Doctoral thesis

Functional and epigenetic changes of senescent immune cells in patients with rheumatoid arthritis

Submitted by

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“Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig angefertigt und abgefasst, und jene Personen und Institutionen, die am Zustandekommen der Forschungsdaten beteiligt waren, namentlich genannt habe. Andere als die angegebenen Quellen habe ich nicht verwendet und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht. Die Arbeit an der Dissertation und daraus entstandener Publikationen wurde gemäß den Regeln der „Good Scientific Practice“ durchgeführt.

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Declaration

„Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”

Date…”

Acknowledgement

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Contents

Abbreviations

RA	rheumatoid arthritis
ACPA	anti-citrullinated protein antibody
MHC	major histocompability complex
HLA-DR4	human leukocyte antigen DR4
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
CTLA-4	cytotoxic T lymphocyte antigen 4
T_H cell	T-helper cell
IFN	interferone
TNF	tumor necrosis factor
IL	interleukin
TCR	T-cell receptor
CD	cluster of differentiation
CCR	chemokine receptor
NKG2D	natural killer group 2D
CX3CR1	C-X3-C chemokine receptor 1
Treg	regulatory T lymphocyte
GITR	glucocorticoid-induced TNFR-related protein
FoxP3	forkhead box P3
nTreg	natural Treg
TRECS	T-cell receptor excision circles
SLE	systemic lupus erythematosus
HC	healthy controls
SDAI	simplified disease activity index
DAS28	Disease Activity Score 28
HAQ	health assessment questionaire
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death receptor 1
γH2AX	histone H2A family member X
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum

RT-PCR	real time polymerase chain reaction
CFSE	carboxyfluorescein succinimidyl ester
PBS	phosphate-buffered saline
PHA	phytohaemagglutinin
ESR	erythrocyte sedimentation rate
CRP	C-reactive protein
DMARD	disease-modifying anti-rheumatic drug
NSAID	non-steroidal anti-inflammatory drug
Bcl-2	B-cell lymphoma 2
DNMT	DNA methyltransferase

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Zusammenfassung

Ziel und Hintergrund des Projekts

Diese Arbeit beschäftigt sich mit dem Zusammenhang zwischen der Alterung von Immunzellen und der Entstehung oder Perpetuation von Autoimmunerkrankungen. Ich habe die Homöostase und Alterung in Subsets der zirkulierenden T Lymphozyten als zentrale Akteure bei der Krankheitsentstehung bei der rheumatoiden Arthritis untersucht. Darüber hinaus habe ich die regulatorischen Eigenschaften eines neuen, seneszenten Lymphozytensubsets beschrieben.

Methoden

Prospektive Querschnittsstudie an 44 Patienten mit RA [Durchschnittsalter 59.3 (± 9.9), 77.3% weiblich, SDAI 7.8 (±7.7)] und 35 gesunden Kontrollen [Durchschnittsalter 56.5 (± 9.4), 82.9% weiblich]. Um die Prävalenz der $CD4^{+}CD28^{-}$ $FoxP3^{+}$ T-Zellen zu erheben sowie für die Bestimmung phänotypischer Änderungen, der Zytokinproduktion, des Proliferations- und Apoptoseverhaltens wurden Methoden der Durchflusszytometrie verwendet. Zur Bestimmung des Seneszenz-Status wurde die T-Zell-Rezeptor (TCR) Diversität mittels RT-PCR gemessen. Die *in vitro* Differenzierung von seneszenten Tregs wurde mittels Zellkultur durchgeführt. Dafür wurden normale $CD4^{+}CD25^{+}CD127^{low}$ Tregs via magnetischer Zellisolation gewonnen und mit anti-CD3/CD28 beads, Interleukin (IL) -2 in der Anwesenheit oder Abwesenheit von TNF-α (100ng/ml) für 7 Tage stimuliert.

Resultate

1.7% [0-14.7] der $CD4^+$ T-Zellen waren $CD28^-FoxP3^+$ in RA Patienten. Diese Subpopulation war in gesunden Kontrollen jedoch kaum vorhanden [0.3 (0-2.8), $p<0.001$]. Die Anzahl von gewöhnlichen $CD4^+FoxP3^+$ regulatorischen T-Zellen war nicht unterschiedlich [32 (0.9-83.5) vs. 27.2 (3.9-83.8), $p=0.416$]. Phänotypische Analysen zeigten dass $CD28^-FoxP3^+$ T-Zellen signifikant mehr PD-1 an der Zelloberfläche exprimieren verglichen mit ihren $CD28^+$ Gegenstücken [17.45% (0-36.4) vs. 5.45% (1.8-13.5), $p=0.034$]. Die Expression von CTLA-4 war nicht unterschiedlich. Zusätzlich zeigten $CD28^-FoxP3^+$ T-Zellen eine vermehrte Produktion der folgenden Zytokine: IL-2, IL-4, IL-10, IL-17, TNF-α und IFN-γ [alle $p<0.05$]. Weiters zeigte diese seneszente Subpopulation ein reduziertes Proliferationspotential [50% (0-93.6) nicht proliferierende Zellen vs. 4.6% (0-30.6), $p=0.001$]. In Übereinstimmung damit zeigte sie auch eine erhöhte Apoptose-Rate verglichen mit $CD28^+FoxP3^+$ T-Zellen [22.1% (0-30.8) vs. 4.4% (0-7.8), $p<0.001$]. Die TCR Diversität in $CD28^-FoxP3^+$ T-Zellen war drastisch reduziert [medianer TCR Diversitäts-Score: 84 (36-104) vs. 115 (109-125), $p=0.037$]. In Zellkulturversuchen führte die Stimulation mit TNF-α zu einer Abnahme an CD28 Expression [mediane MFI: 6097 (4309-9298) vs. 8380.5 (6704-13252), $p=0.036$] und damit zur Generation von seneszenten, regulatorischen $CD4^+CD28^-FoxP3^+$ T-Zellen.

Schlussfolgerung

In dieser Arbeit beschreiben wir ein neues T-Zell Subset welches seneszente sowie regulatorische Eigenschaften aufweist. Dieses Subset kommt gehäuft in Patienten mit

RA vor und weist Abweichungen bezüglich Phänotyp und Funktion auf, die auf eine Rolle bei der Pathogenese oder Perpetuation der RA hinweisen.

Abstract

Background/Purpose

This work aims at the better understanding of the role of immunosenescence in autoimmune diseases. We therefore focus on T lymphocyte development, homeostasis and aging as a central point of disease pathogenesis and investigate signs of advanced age of T-cells in autoimmune disorders like rheumatoid arthritis as well as the properties of so far unknown senescent T-cell subsets ($CD4^+CD28^-FoxP3^+$).

Methods

Prospective, cross-sectional study on 44 patients with RA [mean age 59.3 (±SD 9.9), 77.3% female, SDAI 7.8 (±7.7)] and 35 healthy controls [mean age 56.5 (±9.4), 82.9% female]. To determine prevalences of $CD4^+CD28^-FoxP3^+$ T cells as well as to test for phenotypic changes, proliferative capacity, cytokine production and apoptosis flow cytometry methods were used. To test for the senescence status TCR diversity was determined by RT-PCR. *In vitro* generation of senescent Tregs was performed in cell culture experiments. Therefore, normal $CD4^+CD25^+CD127^{low}$ Tregs were isolated using magnetic cell isolation method and stimulated with anti-CD3/CD28 beads, interleukin (IL) -2 in the presence or absence of TNF-α (100ng/ml) for 7 days.

Results

1.7% [0-14.7] of $CD4^+$ T cells were $CD28^-FoxP3^+$ in RA patients. This subset, however, was almost absent in healthy individuals [0.3 (0-2.8), $p<0.001$]. The number

of ordinary $CD4^+FoxP3^+$ regulatory T cells was not influenced [32 (0.9-83.5) vs. 27.2 (3.9-83.8), p=0.416]. Phenotypic analysis revealed that $CD28^-FoxP3^+$ T cells express significantly more surface PD-1 compared to their $CD28^+$ counterparts [17.45% (0-36.4) vs. 5.45% (1.8-13.5), p=0.034], whereas CTLA-4 expression was not different. In addition, $CD28^-FoxP3^+$ T cells showed increased production of various cytokines including IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ [all p>0.05]. Further this senescent subset showed reduced proliferative potential [50% (0-93.6) non proliferating cells vs. 4.6% (0-30.6), p=0.001] which is in line with increased apoptosis rate in comparison to $CD28^+FoxP3^+$ T cells [22.1% (0-30.8) vs. 4.4% (0-7.8), p<0.001]. TCR diversity was drastically reduced in $CD28^-FoxP3^+$ T cells [median TCR diversity score: 84 (36-104) vs. 115 (109-125), p=0.037]. In cell culture experiments, TNF-α was able to down-regulate CD28 [median MFI: 6097 (4309-9298) vs. 8380.5 (6704-13252), p=0.036] and thus generate senescent regulatory $CD4^+CD28^-FoxP3^+$ T cells.

