Master Thesis

The Influence of Antithymocyte Globulin on CD4+ T Cells from Patients with Rheumatoid Arthritis

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ABSTRACT

Background

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by the production of autoantibodies and chronic synovial inflammation leading to destruction of bone and cartilage tissue in the affected joints. Despite extensive efforts in research to elucidate the mechanisms involved in development and progression of RA, many of these aspects still remain unknown. CD4+CD28- T cells are attributed a role in disease pathogenesis of RA and their elimination might be a promising approach for future therapies. Antithymocyte globulin (ATG) is an immunosuppressive agent acting mainly via depletion of CD4+ T cells. The aim of this thesis is to further elucidate the mechanisms of action of ATG and investigate its effect on CD4+CD28- T cells obtained from RA patients to evaluate a possible usefulness of ATG in RA therapy.

Methods

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of RA patients. Either whole PBMCs or CD4+ T cells isolated via magnetic assisted cell sorting (MACS) were treated with different concentrations of ATG before apoptotic rates and phenotypic changes were analyzed by flow cytometry. Freshly isolated cells were compared to cells that have undergone pre-activation in culture before treatment. Memory (CD45RO+CD28+) and senescent (CD45RO+CD28-) CD4+ T cells were isolated via fluorescence assisted cell sorting (FACS) and compared for changes in expression levels of a selection of pro- and/or anti-apoptotic genes, including nuclear receptor (NR)4A1, using real-time PCR (qPCR). Cells were furthermore treated with ATG and NR4A1-inhibitor C-DIM-8 with subsequent flow cytometric assessment of apoptotic rates and qPCR-assisted analysis of NR4A1 gene expression changes.

Results

Displayed as median and range, treatment with 30µg/mL ATG resulted in an apoptotic rate of 34.3% (27.4-36.6) in pre-activated CD4+ T cells as a part of total PBMCs while 66.5% (58.5-69.2) were apoptotic after treatment with 100µg/mL. Freshly isolated cells, whereas, were less susceptible to the apoptotic stimulus,

displayed by an apoptotic rate of only 36.8% upon 100µg/mL ATG. Furthermore, ATG-induced apoptosis of CD4+ T cells was independent of other PBMCs. MACS-isolated CD4+ T cells showed apoptotic rates of 42.2% (37.9-52.1) and 69.9% (55.4-88.3) in response to 30µg/mL and 100µg/mL ATG, respectively. Moreover, ATG induces phenotypic changes in T cells by down-regulation of surface markers CD3, CD8 and CD28. Interestingly, expression of CD4 remains unaffected. Gene expression analysis revealed a massive up-regulation of nuclear receptor family members NR4A1 (235.5-fold vs. 190.2-fold), NR4A2 (117.9-fold vs. 58.5-fold) as well as NR4A3 (32.8-fold vs. 77.5-fold) in memory and senescent CD4+ T cells, whereas differences between the two subsets were not significant.

In addition, the presence of NR4A1 inhibitor C-DIM-8 did not abrogate ATG-induced apoptosis of CD4+ T cells. The subsequently investigated inability of C-DIM-8 to diminish enhanced NR4A1 gene expression might indicate an overall ineffectiveness of the agent's inhibitory capability.

Conclusion

ATG is a potent inducer of apoptosis in CD4+ T cells, especially when cells are pre-activated. NR4A1 might be involved in the apoptotic mechanism induced by ATG. However, this hypothesis needs further confirmation. Additionally, our data were not able to elucidate the mechanisms behind the recently reported elevated susceptibility of the pro-inflammatory CD4+CD28- T cell subset following ATG treatment. Further investigations will be required in this course to clarify if ATG might be indicated in future therapy of RA and other disease where involvement of CD4+CD28- T cells is described.

ZUSAMMENFASSUNG

Hintergrund

Rheumatoide Arthritis (RA) ist eine Autoimmunerkrankung die durch chronische Gelenksentzündungen sowie die Produktion von Autoantikörpern charakterisiert ist und zur Zerstörung von Knochen- und Knorpelgewebe in den betroffenen Gelenken führt. Trotz großer Bemühungen die zugrunde liegenden Mechanismen der Entstehung und Progression von RA zu erforschen, sind dennoch viele dieser Aspekte bisher ungeklärt. CD4+CD28- T-Zellen wird eine Rolle in der Krankheitspathogenese zugesprochen, weshalb die Eliminierung dieses Zell-Subtyps einen vielversprechenden Therapieansatz für die zukünftige Behandlung von RA liefern könnte. Antithymozytenglobuling (ATG) ist ein Immunsuppressivum dessen Wirkung hauptsächlich auf der Depletion von CD4+ T-Zellen beruht. Ziel dieser Arbeit ist eine genauere Untersuchung der Wirkmechanismen von ATG, sowie dessen Auswirkungen auf CD4+CD28- T-Zellen von RA Patienten, um einen möglichen Nutzen von ATG in der RA-Behandlung zu evaluieren.

Methoden

Mononukleäre Zellen des peripheren Blutes (PBMCs) wurden aus dem Blut von RA-Patienten isoliert. Entweder gesamt-PBMCs oder mittels magnetischer Zellseparation (MACS) isolierte CD4+ T-Zellen wurden mit verschiedenen ATG-Konzentrationen behandelt. Nachfolgend wurden phänotypische Veränderungen sowie die Apoptoserate der Zellen mittels Durchflusszytometrie gemessen. Die Auswirkungen der ATG-Behandlung auf Zellen, die in Kultur vor-aktiviert wurden, und frisch isolierten Zellen wurden verglichen. Gedächtnis- (CD45RO+CD28+) und seneszente (CD45RO+CD28-) CD4+ T-Zellen wurden mittels Durchflusszytometrie (FACS) isoliert und mittels Real-Time PCR (qPCR) auf Veränderungen in der Genexpression bestimmter Apoptosegene (u.a. NR4A1) untersucht. Die Zellen wurden außerdem auch mit ATG und dem NR4A1-Inhibitor C-DIM-8 behandelt, woraufhin sowohl die Apoptoserate mittels Duchflusszytometrie, als auch die Genexpression von NR4A1 mittels qPCR gemessen wurden.

Resultate

Behandlung mit 30µg/mL ATG resultierte in einer medianen Apoptoserate von 34.3% (Spannbreite 27.4-36.6) der vor-aktivierten CD4+ T-Zellen in gesamt-PBMCs, während nach Behandlung mit 100µg/mL ATG bereits 66.5% (58.5-69.2) der Zellen apoptotisch waren. Frisch isolierte Zellen reagierten hingegen weniger sensibel auf den apoptotischen Stimulus (36.8% apoptotische Zellen nach 100µg/mL ATG-Behandlung). Der Mechanismus der ATG-induzierten Apoptose von CD4+ T-Zellen ist unabhängig von anderen PBMCs, da auch in MACS-isolierten CD4+ T-Zellen nach ATG-Behandlung (30µg/mL und 100µg/mL) Apoptoseraten von 42.2% (37.9-52.1) und 69.9% (55.4-88.3) zu sehen waren. ATG führt des weiteren zur verminderten Expression der T-Zell Oberflächenmarker CD3, CD8, und CD28, während jedoch keine Verminderung der CD4-Expression festgestellt werden konnte. Die Genexpressionsanalyse zeigte außerdem eine massiv gesteigerte Expression von NR4A1 (235.5-fach vs. 190.2-fach), NR4A2 (117.9-fach vs. 58.5-fach) sowie NR4A3 (32.8-fach vs. 77.5-fach) in Gedächtnis- und seneszenten CD4+ T-Zellen, wobei die Unterschiede zwischen den beiden Zell-Subtypen nicht signifikant sind.

Die Zugabe des NR4A1-Inhibitors C-DIM-8 konnte keine Verminderung der ATG-induzierten Apoptose hervorrufen. Auch wurde durch C-DIM-8 die erhöhte Genexpression von NR4A1 nicht gehemmt. Beide Beobachtungen weisen darauf hin, dass der Inhibitor nicht effektiv ist.

Schlussfolgerung

ATG ist ein sehr effektives Mittel zur Apopotseinduktion von CD4+ T-Zellen insbesondere wenn diese bereits vor-aktiviert sind. NR4A1 könnte in den Mechanismus der ATG-induzierten Apoptose verwickelt sein. Diese Hypothese muss jedoch noch bestätigt werden. Abgesehen davon, konnten unsere Ergebnisse auch den Mechanismus hinter der kürzlich beschriebenen gesteigerten Apoptose in CD4+CD28- T-Zellen nicht erklären. Weitere Analysen werden daher nötig sein um zu evaluieren, ob ATG einen Nutzen in der Behandlung von RA und anderen Erkrankungen mit CD4+CD28- T-Zell-Involvierung haben könnte.

