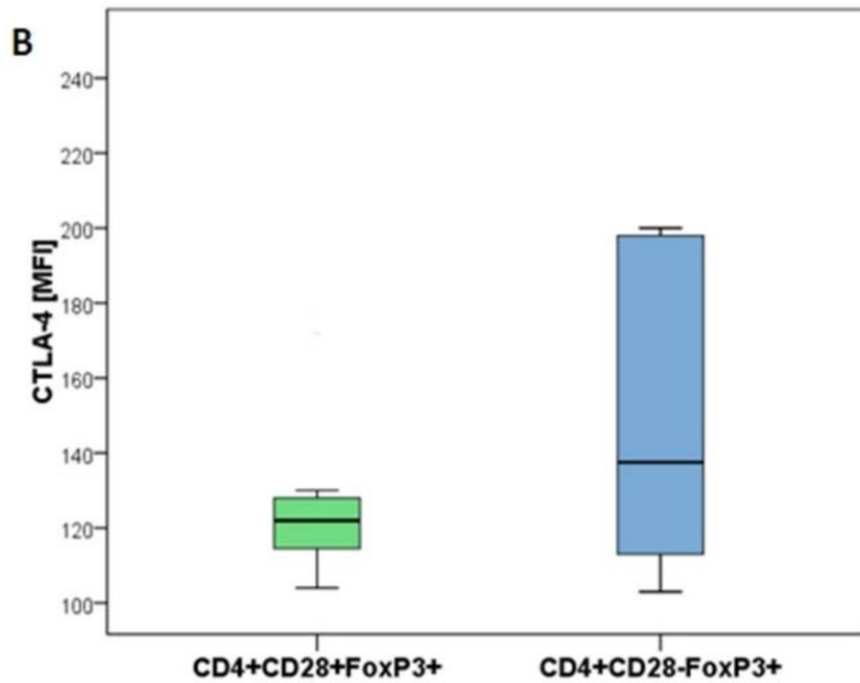

Figure 4b Differential expression of surface protein CTLA-4. Expression of surface molecules of CD4+CD28+FoxP3+ (green) as well as CD4+CD28-FoxP3+cells (blue) **(B)** Box plots show median fluorescence intensity (MFI) of CTLA-4.

3.3 *In vitro* generation of CD4+CD28-FoxP3+ Tregs following stimulation with TNF-α

Since CD28- Tregs occur in RA patients, we tried to generate this subset out of conventional Tregs by stimulation with RA-Typical cytokines *in vitro*. After exposure to TNF-α for 14 days CD4+CD28+ Tregs showed a downregulation of CD28 *in vitro* (median MFI: 3295 [range: 1293-16853] vs. 7423 [3986-132529] p=0.05) **(see Figure 5A)**. In addition, an upregulation of CD25 (27649 [15085-43991] vs. 14779.5 [10119-28332] p=0,025) and CD127 (996.5 [-35-1480] vs. 723.5 [-492-828] p=0,028) on TNF-α treated Tregs in contrast to unstimulated Tregs could be detected. The expression of FoxP3, however, was similar in all groups **(see Figure 5B).** In general, stimulation with IL-15 showed no significant effect.