Conclusion

We discribe 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. Phenotype and function suggest a role or this subset in disease pathogenesis.

Background

Clinical background and relevance of rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common inflammatory joint disease affecting up to 1% of the western population.(1) RA predominately occurs in women of middle and older age and is clinically characterized by pain, swelling and stiffness of small and medium-sized joints. In case of insufficient therapeutic suppression of inflammation, progressive joint destruction occurs, leading to permanent disability and early death.(2) Extra-articular manifestations such as rheumatoid nodules or RA vasculitis are typically observed in RA patients with long-standing disease and cardiovascular events contribute to an elevated mortality in RA patients compared to the general population.(1)

Current guidelines recommend an early and aggressive therapy of RA; nevertheless, remission is achieved in a minority of patients only.(3) Therefore, there is a need of a better understanding of RA.

Relevance of T-cells for the pathogenesis of RA

The cause of RA is still unknown. It's the current understanding of the disease that environmental factors trigger a self-perpetuating immune reaction in people with a specific genetic background.(4) Aberrantly activated immune cells cause synovial

inflammation and autoantibody production (ACPA and rheumatoid factor) finally leading to the destruction of cartilage and bone.

The exact role of T-cells for the pathogenesis of RA is unknown. The strong linkage of genes of the major histocompability complex (MHC), particularly the shared epitope as well as genes involved in T-cell activation (such as HLA-DR4, PTPN22, CTLA-4) with RA indicates that T-cell recognition of a so far undefined antigen is a crucial event.(5) Besides, the clinical efficacy of abatacept, an inhibitor of T-cell co-stimulation supports the central role of T-cells in the evolvement of RA.

RA is mainly a T_H1 driven disease. T_H1 cells are characterized by production of interferon-γ and TNF-α whereas T_H2 cells secrete IL-4 and IL-5 and are involved in allergic diseases. Terminally differentiated T-cells are present in peripheral blood and at inflammatory sites of RA patients; T-cells have been associated with the long-term perpetuation of the autoimmune response as outlined below.(6)

Thymic function and T-cell homeostasis

An increasing number of studies indicate that premature T-cell aging contributes to the pathogenesis of RA and other autoimmune disorders.(7,8) Based on their developmental status, expression of surface molecules and antigen experience three stages of maturation are currently defined: naïve, memory and senescent effector-memory T-cells. The hallmark feature of naïve T-cells in flow cytometry analyses is the expression of both CD28 and CD45RA (=220kDA isoform of the leucocyte common antigen CD45 that is involved in the regulation of T and B-cell antigen receptor signaling). Memory T cells express CD28 and CD45RO (=180kDA isoform of

the leucocyte common antigen CD45) whereas aged effector-memory T-cells are characterized by the down-regulation of CD28 with a variable expression of CD45RA and CD45RO.

T-cell aging as normally observed in individuals of advanced age is determined by:

1. Thymic involution after puberty drastically reducing the output of naïve T-cells(9)
2. Age related changes of the cell membrane leading to altered cellular signaling(10)
3. Recurrent infections leading to exhaustion of the T-cell reserve(11)

As a consequence of thymic involution, the proliferation of peripheral T-cells compensates for the lack of new T-cell generation on order to maintain homeostatic control.(12) Ongoing peripheral T-cell renewal is associated with a decline in telomere length in T-cells, contraction of T-cell receptor (TCR) repertoire, epigenetic changes and accumulation of terminally differentiated effector-memory T-cells.(13)

Aging of a cell is associated with the shortening of chromosomes at the telomere after each cycle of reproduction, this phenomenon starts even before birth. The longevity of an organism requires a fine balance of maintenance and repair with the need to sustain the effector functions. On that account enzymatic machineries exist that maintain telomere length thereby restoring genomic integrity.(14) At a certain point cells succumb to irreparable damage and still preserve some functionality while being rendered senescent. Cellular senescence is associated with a shift in gene expression.(15,16) However, this shift involves only a minority of expressed genes while many effector functions are maintained.(17) Telomere erosion, however, initiates

a program of cell senescence that prevents further cell divisions resulting in protection of cells from excessive telomere loss and cell death.(18,19) In addition, telomerase enzyme is only able to slow down but not fully compensate for telomere loss.(20) As a result, telomere length of human peripheral blood cells is suggested to be a predictive marker for age-dependent mortality.(21)

The cells of the immune system naturally undergo constant self-renewal. Renewal of T-cells is exceptional and distinct from other hematopoietic lineages since T lymphocyte development depends on thymic activity also. As a result, telomere length of newly generated T-cells is determined by the telomere length of hemotopoietic cells and the telomere elongation mechanisms in thymocytes. An effective immune response, however, requires a rapid clonal expansion of individual antigen-specific T- and B-cell precursors. Accordingly, memory T-cells display shorter telomere lengths in general.(22) Senescent T-cells that have been repeatedly stimulated in vivo have the shortest telomeres.(23)

Terminally differentiated effector-memory T-cells are phenotypically characterized by the loss of the most important co-stimulatory molecule CD28.(24) $CD28^-$ T-cells preferentially express T_H1 type chemokine receptors and cytokines, they combine features of short-lived effector memory and long-lived central memory cells (expression of CCR-5 and CCR-7, respectively) allowing the cells to home either to sites of inflammation as well as to lymph nodes.(25)

In RA, an increased occurrence of senescent lymphocytes is observed even in young individuals and several studies found an inadequate accumulation of terminally differentiated effector-memory T-cells in peripheral blood as well as at sites of inflammation.(26) As RA promotes immunosenescence due to chronic immune

activation, it is still a matter of debate whether premature T-cell aging is the cause or the consequence of the disease.(27,28)

Thymic T-cell production is inappropriately reduced in young patients with RA. Even patients with recent onset of disease demonstrated impaired thymus function and a severely contracted TCR repertoire of naïve and memory T-cells.(8,29) Interestingly, also healthy individuals bearing the shared epitope had an inadequate atrophy of thymus and signs of early T-cell aging.(30) These observations strongly argue in favor of immunosenescence preceeding the onset of RA rather than being only a consequence of it.

The role of senescent T-cells for the pathogenesis of RA

The prevalence of circulating senescent $CD28^-$ T-cells is increased in RA. $CD4^+CD28^-$ T-cells are auto-reactive and resistant to apoptosis; they aberrantly express stimulating NK cell receptors such as NKG2D and CX3CR1 and are activated by their corresponding ligands within the synovia.(31,32)

The increased prevalence of $CD28^-$ T-cells is linked with a worse outcome of RA: these patients more commonly have bone erosions and extraarticular manifestations and demonstrate an excess cardiovascular risk compared to RA patients with low levels of senescent T-cells.(33,34) In one study it was found that $CD28^-$ T-cells directly lyse endothelial cells by perforin thus promoting pre-arteriosclerotic vascular damage.(35)

The occurrence of terminally differentiated T-cells is also of relevance for patients receiving abatacept, a costimulation blocker acting at the CD28 level. Patients with a

high number of $CD28^-$ T-cells responded worse to this therapy compared to patients with low $CD28^-$ T-cells counts.(36)

Relevance of regulatory T-cells in RA

Apart from prematurely aged T-cell subsets, an imbalance of regulatory T-cells (Tregs) may contribute to pathogenesis of the RA.(37) The number of Tregs was increased in synovial fluid compared to peripheral blood and these Tregs had an activated phenotype with high levels of CTLA-4, GITR, OX-40 and FoxP3.(38) In addition, natural Tregs (nTregs) had a reduced capacity to inhibit the production of pro-inflammatory cytokines.(39) Synovial fluid non-regulatory T-cells were resistant towards Treg mediated suppression. This deficiency was partially restored by anti-TNF-α treatment. A possible explanation for the reduced effect of Tregs in RA might be a relatively low expression of CTLA-4. The reason for CTLA-4 downregulation is unknown but CTLA-4 gene polymorphisms that are associated with a higher susceptibility to RA, might play a role.(40,41) Artificial induction of CTLA-4 in Tregs from RA patients *in vitro* restored their suppressive capacity.(42) Of note, a lot of current drugs for the treatment of RA also influence Treg frequencies and/or function.(43)

Recent studies indicate that Tregs have the potential to convert into pro-inflammatory IL-17 secreting cells (T_H17). T_H17 cells are the main T effector cell subset involved in the pathogenesis of inflammation and autoimmunity.(44,45) An augmented T_H17 response as indicated by increased serum levels of IL-15, IL-17 and IL-23 was noted in

various autoimmune diseases such as RA directly related to clinical disease activity.(46,47)