CONTENTS

ABBREVIATIONS

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation induced cell death
APC	antigen presenting cell
ATG	antithymocyte globulin
BC	buffy coat
bDMARD	biological disease modifying antirheumatic drug
CD	cluster of differentiation
CRP	C-reactive protein
Ct	cycle threshold
ctl	control treatment
CTLA-4	cytotoxic T lymphocyte associated protein 4
DC	dendritic cell
DMSO	dimethyl sulfoxide
DN	double negative
DP	double positive
EULAR	European League Against Rheumatism
ESR	erythrocyte sedimentation rate
FACS	fluorescence assisted cell sorting
FBS	fetal bovine serum
Fw	forward
GC	glucocorticoid
HLA	human leukocyte antigens
Ig	immunoglobulin
IL	interleukin
IFN-γ	interferon gamma
JAK	janus kinase
KIR	killer cell Ig-like receptors
MACS	magnetic assisted cell sorting
MFI	median fluorescence intensity
MHC	major histocompatibility complex

MMPs	matrix metalloproteinases
MOMP	mitochondrial outer membrane permeabilization
NK cell	natural killer cell
NKRs	natural killer cell receptors
NR	nuclear receptor
NSAID	Non-steroidal anti-inflammatory drug
OKT3	anti-human CD3 antibodies
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
qPCR	real-time polymerase chain reaction
RA	rheumatoid arthritis
RF	rheumatoid factor
Rv	reverse
SASP	senescence associated secretory phenotype
tsDMARD	targeted synthetic disease modifying antirheumatic drug
sDMARD	synthetic disease modifying antirheumatic drug
SEM	standard error of the mean
SNP	single nucleotide polymorphism
TCR	T cell receptor
T_{CM}	central memory T cells
T_{EM}	effector memory T cells
TGF-β	transforming growth factor-β
Th cells	T helper cells
Tregs	regulatory T cells
TNF-α	tumor necrosis factor-α

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1. BACKGROUND

Despite decades of extensive research in the field of autoimmune diseases, many underlying mechanisms of autoimmune-disorders such as rheumatoid arthritis (RA) still remain unclear and curative treatments are lacking. To date, therapeutic management of RA is aimed to impede inflammation and limit the progression of tissue destruction. Thus, the search for new therapeutic approaches to further improve quality of life of the affected individuals is continuous.

An important role in the pathogenesis of the disease is attributed to CD4+ T cells. First of all, these cells contribute to the initiation of the characteristic autoimmune-inflammation upon recognition of self-antigen presented on antigen presenting cells (APCs) including B cells, macrophages and dendritic cells (DCs). Subsequently, CD4+ T cells infiltrate the synovial membranes and start to secrete pro-inflammatory cytokines and chemokines for the recruitment of additional immune cells, resulting in promotion and maintenance of inflammation in the affected joints.

Antithymocyte globulin (ATG), apart from being commonly used for T cell depletion and immunosuppression in transplantation, has also been implicated in the management of several autoimmune disorders including multiple sclerosis, systemic sclerosis, Crohn's disease, systemic lupus erythematosus and severe refractory Wegener's granulomatosis. Nevertheless, little research has been done on ATG with respect to rheumatoid arthritis so far.
In this master's thesis we aimed to investigate effects of ATG on CD4+ T cells from RA patients *ex vivo* to evaluate if ATG-treatment could possibly have a beneficial impact on disease severity and be included in the management of RA in the future.

1.1. The immune system & T cells

The human immune system is a complex, interactive network of various components including humoral factors, cytokines, specialized cells as well as lymphatic organs and tissues for the maturation and differentiation of the latter, all aimed to confer host defense against harmful bacteria, parasites, viruses and other pathogens. While natural killer cells (NK cells), DCs, granulocytes and macrophages are assigned to the innate part of the immune system, there is a second, adaptive part comprising T cells, B cells and the corresponding humoral component of immunoglobulins (Ig).

In contrast to the broad and fast response induced by the innate system, the adaptive immune response is longer lasting and highly specific but, nevertheless, depends on activation by cells of the innate system.[1–3]

1.1.1. T cell maturation

T cell progenitors arise in the bone marrow from where they migrate into the thymus. By the time of migration, progenitor cells do neither express the lineage specific surface molecule CD3, nor T cell receptor (TCR) molecules that can later be found on adult T cells and are crucial for T cell function. In addition, they are yet lacking CD4 and CD8 surface molecules, therefore referred to as “double negative” (DN) thymocytes. After migration into the cortex of the thymus, these DN thymocytes start to proliferate. A somatic recombination process in the genes encoding the TCR is induced, resulting in the expression of a pre-TCR (consisting of a pre-TCR α-chain and a rearranged β-chain) in combination with CD3 on the cell surface. Successful expression further enhances proliferation and induces transition of the cells into a CD4+CD8+ double positive (DP) state. Upon additional rearrangements, the pre-TCR α-chain is replaced by a rearranged α-chain, finally resulting in expression of a functional αβ-TCR on the surface of CD4+CD8+ DP cells.

1.1.2. Positive & negative selection

As the recombination of TCR genes occurs in a random manner, part of the receptors evolving from this process will have antigen-binding regions that react with self-antigens. Therefore, the next maturation steps involve certain selection processes to eliminate potential autoreactive thymocytes and to allow only self-tolerant cells to survive and proliferate.

These selection events depend on specialized APCs in the thymic cortex and medulla that express a high density of major histocompatibility complex (MHC) class I and II molecules associated with self-peptides on their surface. If the newly arranged TCR of a DP thymocyte binds to the self-peptide/MHC complex with too high or too low affinity, a process termed “negative selection” is induced resulting in apoptosis of the affected cells.[4] An appropriate, intermediate binding affinity of the TCR, initiates effective maturation of the cell, a mechanism termed “positive selection”. The fate of the maturing, positively selected thymocytes is furthermore dependent on the class of MHC molecule their TCR interacts with. Interaction with MHC I triggers DP cells to lose their CD4 surface markers, giving rise to CD8+ T cells (cytotoxic T cells), while thymocytes interacting with MHC II are committed to become CD4+ T cells exerting primarily helper function (helper T cells).[5] This is the last step in the thymic T cell maturation process. The arising single positive T cells are then ready to leave the thymus and migrate to the peripheral lymphoid organs. Although a vast majority of T cells can be found within the lymphoid tissues, T cells are not static but continuously circulate from one lymphoid organ to another via the blood or lymph stream.

1.1.3. Priming & activation

Priming is the term to describe the first contact of a naïve T cell with its specific antigen. This contact induces activation, clonal expansion and differentiation into different subsets of effector cells. Effector cells can then migrate to the site of inflammation, release pro-inflammatory cytokines and activate or kill other cells.

T cell priming primarily occurs in the peripheral lymphoid organs where the naïve cell first encounters a foreign pathogen that is presented by certain APCs. For the priming of a naïve T cell, DCs are the type of APC that is of particular importance. Other APCs such as macrophages and B cells may further process the antigens in order to attract and activate already differentiated, antigen-specific cells, thereby inducing an immune response. Antigens are presented in a complex with either MCH class I or II molecules, depending on the type of antigen that is presented. Specialized professional APCs can internalize exogenous pathogenic material at a site of inflammation via endocytosis, migrate to the corresponding down-stream lymphatic tissue thereafter and present the antigen on their surface via MHC II molecules. On the other hand, endogenously produced antigens (for example tumour or virus derived proteins from cancer- or infected cells) are complexed and presented with MHC class I. As predetermined by maturation in the thymus, CD4+ T cells require antigen/MHC II complexes for activation, while CD8+ T cells are only activated by MHC I-bound antigen.[1]

This activation is followed by clonal expansion and differentiation of T cells into various effector cell subsets, each of which exerting certain functions in the following immune response.

1.1.4. CD4+ T cell effector subsets

Major effector subsets of CD4+ T cells are T helper (Th) 1, Th2, Th17 and regulatory T cells (Tregs). The cytokine surrounding has a key impact on the differentiation process, determining into which effector subset a naïve CD4+ T cell will most likely differentiate. Interleukin (IL)-12 and interferon-γ (IFN-γ) induce differentiation into Th1 cells, IL-4 and IL-2 trigger Th2 development, transforming growth factor-β (TGF-β), IL-6, -21 and -23 induce a Th17 phenotype, whereas TGF-β in combination with IL-2 result in Treg development (summarized in figure 1).[6]

Each effector cell subset can furthermore be differed by a characteristic transcription-factor and cytokine profile conferring either pro- or anti-inflammatory, survival or protective functions to the respective subset. For example, Th1 cells are characterized by the release of IFN-γ, IL-2 and tumor necrosis factor-α (TNF-α) and Th2 cells secrete IL-4, IL-5 and IL-13, while Th17 cells produce IL-17, IL-21 and IL-22. The cytokine profile of Tregs is characterized by the release of TGF-β and IL-10 (transcription factors and cytokine profiles also depicted in figure 1).[6,7]