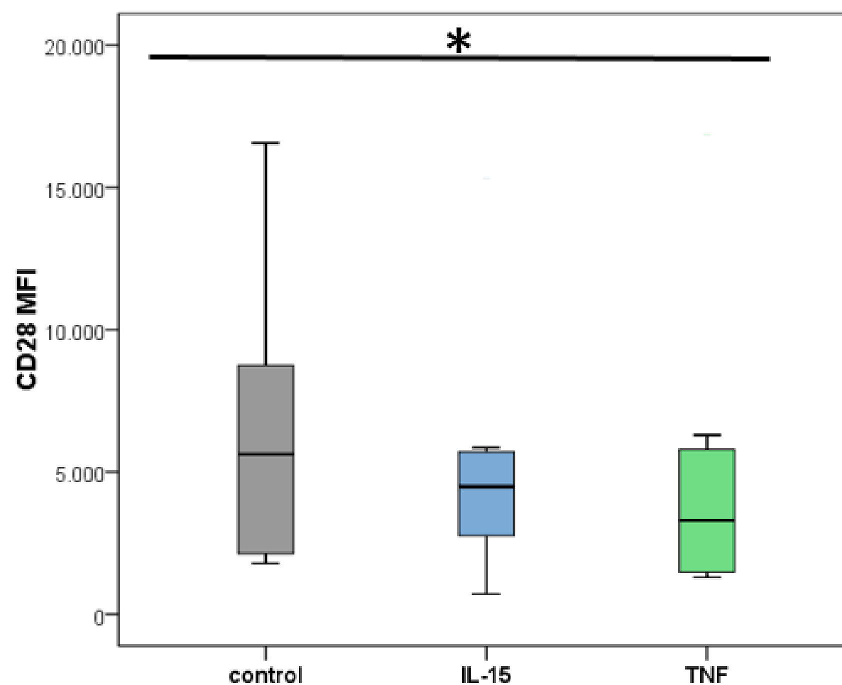

Figure 5a *In vitro* down-regulation of CD28 in Tregs in the presence of TNF-α after 14 days. Box plots show median expression of CD28 (MFI) in Tregs of 8 healthy individuals following TNF-α (green), IL-15 (blue) or IL-2 (control,grey) stimulation. *…p<0.05

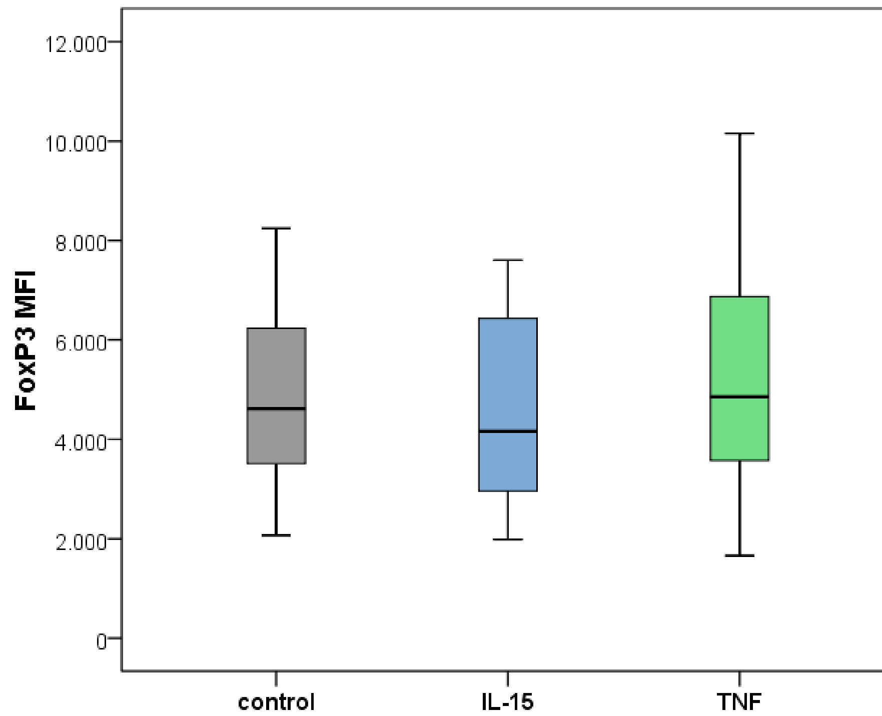

Figure 5b Equal FoxP3 expression after 6 days of stimulation with IL-2, Il-15 or TNF-α. Box plots show median expression of FoxP3 (MFI) in Tregs of 8 healthy individuals following TNF-α (green), IL-15 (blue) or IL-2 (control, grey) stimulation. *…$p<0.05$

3.4 Cytokine production measurement of senescent Tregs

Furthermore, Tregs that were cultured with IL-2, IL-15 or TNF-α for 14 days were investigated compared concerning of the production of the following cytokines: IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ. Interestingly, the presence of IL-15 caused an increased production of IL-4 (637 [414-1247] vs. 461.5 [297-725] p=0.028) **(see Fig 6A)**, IFN-γ (912.5 [644-1120] vs. 828 [566-1075] p=0.028) as well as IL-17 (781.5 [515-1039] vs. 475.5 [356-792] p=0.028) **(see Fig 6B)**. Stimulation with TNF-α did not lead to changes in cytokine profile of Tregs.