Regulatory T-cells and aging

Similar to the developmental stages known for non-regulatory T-cells, different cellular subsets of Tregs were also observed. In humans, $CD4^{+}FoxP3^{+}$ Tregs may have either a 'naïve-like' phenotype characterized by the expression of $CD25^{+}CD45RA^{+}$ or a $CD25^{hi}CD45RO^{+}$ 'memory-like' phenotype.(48)

In humans, the highest prevalence of naïve-like Tregs was found in cord blood and it was assumed that these naïve-like Tregs were produced in the thymus.(49,50) The prevalence of memory-like Tregs increases rapidly during childhood and it was demonstrated that these memory-like Tregs have shorter telomeres and a lower content of T-cell receptor excision circles (Trecs) compared to naïve-like Tregs reflecting a longer replicative history.(48) The mechanisms mediating the transition of a naïve-like Treg into a memory-like phenotype still have to be explored; however, it is believed that antigen experienced dendritic cells migrating to secondary lymphoid tissues are involved. Tregs proliferate upon stimulation with autologous immature and mature dendritic cells.(48,51)

Human adult peripheral blood usually contains both, naïve-like and memory-like Tregs. Parallel to the reduction of total naive T-cells the quantity of naive-like Tregs declines with age whereas the prevalence of memory-like Tregs increases.(52,53) The total pool of circulating Tregs, however, remains unchanged.(54) As naïve-like Tregs exhibit a higher proliferative potential *in vitro* compared to memory-like Tregs it can

be expected that the capacity of the immune system to down-regulate abnormal immune responses declines with age.(48)

Replicative senescence of T-cells is a prominent feature in elderly people resulting from homeostatic proliferation and repetitive antigen exposure.(27) The most important phenotypic feature of senescent T-cells is the loss of the type I transmembrane protein CD28, a major co-stimulatory molecule. Given that Tregs proliferate in the periphery to maintain the total Treg pool after thymic failure it is plausible to hypothesize that Tregs may undergo terminal differentiation as well.(37,55)

Interestingly, a proportion of Tregs from aged mice showed decreased expression of CD25.(56,57) These $CD25^{low}$ Tregs occurred predominantly in the spleen(58) but had comparable functional properties to $CD25^{+}$ Tregs. A similar $CD4^{+}CD25^{-}FoxP3^{+}$ Treg population has been observed in SLE patients.(59) SLE patients are known to have a prematurely aged immune system with accumulation of $CD28^{-}$ T-cells. A detailed characterization of $CD4^{+}CD25^{-}FoxP3^{+}$ Tregs regarding the expression of naïve/memory T-cell markers or determination of telomere lengths was unfortunately not performed. Further evidence for the occurrence of Treg senescence was found in a study on healthy aged individuals reporting the occurrence of a $CD8^{+}CD25^{+}$ Treg population lacking CD28 expression. These regulatory cells shared phenotypic and functional features with $CD4^{+}$ Tregs from the same population.(60) Strikingly, Zhang et al reported that mice with a conditional knock-out of CD28 developed a severe autoimmunity. They suggest that CD28 is of enormous implication for adequate immune-modulating function of Tregs.(61) The occurrence and possible characteristics of terminally differentiated $CD4^{+}$ Tregs is an interesting issue that has to be investigated by future studies.

Synopsis

Premature T-cell aging contributes to the pathogenesis of RA. The impact on other autoimmune diseases such as axial spondyloarthritis is more obscure. Senescent T-cells, however, are characterized by the loss of CD28 and expression of pro-inflammatory molecules. $CD28^-$ T-cell prevalences correlate with disease severity in RA. Increasing evidence suggest a pivotal role of Tregs in the disease pathogenesis as well. Numbers and function of Tregs are altered in RA and lead to an elevated T_H17 response.

Mice experiments indicate a pivotal role for CD28 in the functionality of Tregs. A lack of CD28 in Tregs led to severe autoimmunity in these models. In human naïve as well as memory Treg subsets were described.

Whether senescent $CD28^-$ Tregs occur in human and their role in the pathogenesis of autoimmune diseases is unknown.

Based on our expertise we have already published our observations with senescent T-cells in spondyloarthritis.(62)

Aims of the study

This work aims at the better understanding of the role of immunosenescence in autoimmune diseases. We therefore focus on T lymphocyte development, homeostasis and aging as a central point of disease pathogenesis.

This study is concerned with the characterization of a novel T-cell subset that combines regulatory and senescent properties - $CD4^{+}CD28^{-}FoxP3^{+}$ T-cells. We investigate whether $CD4^{+}CD28^{-}FoxP3^{+}$ T-cells are associated with the occurrence and disease severity of RA and survey this novel T-cell subset regarding phenotype, function and epigenetic alterations.

Patients, materials and methods

Study design

This was a prospective study on consecutive patients with RA as well as healthy individuals.

Patients

44 consecutive patients with a final diagnosis of RA based on the 2010 criteria of the American College of Rheumatology – European league against rheumatism and 35 age- and sex-matched healthy controls (HC) were prospectively enrolled. Detailed family and medical history including disease duration, prior and current treatments were obtained from each patient. We performed the SDAI, the DAS28 and the HAQ and we investigated patients for the presence of extra-articular manifestations (particularly rheumatoid nodules, RA-associated lung disease, RA-associated vasculitis etc.). In addition, we recruited 35 healthy controls out of lab personnel and relatives of the investigators. This study was approved by the Institutional Review board of the Medical University Graz and written informed consent was obtained from each individual.

Peripheral blood mononuclear cells and cell culture

Peripheral venous blood was drawn and peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque density gradient centrifugation. Cells were washed twice with RPMI 1640 containing 10% fetal calf serum, 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The total cell number was determined by a Beckmann Coulter.

Antibodies and flow cytometry

Surface and intracellular staining of $1*10^6$ freshly isolated PBMCs was performed using appropriate combinations of antibodies for detection of CD3, CD4, CD28, (T-cell marker) CD25, CD127, FoxP3, (Treg marker) CTLA-4 (marker for Treg function), IL-2 (T_H1), IL-4 (T_H2), IL-10, IL-17 (T_H17), TNF-α, IFN-γ (Th1; all Becton Dickinson, San Diego, USA) and γH2AX (marker of senescence). Surface staining for 30 minutes at 4^oC at dark was followed by permeabilization for 30 min at room temperature and intracellular staining for 30 minutes at 4^oC at dark according to a routine protocol.

As a control, appropriate isotype controls were used. Stained cells were analyzed on a FACS Canto II (Becton Dickinson). 300.000 events were counted at each acquisition and data are analyzed with DIVA software.

Separation of T-cell subsets

For functional assays, $CD4^+$ T-cells were isolated by positive selection of PBMCs labeled with magnetic-bead conjugated anti-human CD4 mAbs using MACS MultiSort Kit and LS columns (Midi-MACS) according to manufacturer's instructions (Miltenyi Biotech, Amsterdam, The Netherlands). Purified $CD4^+$ T-cells were then separated into the $CD28^+CD25^+CD127^{dim}$, $CD28^+CD25^-$, $CD28^-CD25^+CD127^{dim}$ and $CD28^-CD25^-$ fractions by another sorting step using FACS Sorting technology (FACS Aria). Therefore, cells were labeled with PerCP-Cy5.5 anti-CD28,PE-Cy7 anti-CD25 and PE anti-CD127 antibodies according to cell number. Flow cytometry was then performed to determine purity of selected cells.

For *in vitro* aging, Tregs were isolated using $CD4^+CD25^+CD127^{dim/-}$ Reg. T Cell isolation Kit II (Miltenyi Biotech, Amsterdam, The Netherlands) and autoMACS Pro.

For validation, cells were intracellularly stained for FoxP3 expression and analysed on a FACS Canto II.

Cell culture: *In vitro* aging of Tregs

After isolation of $CD4^+CD25^+CD127^{dim/-}$ regulatory T-cells via AutoMACS (Miletnyi) the cells were cultured as described in(63) with some modifications. In brief, $1x10^5$ cells/ml are given 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 and anti- CD3/CD28 MoAb- coated microbeads (life sciences) at a 4:1 bead-to-cell ratio and were stimulated with 200 U/ml human recombinant (rHU) IL-2 (SIGMA), with or without 100 ng/ml TNF-α (SIGMA).

After six days of cultivation, the cells were harvested, washed and given in medium plus 20U/ml for 2 days. After the resting phase, the expanded Tregs were restimulated 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.

During the expansion every 2-3 days fresh medium, Il-2 and TNF-α were added.