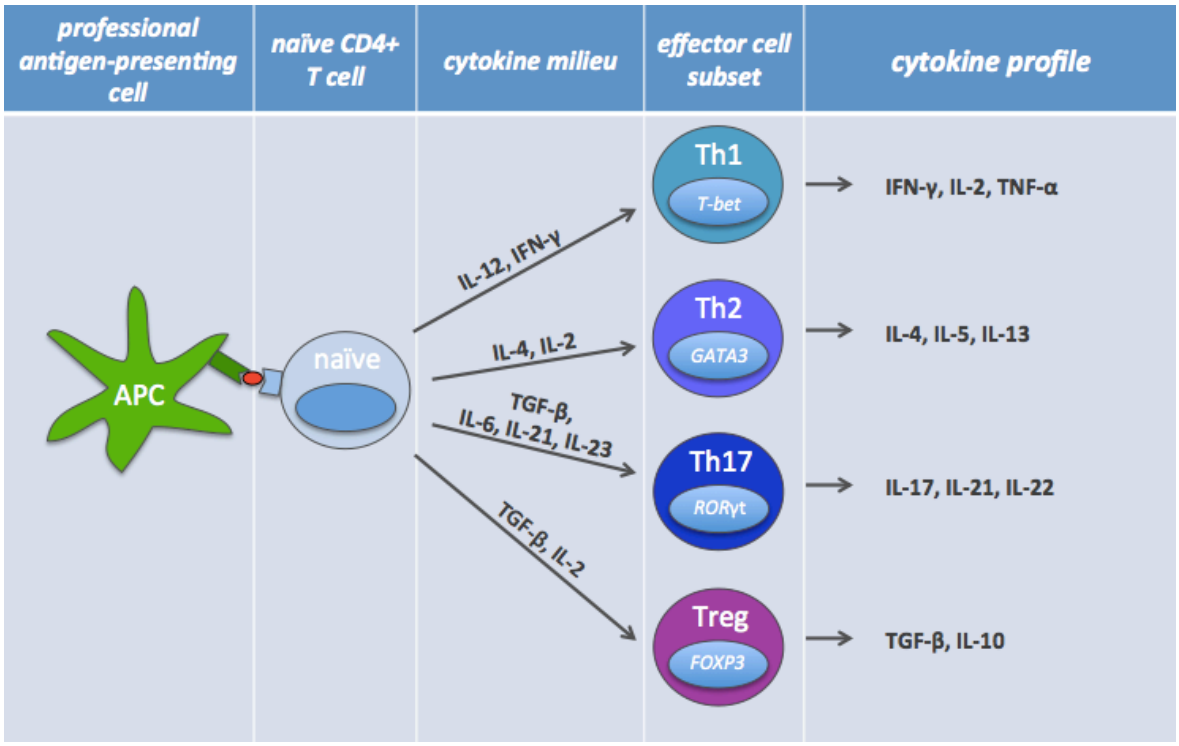

Figure 1: Overview of cytokines involved in or resulting from CD4+ T cell differentiation

Since the life span of such evolving effector cells is rather short, a second set of cells with potential long-term survival is formed, the so-called memory T cells. Memory cells can generally be divided into central memory T cells (T_{CM}), which reside in the secondary lymphoid organs, and effector memory T cells (T_{EM}) that migrate to and persist in the recently infected tissue. The main function of memory T cells is the induction of a faster and more effective immune response during re-exposure to an antigen the immune system was already confronted with previously.[8]

1.2. Immune homeostasis

Immune homeostasis is a term to describe the mechanisms the immune system has invented for the maintenance of its functional stability in steady-state conditions. Impairment or dysfunction of these homeostatic processes may entail significant malignant consequences.

1.2.1. Immunological tolerance

As mentioned before, a pivotal role of the immune system lies in its ability to distinguish foreign, pathogenic structures from self-antigens.
Lymphocyte tolerance mechanisms that are essentially involved in this process can generally be divided into two categories: central and peripheral tolerance.

1.2.1.1. Central tolerance

This comprises the processes of eliminating immature but potentially autoreactive T and B cells during their maturation process in their primary (or central) lymphoid organs, the thymus and bone marrow, respectively.[9]
Elimination of self-reactive T cells is mainly achieved via clonal deletion and has been described in section *1.1.1* in more detail.
For immature B cells that react with ubiquitous membrane-bound autoantigens in the bone marrow a distinct tolerance mechanism is described. B cells can induce receptor gene rearrangement resulting in the generation of receptors with different specificities that may replace their autoreactive precursors. However, when receptor editing has been exhausted, clonal deletion is the default pathway to erase remaining self-reactive B cell clones.[10]

Although central tolerance mechanisms ensure eradication of the majority of self-reactive lymphocytes, the process is incomplete and a small number of self-reactive cells can somehow escape into the periphery. The immune system has therefore developed additional mechanisms for the maintenance of tolerance in the peripheral lymphoid organs.

1.2.1.2. Peripheral tolerance

The main pathways of peripheral tolerance are anergy and suppression of self-reactive cells. For example, T cell activation does not only require recognition of an antigen/MHC-complex but also needs a co-stimulatory signal provided by the APC. If this co-stimulatory signal is absent, the T cell becomes anergic and cannot be activated anymore. Continuous attempts to activate a T cell can furthermore trigger the cell to increase the expression of death receptors Fas and its ligand Fas-L, allowing the cell to undergo activation-induced cell death.[11]

Suppression of autoreactive cells, whereas, requires specialized CD4+ Treg cells characterized by the additional expression of the surface marker CD25 and the transcription factor Foxp3. Suppression is on the one hand achieved via cytokines such as IL-10 and TGF-β, but may also involve direct interactions between cells.[12] However, apart from CD4+ Tregs there is also a subset of CD8+ Tregs involved in the mechanisms of peripheral tolerance, first described decades ago in the early 1970s.[13,14]

The simplest mechanism concerning B cell tolerance is the absence of the T cell help for the activation of autoreactive B cells. However, B cells might also be rendered anergic if they encounter an antigen that is not capable of cross-linking their surface immunoglobulin receptors.[15]

1.2.2. Lymphocyte apoptosis

The mechanism of apoptosis – also termed programmed cell death – play a pivotal role in the homeostatic regulation of the immune system. While enhanced lymphocyte apoptosis may lead to immunodeficiency, impairment of the programmed cell death can result in the development of malignancies such as lymphoid cancer or autoimmunity.[16,17] Accordingly, these processes must be tightly regulated to ensure a reasonable number of T cells even when the immune system is challenged.

Activation induced cell death (AICD) is one of the major mechanisms that allows to control lymphocyte homeostasis and prevents hyperplasia of lymphoid tissues despite repetitive antigen-stimulation. This pathway depends on the engagement of death receptor Fas with its ligand Fas-L and was shown to be enhanced upon continuous TCR stimulation.[18,19] Another example of receptor-mediated apoptosis is represented by the ligand TRAIL, a member of the TNF family of cytokines known to elicit apoptosis following recognition by its specific death receptors TRAIL-R1 and TRAIL-R2.[20]

Alternatively to this "death-by-instruction" via receptor signalling, cells can be subject to so-called "death-by-neglect" if they are denied access to extrinsic signals they require to maintain viability. Members of the Bcl-2 protein family represent only some of the players involved in cell death by neglect. More precisely, the function of proteins like Bcl-2 and Bcl-xL is to prevent initiation of mitochondrial outer membrane permeabilization (MOMP) and the resulting cytochrome C release that would trigger apoptosis.[16]

In contrast, members of the BH3 family of proteins (e.g. Bad, Bid, PUMA, NOXA) antagonize the anti-apoptotic function of Bcl-2 and Bcl-xL and initiate the mitochondrial pathway of apoptosis.[21,22]

Furthermore, the nuclear receptor 4A family member NR4A1 was investigated to successfully induce apoptosis upon nuclear export and translocation to the mitochondrial membrane where it sequesters Bcl-2.[20,23]

However, multiple pathways of apoptosis are known to date and there are a tremendous number of other genes and molecules substantially involved in the regulation of immune homeostasis.

1.2.3. Autoimmunity

Multiple factors are supposed to somehow impair immune homeostasis and the crucial self-tolerance mechanisms, misleading the immune system to attack tissue and organs of the affected individual's own body, culminating in autoimmunity.