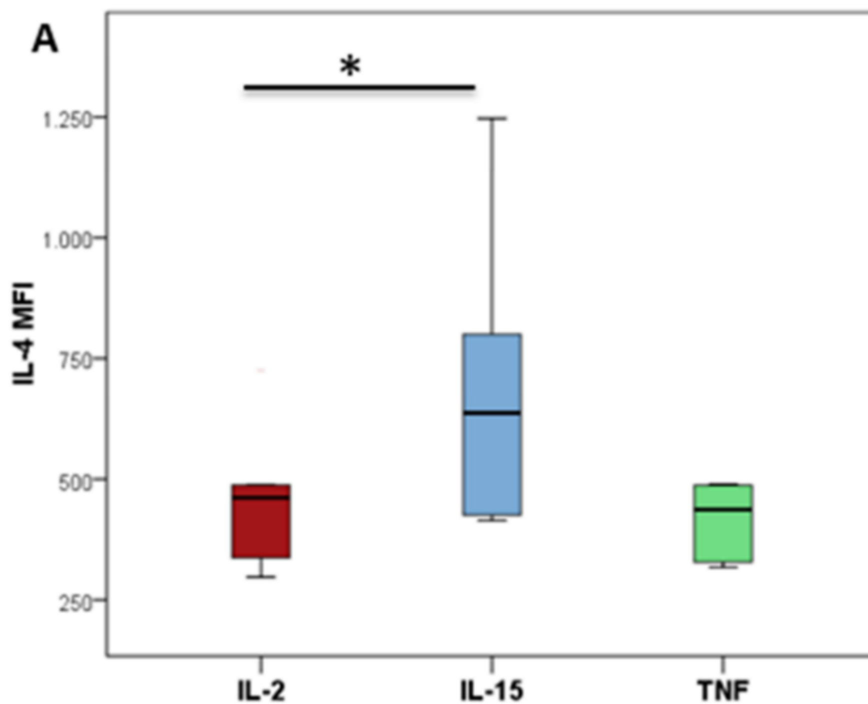

Figure 6a Elevated IL-4 Cytokine production of IL-15 treated Tregs after 14 days of stimulation. Box plots show median fluorescence intensity (MFI) of IL-4.

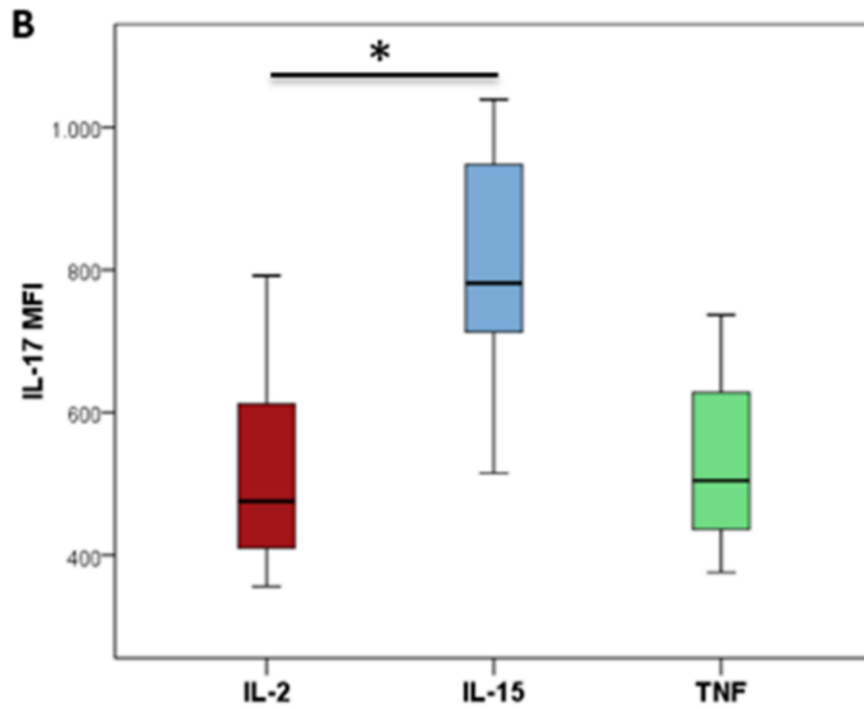

Figure 6b Elevated IL-17 cytokine production of IL-15 treated Tregs after 14 days of stimulation. Box plots show median fluorescence intensity (MFI) of IL-17.