Determination of telomere length

Telomere lengths were measured using DNA from PBMCs as well as T-cell subsets by quantitative real-time PCR analysis and LightCycler FastStart DNA Master SYBR Green I (Roche) according to manufacturer's instructions. The following primers were used: 5´CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT 3´ and 5´GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3´. For normalization served a single copy gene (36D4) and appropriate primers (fw: 5´CCC ATT CTA TCA TCA ACG GGT ACA A 3´; rev: 5´CAG CAA GTG GGA AGG TGT AAT CC 3´). Ct-values of test samples and control single copy genes were calculated and $2^{\Delta Ct}$ value was determined as described previously.(64)

Determination of TCR diversity

RNA from enriched subsets was extracted using RNeasy Protect Mini Kit (Qiagen) according to manufacturer regulations. The amount of RNA was determined with Eppendorf Biophotometer plus. Finally, a concentration of 1 µg RNA was used for reverse transcription with First Strand cDNA Synthesis Kit for RT-PCR AMV (Roche) according to manufacturer regulations. cDNA was diluted 1:5 for PCR using AmpliTaq

Gold™ DNA Polymerase (Applied Biosystems), 1X PCR Gold Buffer (Applied Biosystems), 2,5mM MgCl2 (Applied Biosystems), 0,4 mM dNTP Polymerisation Mix (GE Healthcare), 0,5 µM TCR C β 5´FAM labelled primer (Ingenetix) and 0,5 µM unlabelled TCR V β primer (Ingenetix) according to Hingorani et al. 1996. (65) This resulted in 25 reactions per sample. Primer sequences are listed in table I. Cycle conditions were a denaturation step at 94°C for 6 min, 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min, a final annealing step at 72°C for 7 min. After amplification 1 µl of pcr-product was supplemented with 0,5 µl of GeneSCan™-500 TAMRA™ Size Standard (Applied Biosystems) and 12 µl HI-DI Formamide (Applied Biosystems). Electrophoresis was performed with ABI Prism 310 Genetic Analyzer (Applied Biosystems) and 310 Data Collection Software. Analysis was done by GeneScan® Software (Applied Biosystems). Calculations included peak count (Complexity score) and single peak area in percent of whole peak area.

Table I: Spectratyping primer and probe sequences:

BV1	CAA CAG TTC CCT GAC TTG CAC
BV2	TCA ACC ATG CAA GCC TGA CCT
BV3	TCT AGA GAG AAG AAG GAG CGC
BV4	CAT ATG AGA GTG GAT TTG TCA TT
BV5.1	TTC AGT GAG ACA CAG AGA AAC
BV5.2	CCT AAC TAT AGC TCT GAG CTG
BV6	AGG CCT GAG GGA TCC GTC TC
BV7	CTG AAT GCC CCA ACA GCT CTC
BV8	TAC TTT AAC AAC AAC GTT CCG
BV9	AAA TCT CCA GAC AAA GCT CAC
BV10	CTC CAA AAA CTC ATC CTG TAC CTT
BV11	ACA GTC TCC AGA ATA AGG ACG
BV12	GAC AAA GGA GAA GTC TCA GAT
BV13.1	GAC CAA GGA GAA GTC CCC AAT
BV13.2	GTT GGT GAG GGT ACA ACT GCC
BV14	GTC TCT CGA AAA GAG AAG AGG AAT
BV15	GTC TCT CGA CAG GCA CAG GCT
BV16	GAG TCT AAA CAG GAT GAG TCC
BV17	CAC AGA TAG TAA ATG ACT TTC AG

BV18	GAG TCA GGA ATG CCA AAG GAA
BV19	TCC TCT CAC TGT GAC ATC GGC CA
BV20	TCT GAG GTG CCC CAG AAT CTC
BV21	GAT ATG AGA ATG AGG AAG CAG
BV22	CAG AGA AGT CTG AAA TAT TCG A
BV23	TCA TTT CGT TTT ATG AAA AGA TGC
BV24	AAA GAT TTT AAC AAT GAA GCA GAC
BC-R	CTT CTG ATG GCT CAA ACA C

Functional assays

Proliferation assay

A CFSE stock (10 mM in DMSO; Invitrogen) stored at − 20 °C, was thawed and diluted in phosphate-buffered saline (PBS) to the desired working concentrations. For all experiments, freshly purified PBMCs were resuspended in PBS at 5-10 × 10^6 cells/ml and incubated with CFSE (final concentration: 1 µM) for 7 min at 37 °C. Cells were washed three times and resuspended in culture medium. Cells were stimulated with plate-bound anti-CD3 (10 µg/ml, 18h) or PHA (1 µg/ml) and cultured for 72 hours. Afterwards cells were stained according to our flow cytometry protocol.

Cytokine production measurement

Freshly isolated PBMCs were stimulated with plate-bound anti-CD3 (10 µg/ml, 18h) or PHA (1 µg/ml) and cultured for 72 hours. Golgi transport was inhibited by brefeldin A (10 ng/ml) 4 hours prior to cytokine staining. Afterwards cells were harvested and stained intracellularly for IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ (all BD bioscience) as well as surface markers according to flow cytometry protocol.

Determination of suppressive activity

$CD25^-$ Effector T cells (Teff) were seeded in an U-bottom 96-well plate at a concentration of $2.5x10^4$/well and cultivated in the presence or absence of autologous $CD28^+CD25^+$ or $CD28^-CD25^+$ T-cells at a 1:1 ratio and the presence of irradiated autologous PBMC ($2.5x10^4$/well). Cells were stimulated by adding anti-CD3 (10 µg/ml) and incubated at 37°C and 5% CO_2 for 96h. During the last 18h responder cell proliferation was monitored through the analysis of ^{3}H-thymidine uptake (Biotrend, Köln, Germany) measured on a MicroBeta Trilux Counter (Perkin Elmer, Wellesley, USA).

Epigenetic investigations

Methylation assay

DNA of T-cell subsets was obtained from 5 RA patients that indicated and high frequency (>5%) of $CD4^+CD28^-$ T-cells. Methylation status was assessed using MethylFlash Methylated DNA Quantification Kit (Epigentek) following manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using the SPSS program, version 20.0 (Chicago, IL, USA). Results were described as median and range or mean and standard deviation as appropriate. The Kolmogorov-Smirnov test was used to analyze the distribution of the variables. In case of a normal distribution of the continuous

variables, the two-sided Student´s t-test (comparison of two groups) or ANOVA (comparison of three or more groups) were applied and in case of non-parametric distribution, the Mann-Whitney-U and the Kruskal-Wallis tests were performed. Correlation between variables was evaluated by the Spearman´s rank correlation coefficient. Paired data were compared with the Wilcoxon test.

Results

Patients' characteristics

Clinical characteristics are depicted in table II. In the RA cohort, 2 (6.9%), 2 (6.9) and 17 (58.6) out of the 29 patients with available SDAI values had high, moderate or low disease activity, respectively, and 8 (27.6) patients were in remission.(66) Seven (15.9%) RA patients had early disease (≤2 years' duration).

Table II: Patients' characteristics:

	HC	**RA**	**p-value**
Number	35	44	
Age [years] †	56.5 (±9.4)	59.3 (±9.9)	0.199
Female, n (%)	29 (82.9)	34 (77.3)	0.542
Disease duration [years] †	n.a.	7.8 (±7)	
Disease activity scores:			
SDAI†	n.d.	7.8 (±7.7)	
DAS28†	n.d.	2.8 (±1)	
Laboratory data:			
ESR (mm/1sth) ‡	n.d.	11 (1-41)	
CRP [mg/l] ‡	n.d.	3 (0-22)	
Current medication:			
Corticosteroids, n (%)	0	9 (20.5)	
Biologicals, n (%)			

anti-TNFα	0	18 (40.9)	
Tocilizumab	0	9 (20.5)	
Abatacept	0	8 (18.2)	
Rituximab	0	2 (4.5)	
DMARDs, n (%)			
Methotraxate	0	25 (56.8)	
Leflunomide	0	8 (18.2)	
Sulfasalazine	0	2 (4.5)	
Other	0	1 (2.3)	
NSAIDs, n (%)			
Regularly	0	5 (11.4)	
on demand	0	32 (72.7)	

†mean (±standard deviation); ‡median (range)

CRP, C-reactive protein [0-5 mg/l]; DAS28, Disease Activity Score 28; DMARD, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate [0-30 mm/h]; HCs, healthy controls; n, number; n.a., not applicable; n.d., dot determined; NSAID, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; sDAI, simplified disease activity index

Increased prevalence of $CD4^+CD28^-FoxP3^+$ T-cells in RA

To estimate if the novel T-cell subset $CD4^+CD28^-FoxP3^+$ is relevant for the disease pathogenesis of RA, we analyzed occurrence of these cells in RA patients and healthy controls. We found elevated numbers of $CD4^+CD28^-FoxP3^+$ T-cells in RA patients

compared to healthy individuals (1.7% of total $CD4^+$ [range 0-14.7] vs. 0.3% [0-2.8]; $p<0.001$; figure I), whereas the frequency of $CD4^+CD28^+FoxP3^+$ T-cells was equal between groups (32% of total $CD4^+$ [range 0.9-83.5] vs. 27.2% [3.9-83.8]; p=0.416; figure II).