Development of autoimmunity can lead to a spectrum of autoimmune disorders that range from organ specific disorders, where T cells and B cell derived antibodies react to self-antigens localized in a specific tissue, to systemic manifestations, in which the involved autoantigens are spread throughout various tissues in the whole organism.[24] The overall prevalence of such autoimmune disorders in the general population is approximately 3-5%.[25–27]
Notably, autoimmune disorders do not only vary in age distribution but also show a gender bias with a female-to-male ratio ranging from 1:1 to 10:1. An exception to this predominant occurrence of autoimmunity in women is Crohn's disease, with a female-to-male ratio of 1:1.2.[28]

One hypothesis is for example based on the existence of "hidden" self-antigens that are sequestered by barrier membranes in certain tissues and therefore inaccessible for autoreactive T cells under physiological conditions. However, organ-specific autoimmune disorders may result from damage of these certain tissues due to sudden availability of the previously isolated antigens.[29] Another hypothesis, called molecular mimicry, relies on the structural resemblance of peptide fragments derived from certain infectious agents with organ specific host-proteins.[30] However, the most prominent finding documented in numerous studies is the correlation between genetics and autoimmunity.[31]

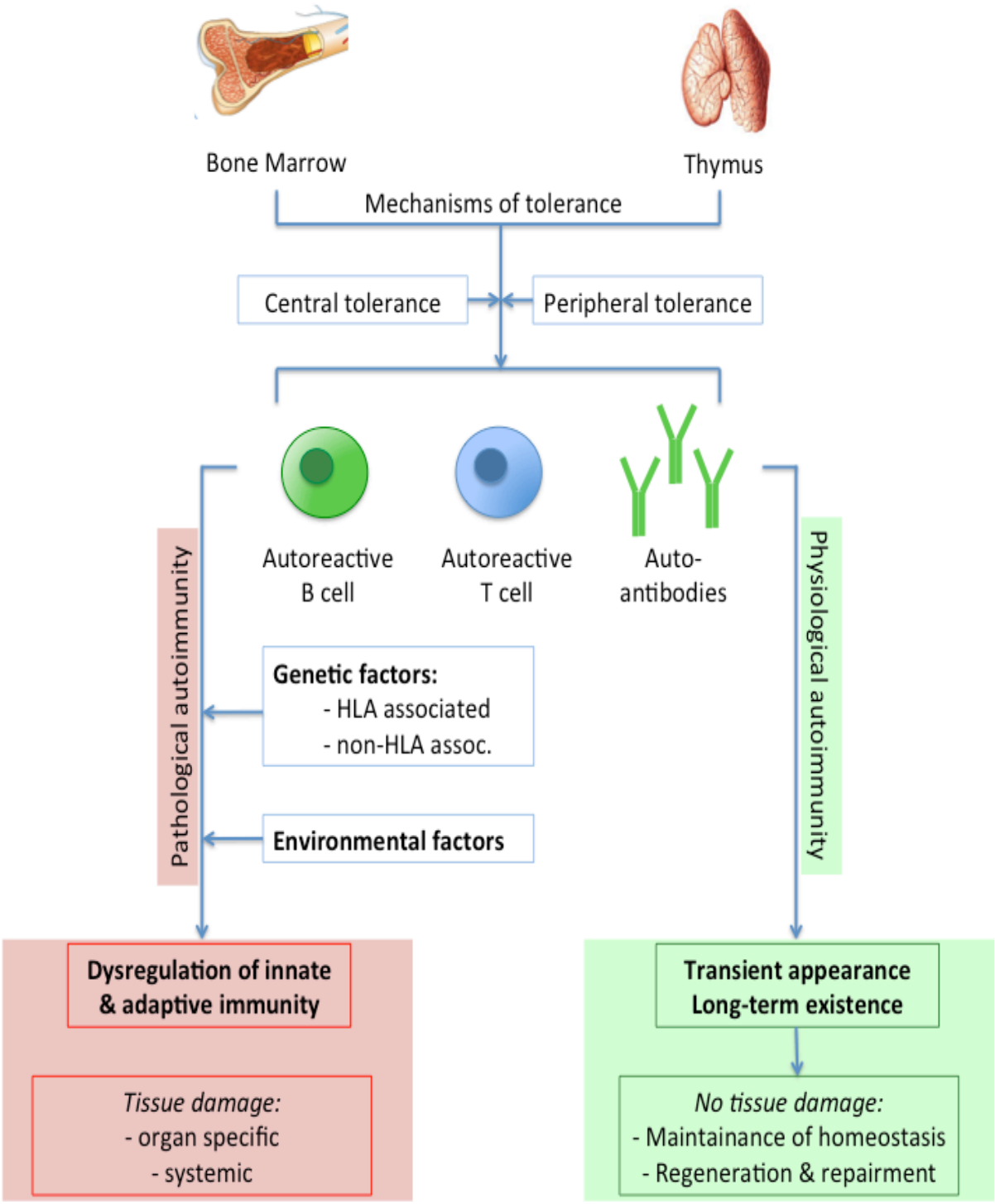

Figure 2: Overview of the development of autoimmunity

Despite broad control by central and peripheral tolerance mechanisms, a small number of autoreactive B and T cells manage to escape into the periphery in healthy individuals. However, these cells remain harmless unless genetic predisposition and environmental factors induce a

1.3. Rheumatoid arthritis

Rheumatoid arthritis is a multifactorial, polygenic and chronic auto-inflammatory disease that is characterized by production of autoantibodies and chronic synovial inflammation which causes destruction of cartilage and bone tissue of small and large joints and results in pain, joint failure impaired mobility and eventually disability.[32]

1.3.1. Epidemiology

RA affects approximately 0.3 – 1% of the adult population in industrialized countries and, as several other autoimmune diseases, occurs predominately in women with a female-to-male ratio of about 3:1.[33] The incidence and prevalence of RA reportedly varies in different populations and geographic areas with a lower prevalence of 0.3-0.7% found in Southern Europe compared to 0.5-1.1% in Northern Europe and North America.[33–37]

Various factors have been reported to increase the risk of developing RA and a majority of these factors are of genetic nature. To date, polymorphisms in more than 30 distinct genetic regions have been linked to increased risk of RA development. Apart from genetics, a role for several environmental factors has been reported. These include the use of oral contraceptives, high alcohol and caffeine consumption, low vitamin D status as well as low socioeconomic status and, above all, smoking. A pathogenic role for hormonal factors is furthermore suggested since RA prevalence also correlates with increasing age and is highest in females older than 65 years.[38,39]

1.3.2. Clinical presentation & diagnosis

Characteristic clinical symptoms of RA are swelling, pain and stiffness of large and – to a higher extent – small joints. Without treatment, RA results in progressive destruction of cartilage and bone tissue of the affected joints, reducing mobility and thereby life quality of the affected individuals.[32]

Progression of the inflammatory processes can furthermore cause various extra-articular systemic complications. These complications may affect the cardiovascular system (atherosclerosis, heart failure and myocardial infarction)[40,41] as well as the brain (fatigue), muscles (sarcopenia), liver (anemia and elevated acute-phase response), exocrine glands (secondary Sjögren's syndrome) and the skeletal system (osteoporosis).[42] Moreover, patients suffering from RA have an increased risk for the development of lung and lymphoma malignancies compared to the general population.[43] These extra-articular manifestations lead to increased mortality and favor premature death in affected individuals, making an early diagnosis and initiation of treatment extremely important.

The diagnosis of RA is primarily based on the recognition of occurring symptoms, followed by the assessment of laboratory markers such as the erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and C-reactive protein (CRP) and is furthermore supported by observations with imaging techniques.[44] The most recent classification criteria for RA have been published in 2010 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR).[45] These criteria include joint involvement, antibody status, acute-phase response and duration of symptoms. Accordingly, RA is diagnosed if an individual suffers from definite clinical synovitis in at least one joint, that cannot be better explained by any other disease and additionally achieves 6 or more points out of the scoring system presented in table 1.

Table 1: Classification criteria for RA released by the American College of Rheumatology and the European League Against Rheumatism in 2010.[45]

A	*Joint involvement*	*Score (0-5)*
	1 large joint	0
	2-10 large joints	1
	1-3 small joints	2
	4-10 small joints	3
	>10 joints; at least 1 small joint	5
B	***Serology***	***Score (0-3)***
	RF and ACPA negative	0
	Either RF or ACPA low-positive	2
	Either RF or ACPA high-positive	3
C	***Acute-phase reactants***	***Score (0-1)***
	CRP and ESR normal	0
	CRP or ESR abnormal	1
D	***Symptom duration***	***Score (0-1)***
	<6 weeks	0
	≥6 weeks	1
For diagnosis of RA an overall score of ≥6 is required.		

1.3.3. Pathogenesis

The underlying cause triggering RA development remains unclear. In general, both parts of the immune system – innate and adaptive – share responsibility for disease initiation and the subsequent preservation of the auto-inflammatory process. Genetic predisposition as well as environmental factors are described to act in concert triggering the immune system to mistakenly recognize self-antigen and thereafter induce self-destructive action against the affected individual's own tissue.

The hereditary component of RA is mainly represented by single nucleotide polymorphisms (SNPs) in the genetic region of the MHC, which encodes the human leukocyte antigens (HLA).

The strongest disease-association is assigned to certain SNPs in the HLA-DRB1 locus. About 80% of RA patients were found to express at least one of 4 defined variants of the HLA-DRB1 allele (DRB1*0101, DRB1*0401, DRB1*0404, DRB1*0405).[46] To date, more than 30 risk alleles for RA have been identified including 3-5% that are not MHC associated.[47] The exact molecular basis of the association between these gene variants and the increased risk of RA development is elusive.