3.5 Determination of suppressive activity

After stimulation with IL-15 and TNF-α the suppressive activity of was measured in comparison to control Tregs that were only treated with IL-2. The suppressive activity of TNF-α treated Tregs, however, was significantly increased compared to Tregs stimulated with the standard protocol (12.8% [-50.8- 48.3] vs. -26% [-58-25] p=0.28). Stimulation with IL-15 did not influence suppressive activity. **(see Figure 7)**

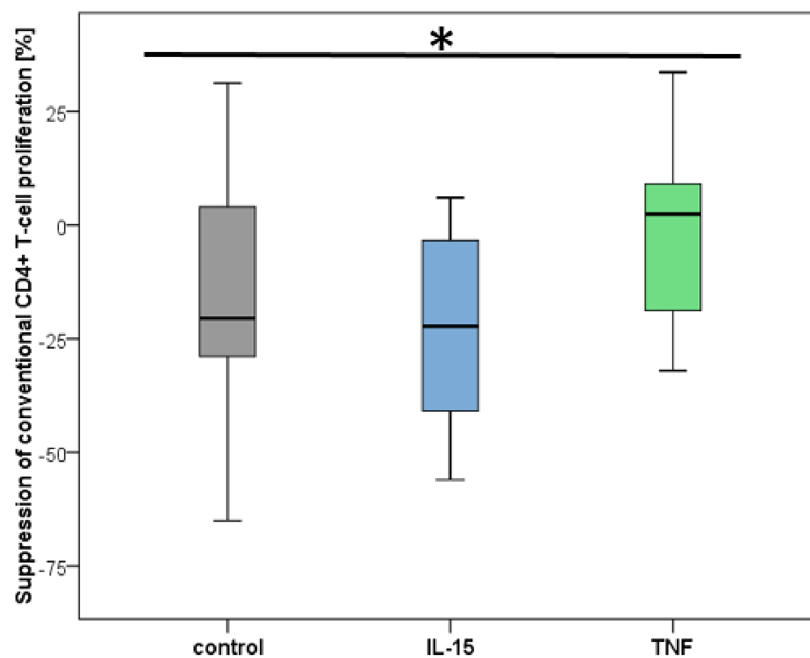

Figure 7 Elevated suppressive activity of Tregs treated with TNF-α. Box plots show suppressive capacity of Tregs treated with IL-2 alone (control, grey), IL-15 (blue) and TNF-α (green) of 6 healthy individuals.

4 Discussion

In the present work we show the occurrence of T-cells that combine features of senescence (loss of CD28) and of immune regulation (expression of FoxP3). These cells are increased in RA patients compared to healthy controls and indicate alterations in phenotype and expression of proteins linked to Treg activity. Moreover, we were able to generate Tregs that lack CD28 expression in vitro by treatment with TNF-α and characterized these cells also.

The existence of senescent regulatory T-cells has long been suggested [36, 45] and has the potential to explain some discrepancies in the role of Tregs in RA pathogenesis and possibly other autoimmune diseases. On the one hand, it is conceivable that the occurrence of these cells influence the number of ordinary Tregs and thus cause contradictory data in studies investigating Treg prevalences. On the other hand, the function of Tregs can be altered in patients with high numbers of CD4+CD28-FoxP3+ T-cells due to changes in surface expression of PD-1 and CCR6 as well as suppressive activity following TNF-α stimulation.

In a previous study it was shown that PD-1 is a cell surface ligand which is expressed to some degree on exhausted or resting T-cells and increases following T-cell receptor activation. [116] In accordance with this study, PD-1 was also enhanced in CD4+CD28-FoxP3+ T-cells compared to their CD28+ counterparts. The PD-1–PD-ligand pathway is important in inhibiting self-reactive T-cell responses and suggests that PD-1 inhibitory signals may be a useful strategy for ameliorating autoimmune diseases. The elevated expression of PD-1 in patients with RA, therefore, seems to be a defensive mechanism. [117]

The expression of CTLA-4 remained unchanged in RA patients in comparison to healthy individuals. Nevertheless, as already mentioned inhibition of CD28 signaling and thus T-cell activation by CTLA-4 is only one of the possible regulatory mechanisms of Tregs.