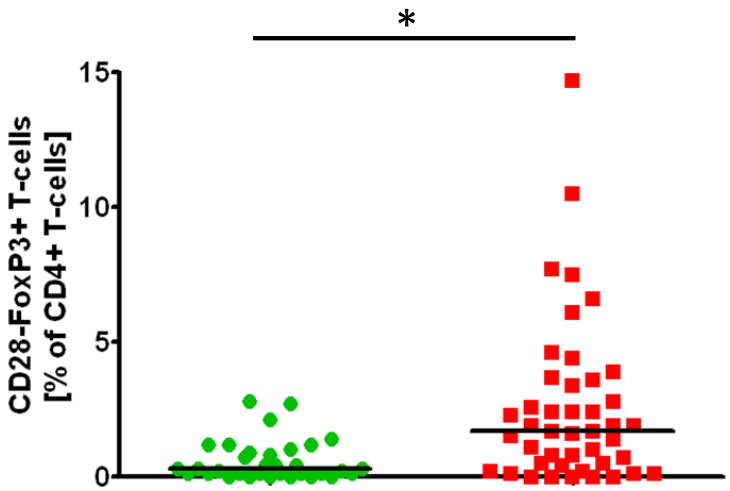


Figure I: Elevated numbers of $CD4^+CD28^-FoxP3^+$ T-cells in RA patients. Prevalences of $CD4^+CD28^-FoxP3^+$ T-cells in healthy individuals (HC, n=35) and RA patients (RA, n=44). *…$p<0.05$

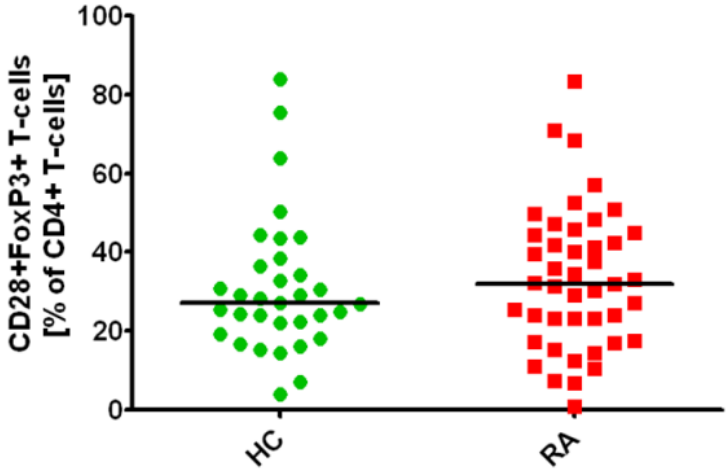


Figure II: Frequencies of $CD4^+CD28^+FoxP3^+$ T-cells are not altered in RA patients. Prevalences of $CD4^+CD28^+FoxP3^+$ T-cells in healthy individuals (HC, n=35) and RA patients (RA, n=44). *…p<0.05

Besides, frequencies of $CD4^+CD28^-FoxP3^+$ T-cells are significantly correlated with age in our RA cohort ($corr_{coeff}$=0.411, p=0.006). In contrast, $CD4^+CD28^+FoxP3^+$ T-cell levels were not linked with age ($corr_{coeff}$=0.213, p=0.164).

No association of $CD4^+CD28^-FoxP3^+$ T-cells with disease activity scores or clinical variables could be observed.

Impaired proliferative capacity of $CD4^+CD28^-FoxP3^+$ T-cells

To test the proliferative potential of the different T-cell subsets we stained isolated PBMCs with CFSE and stimulated them with anti-CD3. $CFSE^{high}$ cells reflect undivided cells. The proliferative capacity of $CD4^+CD28^-FoxP3^+$ T-cells was reduced compared to $CD4^+CD28^+FoxP3^+$ Tregs (53.1% $CFSE^{high}$ [range 14.5-93.6) vs. 5.4% [0.9-30.6]; p=0.008; figure III).

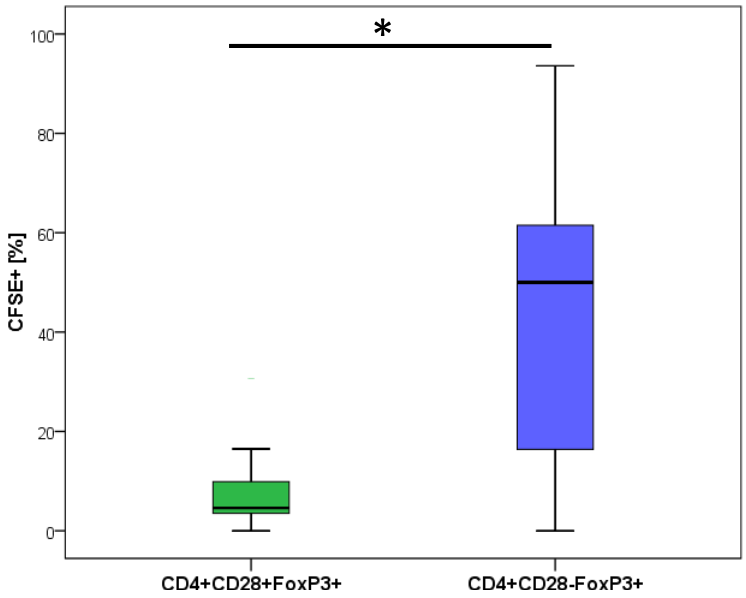


Figure III: Proliferative capacity of different T-cell subsets. PBMCs of 10 RA patients were isolated and cultured in the presence of CFSE and stimulated with OKT-3 (10µg/ml). $CFSE^{high}$ cells represent undivided cells, since CFSE is reduced with each cell division. *…$p<0.05$, n=10 (2x anti-TNFα, 1x Tocilizumab, 3x Abatacept, 2x Rituximab)

Enhanced cytokine secretion of $CD4^+CD28^-FoxP3^+$ T-cells

To test if $CD4^+CD28^-FoxP3^+$ T-cells favor a Th1, Th2 or Th17 milieu we compared $CD28^-$ and $CD28^+$ $CD4^+FoxP3^+$ T-cells in regards to the production of the cytokines IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ. Interestingly, all of these cytokines were more frequently produced by $CD4^+CD28^-FoxP3^+$ T-cells than by $CD4^+CD28^+FoxP3^+$ T-cells (all p=0.005). For detailed analysis see table III.

Table III: Cytokine production of different T-cell subsets:

	$4^+28^+FoxP3^+$	$4^+28^-FoxP3^+$	P-value
IL-2	4.55 (1.1-12)	15.45 (5.1-31.1)	0.005
IL-4	0.3 (0.1-0.8)	4.3 (1.2-7.9)	0.005
IL-10	0.55 (0.3-1.6)	4 (1.5-13.1)	0.005
IL-17	0.7 (0.2-1.7)	4.9 (1.5-14.6)	0.005
TNF-α	11.5 (4.8-18.3)	18 (5.7-34.9)	0.005
IFN-γ	6.8 (2.4-16.1)	16.6 (4.3-40.4)	0.005

Numbers indicate the percentage of positive cells. Median (range) is shown; n=10 (3x anti-TNFα, 2x Tocilizumab, 2x Abatacept)

Altered suppressive function of $CD4^+CD28^-FoxP3^+$ T-cells

Aging of $CD4^+FoxP3^+$Tregs has long been supposed, and naïve as well as a memory phenotype have been described already. So far, no study dealt with end-differentiated senescent Treg cells. $CD4^+CD28^-FoxP3^+$ T-cells, however, may represent this subpopulation. As the isolation of cells expressing the intracellular protein FoxP3 is not feasible in this experimental setup, we detected CD25 positivity in combination with absence of CD127 as the most efficient surrogate markers for FoxP3 expression. Consequently, we studied the suppressive capacity of $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells. In initial experiments we observed that normal $CD4^+CD28^+CD25^+CD127^{dim}$ Tregs indicated good suppressive function (suppression of ~27%, figure IV), whereas

$CD4^+CD28^-CD25^+CD127^{dim}$ T-cells lost the ability of inhibiting proliferation of conventional $CD25^-$ T-cells.