It is the current understanding that APCs (including activated B cells, DCs and macrophages) carrying disease-associated HLA-DR alleles may present arthritis-related auto-antigens to CD4+ T cells. In consequence, these T cells are aberrantly activated and infiltrate the synovial membrane.[48] Moreover, activated B cells differentiate into plasma cells and additionally contribute to RA pathogenesis via secretion of RF and ACPA autoantibodies.[38] T and B cell activation furthermore results in increased production of chemokines and cytokines, promoting inflammation and inducing a feedback mechanism for the recruitment of additional B cells, T cells and macrophages. The latter secrete TNF, IL-6 and IL-1 and are responsible for the progressive destruction of bone tissue as they promote osteoclastogenesis. Additionally, macrophages activate synoviocytes and chondrocytes, which in turn produce and release matrix metalloproteinases (MMPs) inducing degradation of cartilage tissue.[49]

1.4. T cell involvement in RA

Synovitis in RA is thought to be a result of increased endothelial expression of adhesion molecules and chemokines, enabling leukocyte infiltration into the synovial compartment.[48] Invading cells include NK cells, granulocytes, macrophages, B cells and particularly CD8+ and CD4+ T cells.[48,50–52]

T cell infiltration into the synovial membrane in RA patients has already been shown in 1992[53] and the strongest evidence for their implication in RA is the link between an increased RA susceptibility and certain variants of the HLA-DRB1 allele and other genes involved in T cell activation.[46,47]

1.4.1. Th cell subsets vs. Treg cells

An important role in the pathogenesis of the disease is attributed to CD4+ T cells. As previously discussed (*1.1.4. CD4+ T cell effector subsets*), activated CD4+ T cells differentiate into various effector subsets, such as Th1, Th2, Th17 and others. Many studies in the past have indicated that the major driving force of RA is represented by an imbalance in the ratio of Th1 to Th2 cells and the consequential increase in pro-inflammatory Th1 cytokines (e.g. IFN-γ and TNF-α) compared to anti-inflammatory Th2 cytokines (e.g. IL-4 and IL-13).[54] The establishment of this Th1/Th2 paradigm led to major improvements in the treatment of RA (e.g. TNF-α-inhibitors). Although augmented Th1 response makes a major contribution to RA progression it is certainly not the only player involved.

The focus of research in RA and other autoinflammatory diseases has subsequently shifted to the interplay of Tregs and Th17 cells, the latter characterized by the production of IL-17, amongst other pro-inflammatory cytokines.[55,56] IL-17 is nowadays known to promote inflammatory pathology in various autoimmune disorders.[57] On the contrary, CD4+FoxP3+ Tregs appear to play a reciprocal role in regulating Th17 cells and counteracting the production of pro-inflammatory IL-17. Interestingly, the presence of pro-inflammatory cytokines (e.g. IL-6) can trigger differentiation of Tregs into Th17 cells.[58] Studies have shown alleviation of arthritis in animal models upon treatment with anti-IL17 antibodies as well as in IL-17A deficient mice.[59,60] Nevertheless, IL-17-blocking strategies for the treatment of human RA have thus far been ineffective.[61]

Apart from counteracting Th17 cells, Tregs were recently shown to selectively inhibit Th1, but not Th2 cell responses.[62] Importantly, their suppressive effect on effector T cells was shown to be impaired in patients with RA. Abolishment of Treg suppression is described due to the pro-inflammatory cytokine environment in RA joints and decreased sensitivity of T cell effector subsets.[63–65]

1.4.2. Senescent CD4+CD28- T cells

Elucidating the causal relation between immune aging and chronic inflammation could provide valuable information for various autoimmune disorders. Therefore, increased attention has also been drawn to the processes of accelerated immune aging and senescence in patients with RA.[66,67]

1.4.2.1. Emergence & implication in RA

In general, aging of T cells is favored by various determinants including the physiological involution of the thymus, the organ where naïve T cells derive from. Premature thymic involution, indicated in several autoimmune diseases, may thus cause premature senescence of T cells due to increased replicative stress in the periphery. This is addressed to as replicative senescence.[68] Additionally, continuous antigen exposure of the cells at sites of chronic inflammation further increases proliferative stress, contributing to premature T cell aging and senescence.[67]

The accumulation of terminally differentiated effector T cells characterized by remarkable changes in gene expression and function is a hallmark of immune aging. For example, they typically lack expression of the surface receptor CD28 that is required for co-stimulation and full activation of T cells via interaction with protein B7 expressed on APCs. A progressive decline in CD28 expression can be seen after replicative senescence, for example due to continuous autoantigen-stimulation.[69,70] This aging-associated end-stage of differentiation is described for both, CD8+ and CD4+ T cells, although the latter are typically found at a lower frequency.[66] Notably, CD4+CD28- T cells were identified to occur with a higher prevalence in patients with RA compared to healthy individuals and are suspected to contribute to RA pathogenesis.[71,72]

The long replicative history of CD4+CD28- T cells found in RA is confirmed by their highly shortened telomeres.[73] Moreover, a decline in TCR diversity was observed in clonally expanded CD4+CD28- T cells of RA patients.[74,75]

However, these cells have established characteristic additional features (described in the following paragraph) that are unusual for common CD4+ T cells and contribute to the hypothesis of a pathogenic role for CD4+CD28- T cells in RA.

1.4.2.2. Distinctive features

CD28-independet activation:

Typically, lack of the CD28/B7 co-stimulatory signal would trigger a CD4+ T cell to undergo anergy resulting in inability to react to subsequent antigen-presentation. Interestingly, this does not apply to CD4+CD28- T cells. CD4+CD28- T cells have acquired alternative co-stimulatory molecules to maintain capability of being activated and thereby escape their actual fate of entering anergy.

Expression of NK cell receptors:

Surface expression of various NK cell receptors (NKRs) on CD4+CD28- T cells is another feature to distinguish these cells from conventional CD4+ T cells. Several studies have observed various NKRs on CD4+CD28- T cells to act as co-stimulatory molecules for the activation of the cell upon TCR stimulation.[76–78] Moreover, NKR expression is thought to lower the threshold for TCR signaling and enhance activation of the cells.[79] In addition, expression of NKRs such as activating killer cell Ig-like receptors (KIR) was reported to correlate with severe disease manifestations in individuals affected by RA.[76]

Cytotoxic features:

In addition, CD4+CD28- do not only produce extensive amounts of IFN-γ but can also synthesize and release cytotoxic molecules like granzyme B and perforin.[80] In this respect, an *in vitro* model could show their capacity to kill endothelial cells.[81] Intriguingly, the observed cytotoxic features of this particular CD4+ T cell subset do rather resemble NK cells or cytotoxic CD8+ T cells, contradicting typical features of conventional CD4+ T cell subsets which predominately exert regulatory or helper cell function.

Senescence associated secretory phenotype

Further evidence for a pathogenic role of senescent cells is their development of a so called “senescence associated secretory phenotype” (SASP) characterized through their release of SASP-proteins exerting autocrine, paracrine and juxtacrine function.[82] Amongst others, SASP proteins involve multiple MMPs and cytokines (e.g. IL-6, TNF-α), which promote damage of neighboring cells and tissues by eliciting and sustaining an inflammatory response.[83] Thus, a contribution of senescent T cells to tissue destruction and pannus formation in arthritic joints is suggested.[66]

Resistance to apoptosis:

Apoptosis, or programmed cell death, is usually tightly controlled by a balance of pro- and anti-inflammatory proteins expressed in response to environmental stimuli.[84] Interestingly, CD4+CD28- T cells contribute to their own accumulation as they have developed mechanisms to exhibit resistance to apoptotic stimuli.[85] For example, their prolonged proliferative response and resistance to activation-induced cell death might partly be due to expression of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) which induces phosphorylation of the pro-apototic Bcl-2-family protein Bad.[86]

The family of B cell lymphoma-2 (Bcl-2) proteins, however comprises not only pro-apoptotic (e.g. Bax, Bad and Bid), but also anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL and Mcl-1) and members of both groups are substantially involved in the mitochondrial pathway of apoptosis.[87] Thus, the elevated expression of anti-apoptotic proteins such as Bcl-2 is another proposed mechanism for the increased resistance to apoptotic stimuli that is observed in CD4+CD28- T cells.[88]

Overall, CD4+CD28- T cells are another cell subset implicated to contribute to the pathogenesis of RA due to various features including declined TCR diversity, CD28-independent activation, development of SASP, as well as unusual cytotoxic features. Unlike other potentially harmful T cells that can be eliminated via induction of apoptosis, enhanced apoptotic resistance saves CD4+CD28- T cells from natural eradication. A therapeutic approach to counteract this resistance and enhance elimination of the nasty pro-inflammatory T cell subset could therefore be a potential research target to further improve RA treatment.

1.5. Current management of RA

To date, the main objective in treatment therapies of RA is a full-fledged suppression of inflammation, amelioration of symptoms and beneficial intervention in the disease course. Curative treatment, as for many other autoimmune disorders, is still lacking. Individual combination of various medical therapies is needed in order to obtain optimal results for each patient.