As normal T-cells, Tregs also undergo different phenotypic and functional changes with advancing age. In this work the influence of IL-15 and TNF-α on Tregs has been examined. The treatment of Tregs with TNF-α downregulates the level of cell surface expression of CD28 on Tregs. In a study Bryl et al. have already shown the

downregulation of CD28 on T-cells [48], however, the occurrence of CD28- Tregs has not been reported so far. The consequence of chronic exposure of Tregs to TNF-α may be an important issue in RA. It is known that patients with RA have high levels of TNF-α and it would be of interest what phenotypical and functional differences of the Treg pool occur in these patients. Besides, Anti- TNF- alpha therapies are common in RA and it would be interesting to examine whether anti-TNF therapies in RA involve a resurgence of Tregs that express CD28.

In contrast, IL- 15 did not lead to a downregulation of CD28 on Tregs. Gegniat el al. showed that IL-15 does also not trigger a CD28 downregulation on CD4+ T-cells, however IL-15 may play a role in the CD4+ T-cell proliferation. [66] The most important role of IL-15 is its effect of the release of proinflammatory cytokines such as IFN-γ production. [69] In the cell culture experiments of this work the treatment of Tregs with IL-15 lead to an increased production of cytokines like IFN- γ, IL-4 and IL-17.

In terms of FOXP3, human studies corroborate that human CD4+FoxP3+ Tregs from the elderly show enhanced FOXP3 expression compared to Tregs from young individuals.[118] Our *in vitro* experiments showed no differences in FoxP3 expression following TNF-α or IL-15 stimulation. Though the expression of the Treg markers CD25 and CD127 were changed after exposure to TNF-α. Interestingly, CD25 as well as CD127 were enhanced. CD25, the IL-2 receptor is important for the survival and maintenance of Tregs and is highly expressed on these cells. In contrast the levels CD127 are normally reduced, but in a study of 2010 Simonetta et al. showed that Tregs that were activated *in vivo* can also exhibit higher levels of CD127 expression.

Consequently, TNF-α treated Tregs showed increased suppressive capacity also. Moreover, in a study from Garg et al. it was shown that Tregs from aged mice also have a greater ability to suppress effector T- cells in vitro than Tregs from young mice. [118] These findings suggest that senescent Tregs show an elevated capacity to inhibit T-cell proliferation. To evaluate the exact meaning of these findings further evaluations are needed.

In conclusion, the data of this work clearly show that there is a need to investigate aging of Tregs in autoimmune diseases like RA. CD4+CD28-FoxP3+ T-cells probably contribute to a misbalance in immune regulation in a setting of premature immunosenescence and TNF-α seems to alter Treg phenotype and function. However, more studies are needed to address this question in a more detailed way.

4.1 Next steps

In cell culture experiments we want further explore the functional and phenotypical characteristics of Tregs in patients with RA. Therefore, an extended analysis of surface markers on senescent T-cells as well as *in vitro* generated CD4+CD28-FoxP3 Tregs is needed. Besides, the suppressive activity of cultured Tregs from RA patients should be determined by functional assays to exclude the influence by disease pathogenesis. To elucidate the senescence properties of Tregs in patients with RA staining of senescence markers (e.g.HP1-γ, H2AX…) would be advisable.

4.2 Conclusion

In this project the aim was to investigate the occurrence and phenotypical and functional characteristics of terminally differentiated Tregs in RA patients. In these preliminary data it has been found out that CD4+CD28- Tregs occur more frequently in patients with RA than in healthy individuals. The expression of certain surface molecules like PD-1 was higher on in patients with RA compared to healthy individuals whereas the expression of CTLA-4 was similar. The exposition of Tregs to TNF-α leads to a downregulation of CD28 and enhanced their suppressive activity. The treatment of Tregs with IL-15 does not lead to a down regulation of CD28 but increases the production of IL-4, IL-17 and IFN-γ certain cytokines.

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