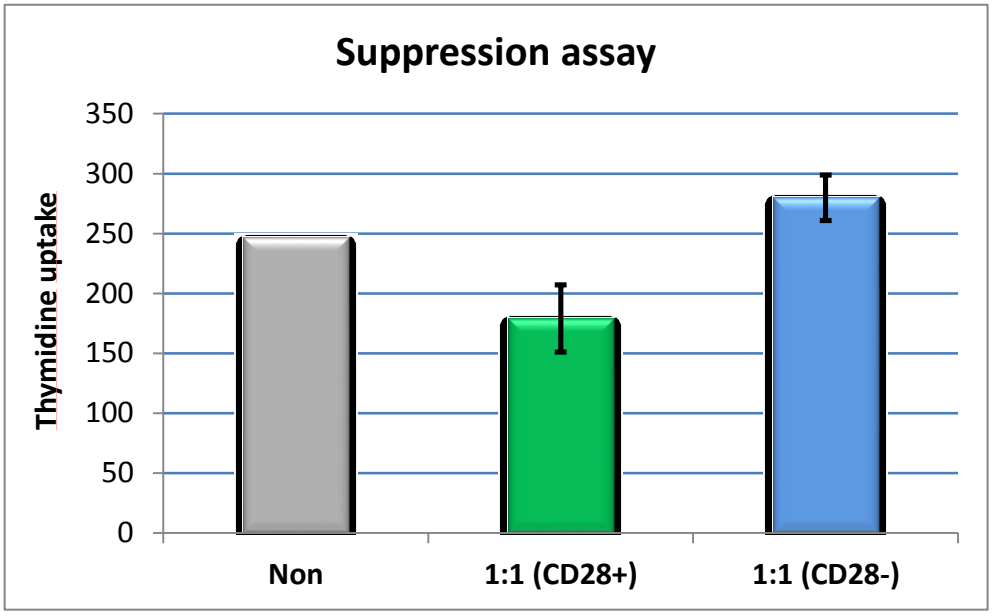


Figure IV: Suppressive capacity of $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells is reduced. $CD4^+CD28^+CD25^+$ $CD127^{dim}$, $CD4^+CD28^-CD25^+$ $CD127^{dim}$ as well as $CD4^+CD25^-$ T-cells (Non) were sorted using FACS technology and cultured alone or in a mixture of 1:1 in X-Vivo Medium with stimulation of OKT-3 (10µg/ml). Responder cell proliferation was monitored through the analysis of ^{3}H-thymidine uptake; n=3 (no biologicals)

CTLA-4 expression is unchanged in $CD4^+CD28^-$ $FoxP3^+$ T-cells

In addition, to the standard suppression assay we performed flow cytometry analysis to investigate the expression of surface molecule CD152 (cytotoxic T lymphocyte associated Ag-4; CTLA-4) that is linked to Treg suppressive function also.

The expression of CTLA-4, however, was similar in both subsets (MFI: 123.5 [103-350] vs. 122 [104-648], p=0.034, figure V).

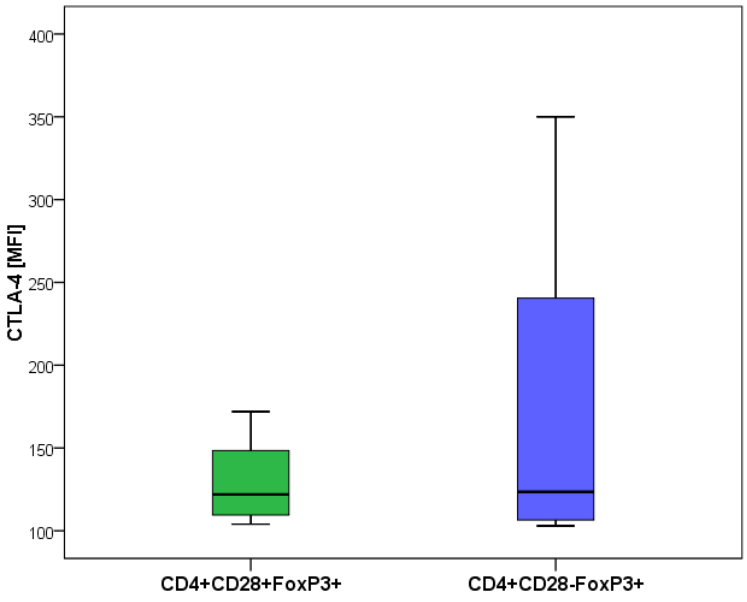


Figure V: CTLA-4 expression is similar in $CD28^+$ and $CD28^-$ T-cell subsets. Expression of CTLA-4 on $CD4^+CD28^+FoxP3^+$ (green) as well as on $CD4^+CD28^-FoxP3^+$cells (blue). Box plots indicate median fluorescence intensity (MFI) of CTLA-4. *…p<0.05, n=8 (2x Tocilizumab, 1x Rituximab)

No differences in telomere lengths $CD28^+$ and $CD28^-$ $FoxP3^+$ T-cells

Telomeric erosion is a general sign for advancing replicative senescence.(67) To address the question if $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells have a higher "immunoage" than their $CD28^+$ counterparts we performed telomere length analysis of T-cell subsets. Interestingly, we were not able to notice statistically significant differences in telomere lengths of $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells (6.11 kbp [5.46-6.19]) and $CD4^+CD28^+CD25^+CD127^{dim}$ T-cells (5.89 [5.6-6.17], p=0.373, figure VI). Besides, conventional young $CD4^+CD28^+CD25^-$ (5.94 [5.53-6.24]) and old $CD4^+CD28^-CD25^-$ T-cells (5.74 [5.5-6.32]) showed similar results.

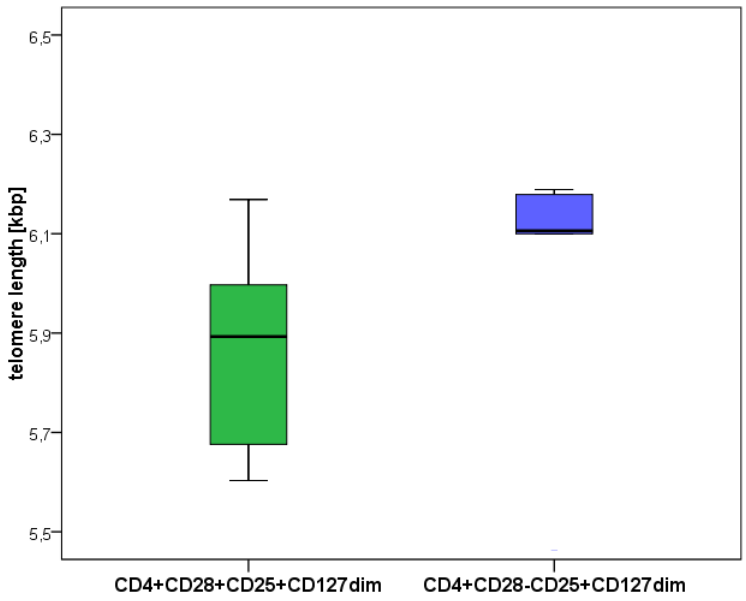


Figure VI: Telomere length of T-cell subsets. T-cell subsets of 5 RA patients were sorted using FACS technology and DNA was extracted. Telomere lengths were assessed using quantitative RT-PCR technique. n=5 (3x anti-TNFα, 1x Tocilizumab, 1x Abatacept)

TCR diversity is reduced in $CD4^{+}CD28^{-}FoxP3^{+}$ T-cells

TCR diversity is known to decrease with T-cell aging and thus $CD28^{-}$ T-cells are manifested as oligoclonal.(68) In accordance, we found significantly decreased TCR diversity in $CD4^{+}CD28^{-}CD25^{+}CD127^{dim}$ (84 [36-104]) compared to their $CD28^{+}$ fellows (115 [109-125]; p=0.037; figure VII).