1.5.1. Symptomatic treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics are two groups of drugs widely used to relieve pain.[89] The use of NSAIDs additionally reduces stiffness but is contradicted by cardiac and gastrointestinal toxicity and the inability to modify long-term course of the disease.[90–92]

1.5.2. Glucocorticoids

Glucocorticoids (GC) have found use in the treatment of RA already more than 60 years ago. Reduction of synovitis was observed in GC short-term use whereas prevention of joint damage was a result of GC long-term use.[93] Furthermore, administration of intra-articular GCs is highly effective for the targeted treatment of individual, active joints.[94] Due to substantial adverse effects, such as avascular necrosis, cataract, cutaneous modifications, increased atherosclerosis, infections and osteoporosis, the use of GCs is only recommended in certain settings.[95]

1.5.3. Synthetic disease-modifying antirheumatic drugs (sDMARDs)

The heterogeneous group of conventional synthetic disease-modifying antirheumatic drugs (sDMARDs) is currently the mainstay of RA management. DMARDs comprise a wide range of drugs with various chemical structures and diverse mechanisms of action, some of which not fully understood yet. Among their therapeutic actions a decrease in acute-phase reactants, reduction of joint swelling, damage progression and pain, and improvement in joint function are observed. Most commonly used in RA treatment is the small-molecule sDMARD methotrexate.[96,97]

Furthermore, sulfasalazine, leflunomide and hydroxychloroquine are also frequently in use for RA treatment,[98–102] whereas in special situations the use of cyclophosphamide, azathioprine or cyclosporine A was described to have beneficial consequences.[103]

These synthetic DMARDs are often used in combination, for example as "triple therapy" methotrexate, hydroxychloroquine and sulfasalazine. However, diverse combinations of sDMARDs have proven efficacy.[104] The most serious adverse effects include interstitial lung disease, hepatotoxicity and blood dyscrasia, conferring particular importance to the monitoring of liver function and blood counts.[104–106]

1.5.4. Biological agents

Novel therapeutic agents have been developed with increasing knowledge about the immunopathogenic mechanisms involved in RA. Targets of these novel biological DMARDs (bDMARDs) are manifold, including B cells, T cells and the involved pro-inflammatory cytokines. This allows a classification into two groups: cytokine-targeting and non-cytokine targeting bDMARDs. However, combined therapy of bDMARDs with methotrexate, or alternatively leflunomide, is conventionally applied to increase efficacy and reduce antibody formation.[107]

1.5.4.1. Cytokine-targeting bDMARDs

TNF-α inhibitors:

Elevated levels of TNF-α are found in synovial fluid and tissue of RA patients and the cytokine is strongly implicated in immune response, cell infiltration, inflammation, angiogenesis, and bone and cartilage damage.[108] Due to this implication in RA pathogenesis TNF-α is one of the major cytokine targets of bDMARDs. By now, four different TNF-α-targeted monoclonal antibodies (infliximab, adalimumab, golimumab and certolizumab) and one soluble receptor (etanercept) are available for RA therapy.

Serious adverse effects include for example increased risk of infections and lymphoma, allergic reactions, lupus-like syndrome and development of anti-drug antibodies.[109,110] However, despite reported excellent overall efficacy 35-40% of RA patients are not responsive to anti-TNF therapy and require alternative treatment.[103]

IL-6 blockade:

IL-6 stimulates differentiation of cytotoxic and Th17 cells, thereby IL-17 production. Furthermore it triggers activation, differentiation and proliferation of B cells, IgG synthesis and contributes to acute and chronic inflammation. With regard to joints, proliferation of synovial cells, angiogenesis and destruction of bone and cartilage tissue are pathogenic events additionally enhanced by IL-6.[103]

The principle of targeting IL-6 in RA treatment is based on the binding of the monoclonal antibody tocilizumab to its target, the IL-6 receptor, thereby blocking the effects of IL-6. The efficacy of tocilizumab has been proven in different settings, including monotherapy and combined therapy with methotrexate. Effects are improvement of clinical symptoms as well as impeding progressive joint destruction while main adverse risks include elevated levels of hepatic enzymes, dyslipidemia, infections and neutropenia.[103,111,112]

IL-1 blockade:

Many effects of the pro-inflammatory cytokine IL-1 resemble those of TNF-α, rendering IL-1 another promising cytokine target in the therapy of RA.
However, anakinra is the only IL-1 blocker currently approved for RA treatment. Since it is administered via daily injections, adverse events include reactions at the injection site as well as infections.[113]

1.5.4.2. bDMARDs with non-cytokine targets

T cell activation blockade:

The co-stimulatory signal via interaction of the protein B7 (expressed on APCs) with CD28 (expressed on the T cell) is required for full T cell activation. In contrast, interaction of B7 with the membrane-bound CTLA-4 on T cells leads to inhibition of the latter.

The biologic agent abatacept is a fusion protein composed of the CTLA-4 extracellular domain and the Fc fragment of human IgG1 that binds B7 on APCs with a greater affinity than CD28. Thereby it blocks the co-stimulatory signal required for the full activation of T cells. Efficacy of abatacept in combination with methotrexate was demonstrated in various clinical trials and improvement of clinical symptoms and limitation of joint damage are reported benefits.[114,115] Adverse effects due to abatacept therapy include increased susceptibility to infections and acute infusion-related reactions.[116,117]

B cell depletion:

B cells substantially contribute to the pathogenesis of RA via presentation of autoantigen, thus activation of T cells and the production of autoantibodies and cytokines.[47] CD20 represents a surface marker that is present predominately on B cells. Treatment with the anti-CD20 antibody rituximab thus results in a broad depletion of peripheral B cells. Several clinical trials have proven rituximab therapy to be highly effective for RA treatment, once again in combination with methotrexate, blocking radiological progression of joint damage and improving clinical manifestations.[118–123]

1.5.5. Targeted synthetic DMARD (tsDMARD)

Kinase inhibition, for example with the janus kinase (JAK) inhibitor tofacitinib represents a novel therapeutic approach in RA therapy.[103] Tofacitinib (which is not yet licensed in the European Union) is a synthetic chemical compound targeted to interfere with specific pathways in signal transduction and can therefore neither be subsumed with conventional sDMARDs nor bDMARDs. Therefore it is addressed

by the novel term tsDMARD.[124] The efficacy of tofacitinib is comparable to TNF blockers.

However, treatment with tofacitinib is primarily recommended for patients who failed to respond to biological treatment. Reportedly, adverse effects include anemia, lymphocytopenia, gastrointestinal reactions and serious infections (e.g. herpes zoster or tuberculosis infections).[103,124]

1.6. Antithymocyte globulins

Antithymocyte or antilymphocyte globulins (ATG) are polyclonal IgG antibodies with major specificity towards human T lymphocytes. Various preparations of ATG are available on the market, differing in their composition as a result of their different origins and methods of production.[125] ATG-F (Fresenius/Neovii Biotech), Thymoglobulin (Sanofi) and ATGAM (Pfizer) are some of the most extensively used preparations. Either of them are obtained via purification from the sera of rabbits or horses that have been immunized with human thymocytes or, in case of ATG-F, with Jurkat T cells, a human cell line resembling activated T cells.[126]

ATG comprise a multiplicity of antibodies with various targets and their entire mechanisms of action are therefore not fully elucidated yet. A majority of antibodies of ATG is directed against T cell-specific molecules, for example CD3/TCR, CD4, CD8 and CTLA-4. Apart from that, antibodies with multiple specificities for other than T cell associated antigens are also contained within ATG preparations. Specific antibodies contained in ATG do also target B cell markers such as CD19, CD20 and CD21, as well as antigens involved in cell adhesion and trafficking (e.g. CD11a, CD18).[126–129]

Currently, several different mechanisms are proposed to be responsible for the depletion of T cells, including complement dependent lysis, opsonization by complement and immunoglobulins, followed by phagocytosis of opsonized cells by macrophages. Moreover, induction of apoptosis is supposed to be the main mechanism of depletion.[130,131] The exact mechanisms regarding T cell apoptosis upon ATG treatment, however, are still under debate.

In spite of that, ATG has found use as immunosuppressive agent, mainly with respect to its T cell depleting properties, in the field of transplantation decades ago.

Some examples for the use of ATG's immunosuppressive properties in clinical settings are induction therapy and rescue treatment of acute rejection, treatment of graft-versus-host-disease, as well as severe aplastic anemia.[132–135] Furthermore, beneficial effects of ATG treatment were reported in patients with autoimmune disorders such as systemic lupus erythematosus, systemic sclerosis and Wegener's granulomatosis.[136–139] More recently, ATG has been reported to preferentially deplete CD4+CD28- T cells, a cell subset thought to contribute to RA pathogenesis, indicating that ATG treatment might also have beneficial effects in RA patients.[140]

2. Materials & Methods

2.1. Isolation of PBMCs

Blood was either obtained from buffy coats (BC) of healthy blood donors or withdrawn from patients with rheumatoid arthritis (classified according to the ACR/EULAR criteria of 2010) using lithium heparin tubes, depending on the type of experiment we aimed to perform. PBMCs were isolated by density gradient centrifugation. Therefore, 15ml of blood were poured into 50ml Falcon tubes and filled up with phosphate buffered saline [PBS (pH 7.2-7.3)] to a total volume of 35ml. This mixture was carefully layered over 15ml of Histopaque (Sigma-Aldrich) in another Falcon tube. Tubes were centrifuged at 400g for 20 minutes (without break). After centrifugation the buffy coat, containing the PBMCs, was harvested into 15mL Falcon tubes and washed twice with PBS. Finally, the cell pellet was resuspended in PBS and total cell numbers were determined on a Beckman Coulter.