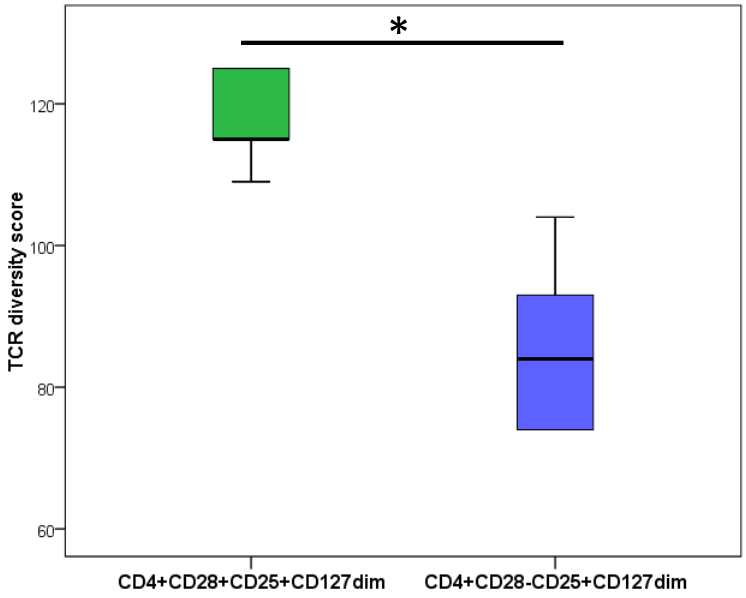


Figure VII: TCR diversity of T-cell subsets. T-cell subsets of 5 RA patients were sorted using FACS technology and RNA was extracted. Spectratyping was determined using PCR method and by constructing a complexity score with a maximum of 125. *…$p<0.05$; n=5 (3x anti-TNFα, 1x Tocilizumab, 1x Abatacept)

γH2AX expression is enhanced in $CD4^+CD28^-FoxP3^+$ T-cells

Senescent cells typically accumulate γH2AX foci which represent repair-proof double-strand breaks in DNA.(69) To further proof the senescent state of $CD4^+CD28^-FoxP3^+$ T-cells we stained fresh cells for the expression γH2AX. $CD4^+CD28^-FoxP3^+$ T-cells showed higher γH2AX mean fluorescence intensity than $CD4^+CD28^+FoxP3^+$ T-cells (MFI: 6422 [2952-258589] vs. 4875 [2875-7743], p=0.046], figure VIII).

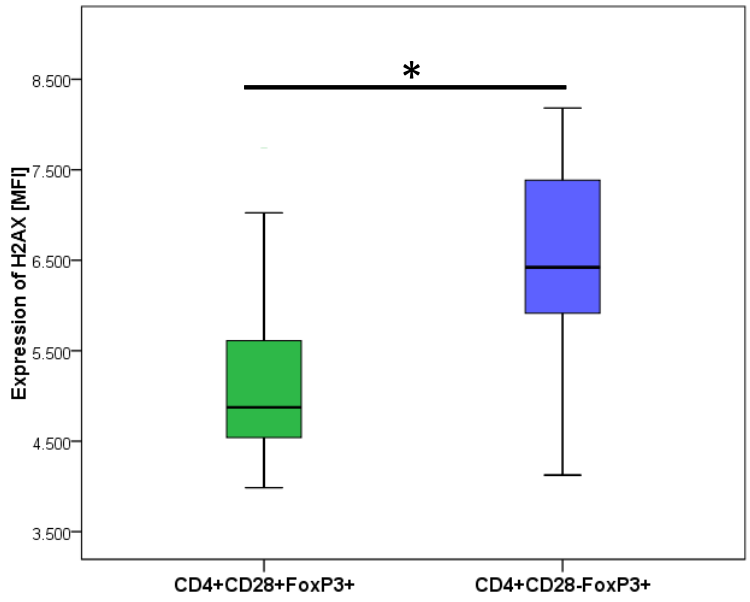


Figure VIII: γH2AX expression is enhanced in senescent $CD4^+CD28^-FoxP3^+$ T-cells. $CD4^+CD28^-FoxP3^+$ (blue) as well as $CD4^+CD28^+FoxP3^+$ T-cells (green) were stained for the expression of γH2AX via flow cytometry. *…p<0.05; n=13 (6x Tocilizumab, 2x Abatacept, 3x Rituximab)

$CD4^+CD28^-FoxP3^+$ T-cells are hypomethylated

Changes in DNA methylation occur with age and increasing evidence suggests an involvement in RA disease pathogenesis. Consequently, we investigated whole DNA methylation status of $CD4^+CD28^-CD25^+$ T-cells. We observed that DNA from $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells was significantly hypomethylated compared to $CD4^+CD28^+CD25^+CD127^{dim}$ T-cell DNA (0.38 [0.17-0.53] vs. 0.61 [0.37-0.86], p=0.036, figure IX). Conventional young $CD4^+CD28^+CD25^-$ and old $CD4^+CD28^-CD25^-$ T-cells, however, did not differ in methylation of total DNA (0.44 [0.31-0.68] vs. 0.45 [0.04-0.51], p=0.405).

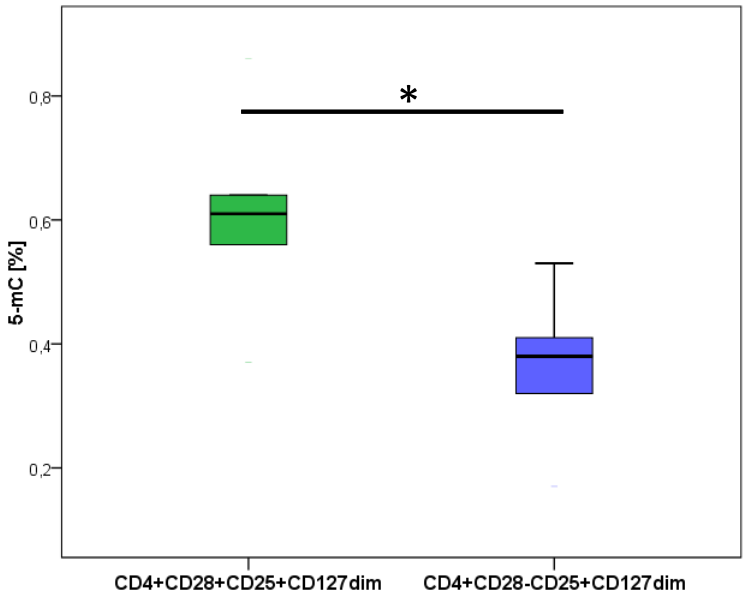
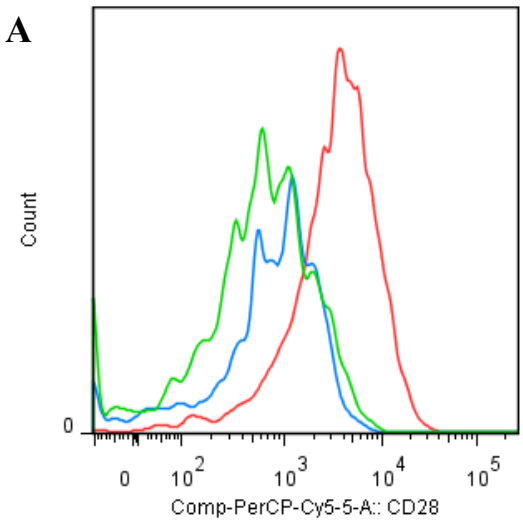


Figure IX: $CD4^+CD28^-CD25^+CD127^{dim}$ cells are hypomethylated compared to their $CD28^+$ counterparts. T-cell subsets of 5 RA patients were sorted using FACS technology and DNA was extracted. Methylation status of $CD4^+CD28^+CD25^+CD127^{dim}$ (green) and $CD4^+CD28^-CD25^+CD127^{dim}$ cells (blue) was determined using MethylFlash Kit. *…$p<0.05$; n=5 (3x anti-TNFα, 1x Tocilizumab, 1x Abatacept)

$CD4^+CD28^-FoxP3^+$ T-cells can be generated *in vitro* by TNF-α

The loss of CD28 is a hallmark feature of immunosenescence in T-cells.(24) It is assumed that the downregulation of CD28 is driven by pro-inflammatory signals such as TNF-α or IL-15.(70,71) To test whether these agents are involved in the generation of $CD28^-$ Tregs, we isolated $CD4+CD25^{high}CD127^{dim/-}$Tregs and cultured them in the presence or absence of IL-15 or TNF-α. We observed a significant decrease of CD28 in

Tregs stimulated with TNF-α (3295 [1293-16853]) compared to controls (5628.5 [1782-16559]; p=0.042, figure X). A decrease of CD28 was also observed following stimulation with IL-15. This difference, however, did not reach significance (4483 [713-15309]; p=0.138). Besides, expression of CD25, CD127 and FoxP3 were not different between groups (data not shown).