2.2. Cell culture conditions

In preparation for short term cell cultivation of PBMCs, all culture dishes were coated with anti-human CD3 antibodies (OKT3) before use, to allow activation and *ex vivo* expansion of T cells within the population of PBMCs. Dishes were therefore incubated with OKT3 (10µg/ml) for 2h at 37°C before the antibody-solution was removed again. Subsequently, the freshly isolated PBMCs were seeded into the dishes and maintained at a density of 1x10^6 – 1.5x10^6 cells/ml and cultured at 37°C and 5% CO_2 in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 2mM L glutamine, 100U/ml penicillin and 100µg/ml streptomycin in all the experiments. Additionally, the medium contained IL-2 at a concentration of 20U/ml in those experiments that aimed an efficient expansion of T cells. Cells were always transferred into new, OKT3-free dishes after 24h of stimulation with OKT3 and substituted with new RPMI medium containing the above described supplements twice a week.

2.3. Treatment of cells

For treatment with ATG (Fresenius) and, in some experiments, the NR4A1 inhibitor C-DIM-8 [solved in dimethyl sulfoxide (DMSO)], either pre-activated cells from short term cell cultures or freshly isolated cells were seeded into untreated 24-well-plates at a density of 1x10^6 cells/ml. Control cells were always treated with unspecific rabbit immunoglobulins (Dako) at a concentration corresponding to the highest concentration of ATG used in the respective experiment. Additionally, control cells in the experiments with C-DIM-8 received an amount of DMSO corresponding to that amount contained in the treatment with the highest concentration of C-DIM-8, to serve as a solvent control. Incubation time was set to 18 hours in all the experiments analyzed by flow cytometry while the incubation time was reduced to 3 hours in the qPCR experiments.

2.4. Annexin V staining and flow cytometric analysis

For the assessment of apoptotic cells via flow cytometry, cells were harvested into FACS tubes after 18h of incubation. Subsequently, cells were washed with 2ml of PBS prior to application of the following staining protocol (table 2). Volumes are intended for the staining at 1x10^6 cells.

1.) Surface staining with following antibodies:

Table 2: Antibodies, fluorescence-dyes, reaction volumes & incubation settings for surface staining

Antibody	Fluorescence-dye	Volume [µl]
CD3*	PE	20
CD4*	V500 (AmCyan)	3
CD8*	V450 (Pacific Blue)	3
CD28*	PerCP-Cy5.5	3
(* BD Biosciences)		
Incubation for 20 minutes at room temperature, protected from light.		

2.) Wash cells twice with 2ml of PBS (300g, 5 min).
3.) Resuspend cells in 1ml of PBS.
4.) Add 1µl of Fixable Viability Dye* (eFluor 780), vortex immediately and incubate for 30 minutes at 2-8°C, protected from light.
5.) Wash cells twice with 2ml of Flow Cytometry Staining Buffer*.
6.) Wash cells once with 2ml of 1x Annexin V Binding Buffer*.
7.) Resuspend cells in 200µl of 1x Annexin V Binding Buffer*.
8.) Add 10µl of Annexin V* (FITC), vortex immediately and incubate for 15 minutes at room temperature, protected from light.
9.) Wash cells once with 2ml of 1x Annexin V Binding Buffer*.
10.) Re-suspend cells in 150µl of 1x Annexin V Binding Buffer*.

(* eBioscience)

Stained cells were then analyzed by flow cytometry on a FACS Canto II (BD Biosciences) recording 100.000 cells per measurement. Obtained data were analyzed with FlowJo vX.0.7.

2.5. Isolation of CD4+ T cells

In preparation for CD4+ T cell isolation using the human CD4+ T Cell Isolation Kit (MACS Miltenyi Biotec) PBMCs had to be isolated via density gradient centrifugation as described before. The subsequent isolation of CD4+ T cells was performed via magnetic assisted cell sorting (MACS) according to following protocol provided by the manufacturer:

1.) Determine cell number, centrifuge cells (300xg for 10 min.) and remove supernatant
2.) Use 40µL of MACS buffer per 1x10^7 cells to resuspend the cell pellet
3.) Add 10µL of CD4+ T Cell Biotin-Antibody Cocktail per 1x10^7 cells
4.) Mix properly and incubate for 5 min. in the refrigerator (2-8 °C)
5.) Add 30µL of MACS buffer and 20 µL of CD4+ T Cell MicroBead Cocktail per 1x10^7 cells.
6.) Mix properly and incubate for another 10 min. in the refrigerator (2-8 °C)

Magnetic separation was performed on an autoMACS® Pro Separator (Miletnyi) using the separation program “depletes”. The resulting negative fraction contains the enriched, unlabeled CD4+ T cells. Purity of isolated CD4+ T-cells reached >95%. Cell number was determined on a on a Beckman Coulter.

2.6. Isolation of senescent & memory CD4+ T cells

Fluorescence assisted cell sorting (FACS) using a FACSAria (BD Bioscience) instrument allowed us to furthermore analyze features of 2 distinct T cell subsets, CD28- (senescent) and memory (CD28+CD45RO+) CD4+ T cells, in response to ATG. Therefore, PBMCs of RA patients were cultured under conditions inducing *ex vivo* T cell expansion and activation until they reached sufficient cell numbers, before isolation of CD4+ T cells was performed (as described before in sections: *Cell culture conditions* & *Isolation of CD4+ T cells*).

After cell number determination of the isolated CD4+ T cells a centrifugation step (300xg, 5 min.) was performed. Supernatant was removed and cells were re-suspended in PBS at a concentration of 1x10^6/100µl prior to application of the following surface staining protocol (table 3). Volumes are intended for the staining of 1x10^6 cells.

Table 3: Antibodies, fluorescence-dyes, reaction volumes & incubation settings for surface staining prior to cell sorting

Antibody	Fluorescence-dye	Volume [µl]
CD45RO*	APC	15
CD28*	PerCP-Cy5.5	3
(* BD Biosciences)		
Incubation for 20 minutes at room temperature, protected from light.		

Stained cells were washed with PBS twice (300xg, 5 min.) and re-suspended in an appropriate volume of PBS for sorting. The sorting process was carried out at 4°C and the cell subsets were sorted into RPMI-containing FACS tubes to provide nutrients and avoid elevated numbers of apoptotic cells already before treatment. ATG-treatment was performed as described before in section *Treatment of cells*.

2.7. RNA isolation

After treatment, cell-suspensions were harvested into FACS tubes, washed with PBS (300xg, 5 min.) once, re-suspended in 1ml of PBS and transferred in into Eppendorf tubes. Another centrifugation step (300xg, 5 min.) resulted in a clearly visible cell pellet at the tube-bottom and allowed for precise removal of supernatant PBS. Subsequently, RNA isolation was performed using RNeasy Mini Kit and QIAshredder (QIAGEN) according to manufacturer's instructions. Lysis of the cell pellet is achieved via a provided lysis buffer. This buffer contains a high concentration of guanidine isothiocyanate, a chaotropic agent that supports the binding of RNA to a silica matrix. The lysis buffer should furthermore be supplemented with β-mercaptoethanol before use, in order to inactivate RNases. After lysis, QIA shredder columns are used to homogenize the samples, prior to addition of ethanol which additionally enhances the binding of RNA to the silica matrix of the subsequently used RNeasy columns. Following washing steps are intended to efficiently remove contaminants and yield in RNA of high purity after elution from the column with RNase-free water. Concentration determination of the obtained RNA was carried out with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo SCIENTIFIC) and samples were stored at -80°C until further usage.

2.8. Reverse transcription

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to reversely transcribe 1µg of RNA into cDNA according to manufacturer's instructions.

Table 4: High-capacity cDNA reverse transcription protocol

10x RT Buffer	2.0 µl
25x dNTP Mix (100 mM)	0.8 µl
10x RT Random Primers	2.0 µl
MultiScribe™ Reverse Transcriptase	1.0 µl
RNA	X µl (≙ 1µg)
Nuclease-free water	14,2 – X µl
Total reaction volume	20 µl

Table 5: Reverse transcription thermal cycling conditions

	Step 1	*Step 2*	*Step 3*	*Step 4*
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 min	∞

The obtained cDNA was stored at -80°C until further usage.

2.9. Real time PCR (qPCR)

GoTaq® qPCR Master Mix was used for qPCR according to manufacturer's instructions. Reaction volume was reduced to 10µl instead of 50µl (Table 6).