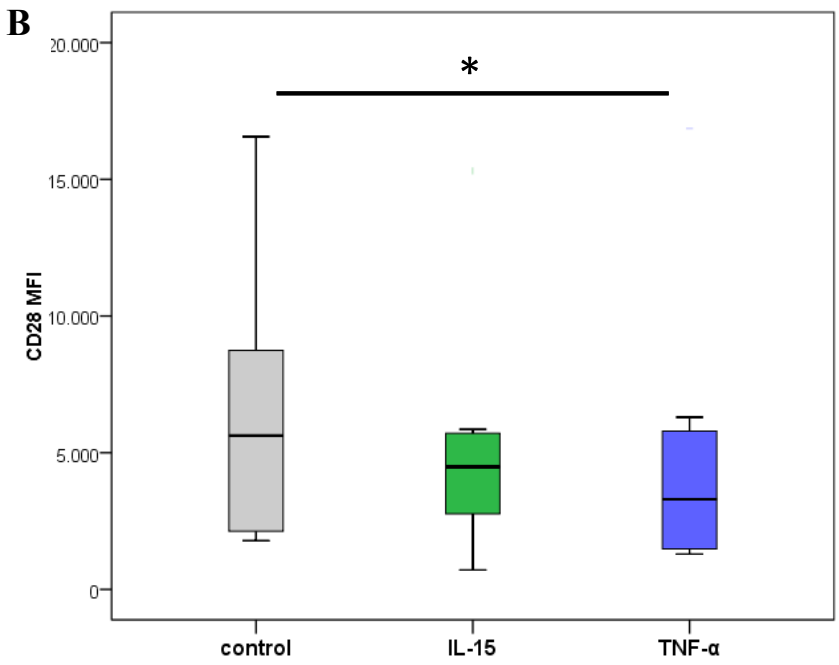


Figure X: *In vitro* down-regulation of CD28 in Tregs in the presence of TNF-α. (**A**) Representative histogram showing CD28 expression of control Tregs (red), following IL-15 stimulation (blue) and following TNF-α stimulation (green). (**B**) Box plots show median expression of CD28 (MFI) in Tregs of 8 healthy individuals. *…p<0.05; n=8

Discussion

In this work we describe the existence of a novel T-cell subpopulation of $CD4^+CD28^-$ $FoxP3^+$ T-cells. These cells occur more frequently in RA patients than in controls and show altered phenotypical and functional properties including diminished suppressive function compared to their $CD28^+$ counterparts. Moreover, this cell subset can be generated *in vitro* following stimulation with TNF-α.

The occurrence of senescent $CD28^-$ T-cells has long been recognized in RA patients and other autoimmune diseases.(6,72) The existence of an aged $FoxP3^+$ Treg population has been hypothesized as well, but no study examined this assumption so far.(37,73) In this work, we identified a population of cells, which exhibit signs of premature aging (loss of CD28) in combination with regulatory capacity (expression of FoxP3) suggesting to be senescent Tregs.

Interestingly, we were not able to show that $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells indicate shorter telomeres than $CD28^+$ ones. This may result from sampling of T-cells from RA patients, given that in RA even naïve T-cells have eroded telomeres.(62) To prove that $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells are senescent, we tested this cell population for TCR diversity, another possible marker for T-cell senescence(68). $CD4^+$ $CD28^-CD25^+CD127^{dim}$ T-cells showed a distinct reduction in TCR diversity. In addition, we stained cells for H2AX another proteins reported to accumulate with aging(69) and observed that $CD4^+CD28^-FoxP3^+$ T-cells expressed the molecule more frequently than their $CD28^+$ counterparts. Taken together, these data suggest that $CD4^+CD28^-FoxP3^+$ T-cells represent a senescent Treg subset.

Since, FoxP3 is expressed in consequence to stimulation(74), this population also could account for activated senescent T-cells even if they lost the co-stimulatory molecule CD28. Beyond that, phenotypic description of normal Tregs is discussable and thus characterization of Treg subsets is even more difficult. Nevertheless, natural as well as activated regulatory T-cells were reported to inhibit conventional T-cell activity(75,76) and more certainly FoxP3 expression is crucial for regulatory properties of Treg cells.(77)

The lack of CD28 may be responsible for the observed reduction in proliferative capacity in $CD4^+CD28^-FoxP3^+$ T-cells. Nevertheless, $FoxP3^+$ cells showed enhanced proliferation compared to their FoxP3 negative counterparts. This leads to the suggestion that $CD4^+CD28^-FoxP3^+$ T-cells are possibly activated by alternative co-stimulatory molecules such as Toll-like receptors which are known to be up-regulated in senescent T-cells.(78)

$CD4^+CD28^-FoxP3^+$ T-cells showed increased expression of several cytokines. The cytokines selected, however, normally represent different expression patterns (Th1, Th2, Th17). The general increase in cytokine production suggests a cytokine storm released by senescent T-cells as a possible result to vicious or altered T-cell stimulation. Genetic and epigenetic modifications in senescent T-cells as a consequence of critical telomere erosion can also not be excluded. In line with this concept we found that $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells are hypomethylated in comparison with their $CD28^+$ counterparts.

In general, age-dependent demethylation and overexpression of genes normally suppressed by DNA methylation - such as KIR2DL4, perforin and CD70 – have been demonstrated in senescent T-cell subsets.(79) Moreover, decreased DNA

methyltransferase (DNMT) levels were found in this subset in RA patients potentially contributing to its pathologic functions. On the other hand, one factor that is important for stable FOXP3 expression is de-methylation at CpG sites within the proximal promoter region of FoxP3.(80) For that reason the hypothesis arose that inhibition of DNMTs might offer a treatment option in autoimmunity. DNMTs reduce methylation at CpG sites, resulting in the maintenance or even induction of FoxP3 expression. *In vitro* studies demonstrated the emergence of a stable $FoxP3^+$ Treg population in the presence of TGF-β and the DNMT inhibitor 5-aza-2'-deoxycytidine.(81) *In vivo* studies using this substance, however, are still missing. Hypomethylation in senescent cells, as observed in this work, therefore reveals contradictory significance.

FoxP3 is essential for the maintenance of immune-tolerance and ectopic expression of FoxP3 in conventional T-cells by retroviral gene transfer resulted in a Treg-like phenotype and function.(77,82) Interestingly, $CD4^+CD28^-CD25^+CD127^{dim}$ T-cells which best represent $CD4^+CD28^-FoxP3^+$ T-cells were not able to suppress proliferation of conventional T-cells. One possible explanation is that $CD4^+CD28^-FoxP3^+$ T-cells are senescent T-cells which lost their suppressive function. Evidence could be given by *in vitro* suppression assays using TNF-α treated Tregs. Attention should be paid to the fact that *in vitro* generated $CD28^-$ Tregs may not completely account for $CD4^+CD28^-FoxP3^+$ T-cells as observed in peripheral blood. Alternatively, FoxP3 might be transiently expressed by senescent T-cells according to stimulation and these cells have no suppressive capacity at all.

The expression of CTLA-4 another protein linked to the suppressive function of Tregs is unchanged in $CD4^+CD28^-FoxP3^+$ T-cells. This finding would suggest that other mechanisms of suppression are altered in senescent Tregs. Of course, these other

mechanisms of inhibitory capacity such as suppression of cytokine production have to be investigated in further experiments to clarify this issue.

In cell culture experiments we were able to generate Tregs with a down-regulated expression of CD28 in the presence TNF-α. The cytokine is closely associated with the pathogenesis of RA and therefore this observation is consistent with the appearance of $CD4^+CD28^-FoxP3^+$ T-cells in the peripheral blood of RA patients. If this down-regulation of CD28 is stable and/or depending on TNF-α dose is not known so far. Patients with anti-TNF-α therapy did not show altered prevalences of $CD4^+CD28^-$ $FoxP3^+$ T-cells in peripheral blood compared to patients on conventional DMARDs only.

Current data indicate a combination of quantitative as well as qualitative changes of Tregs in autoimmune diseases. Studies investigating the numbers and suppressive function of circulating Tregs in patients with autoimmune diseases and healthy individuals revealed contradictory results.(39,83–85) The identification of $CD4^+CD28^-$ $FoxP3^+$ T-cells as a new subset of Tregs with altered phenotype and function may help to understand the discrepancies noted in these studies. Certainly, further studies will shed light on this topic.

This study has a few limitations: First, the most important uncertainty regarding human Tregs is their unreliable identification by flow cytometry. A variety of cell surface molecules have been proposed to better identify Tregs, however, FoxP3 is still deemed as the most specific Treg marker even if activated T-cells without suppressive function may express FoxP3, too. Second, RA is a very heterogeneous disease and thus medication of RA patients is manifold. The prevalences of $CD4^+CD28^-FoxP3^+$ T-cells of were not associated with dose or type of medication in our small RA cohort,

although this needs to be explored in larger studies. Nevertheless interferences in functional assays cannot be excluded totally. Third, the importance of $CD4^+CD28^-$ $FoxP3^+$ T-cells for the pathogenesis of RA is elusive. It is possible that the occurrence of this subset is a secondary effect due to vigorous inflammation. Experiments in humanized mice might be able to clarify this subject. Fourth, we need a more substantial examination of *in vitro* generated $CD4^+CD28^-FoxP3^+$ T-cells to test whether they can be contributed equally with peripheral $CD4^+CD28^-FoxP3^+$ T-cells.

In conclusion, in this work we describe a novel T-cell subset which combines signs of T-cell senescence and immune suppression providing a possible link to disease pathogenesis.

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