Table 6: qPCR reaction

Component	*Volume per 10µl reaction*	*Final concentration*
GoTaq® qPCR Master Mix, 2x	5µl	1x
Forward & Reverse Primer Mix (2µM)	1µl	0.2µM
cDNA (2.5ng/µl)	4µl	10ng/10µl
Final reaction volume:	***10µl***	

Analyzed was the mRNA expression of 14 apoptosis-related genes of interest. Sequences of forward (Fw) and reverse (Rv) primers are listed in Table 7. HPRT (Fw ATG GGA GGC CAT CAC ATT, Rv ATG TAA TCC AGC AGG TCA GCA A) and GAPDH (Fw AAG GTC GGA GTC AAC GGA TTT, Rv ACC AGA GTT AAA AGC AGC CCT G) served as housekeeping genes. All samples were run in triplicates.

Table 7: Sequences of primers used for qPCR

Gene	Direction	Sequence (5´- 3´)
NR4A1	Fw	AGC ATT ATG GTG TCC GCA CAT
	Rv	TGC ACT GTG CGC TTG AAG A
NR4A2	Fw	CGA TTT CAG AAG TGC CTG G
	Rv	TAA ACT GTC TGT GCG AAC CAC
NR4A3	Fw	CCC TTT CAG ACT ATC TGT ACG GAC
	Rv	CTC AGT GTT GGA ATG GTA AAA GAA G
BCL-2	Fw	GGA GGA TTG TGG CCT TCT TTG
	Rv	GCC GGT TCA GGT ACT CAG TCA T
BCL-XL	Fw	CAG TGA CCT GAC ATC CCA GC
	Rv	CCC ATA GAG TTC CAC AAA AGT ATC C
MCL-1	Fw	CCA AGG ACA CAA AGC CAA TG
	Rv	AAG AAC TCC ACA AAC CCA TCC
FAS	Fw	CTG GAC CTT CTT ACC TCT GGT T
	Rv	GGC ATT AAC ACT TTT GGA CGA T
FAS-L	Fw	TGG CCC ATT TAA CAG GCA AG
	Rv	CCA TAG GTG TCT TCC CAT TCC
NOXA	Fw	AGC TGG AAG TCG AGT GTG CT
	Rv	TCC TGA GCA GAA GAG TTT GGA
PUMA	Fw	CGG AGA CAA GAG GAG CA
	Rv	ATG ATG AGA TTG TAC AGG ACC
TRAIL	Fw	ACC AAC GAG CTG AAG CAG ATG
	Rv	CAA GCA ATG CCA CTT TTG GA
BAD	Fw	GGT AGG AGC TGT GGC GAC T
	Rv	CAA GCA TCA TCG CCA GG
BID	Fw	GGA ACC GTT GTT GAC CTC AC
	Rv	GAG GAG CAC AGT GCG GAT
HRK	Fw	CTA GGC GAC GAG CTG CAC
	Rv	AGC CAA GGC CAG TAG GTG

Real time PCR was performed on a LightCycler 480 Instrument II (Roche Diagnostics) using following cycling program:

Table 8: qPCR thermal cycling conditions

	Number of Cycles	*Cycling Program*
Hot-Start Activation	1	95°C for 2 min.
Denaturation	40	95°C for 3 sec.
Annealing/Extension		60°C for 30 sec.
Dissociation	1	60-95°C

Cycle threshold (Ct) values were measured and analysis was done using the ΔΔCt method for relative quantification.

2.10. Statistical analysis

Data are presented as mean values and standard error of the mean (SEM) or median and range. Statistical analyses were performed using SPSS version 23. P-values ≤0.05 were considered to be significant. Due to small sample sizes, none of the observed experiments were proven significant when non-parametrical testing was applied.

3. Results

3.1. ATG induces apoptosis in the CD4+ T cell population of human PBMCs

ATG has been reported to be an immunosuppressive agent with the potential to eliminate various subsets of immune cells via induction of apoptosis.[141] To test this apoptotic effect on T cells of patients with RA we treated pre-activated PBMCs with certain concentrations of ATG for 18 hours *ex vivo.* Displayed as median and range, ATG induced apoptosis in 34.3% (27.4-36.4) at a concentration of 30µg/mL and in 66.5% (58.5-69.2) at 100µg/mL (figure 3A & B).

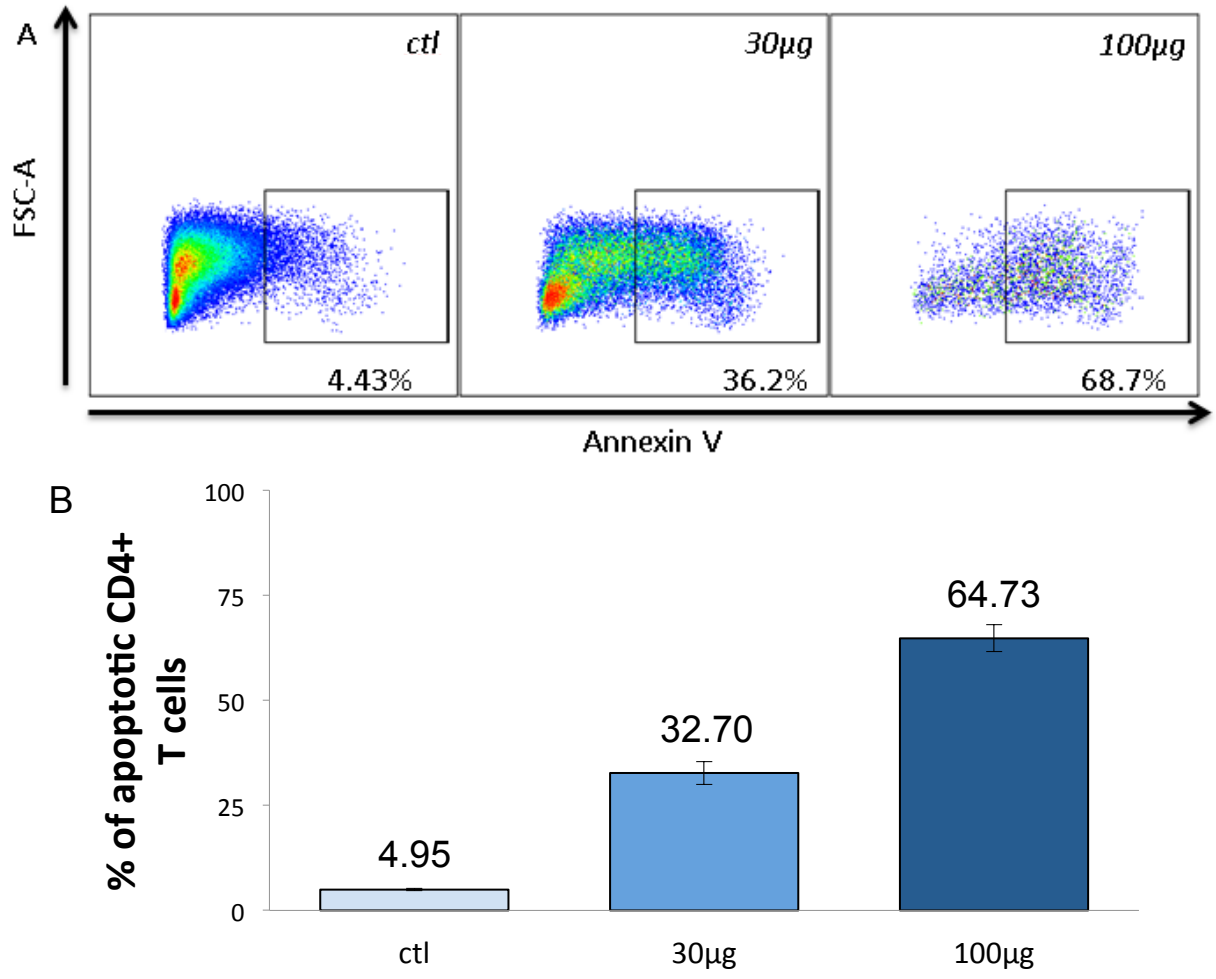

Figure 3: ATG induces apoptosis in a dose-dependent manner in RA CD4+ T cells.
Percentage of viability dye negative (non-necrotic), annexin V positive (apoptotic) CD4+ T cells after 18h of treatment with either ATG (30µg/mL, 100µg/mL) or unspecific rabbit IgG (100µg/mL) to serve as control treatment (ctl) depicted by dot plots from one representative patient (**A**) and as mean values ± SEM from 3 different patients (**B**).

3.2. *Pre-activation renders T cells more susceptible to ATG-induced apoptosis*

The exact mechanisms how ATG induces apoptosis in T cells is elusive. Therefore, we tested the influence of T cell activation in this context. When comparing results of PBMCs cultured under T cell activating conditions for 7-9 days after isolation with freshly isolated PBMCs that have not undergone activation in the presence of OKT3 and IL-2 in advance to ATG treatment, a divergence in susceptibility to the apoptotic stimulus was observed (figure 4A & B).

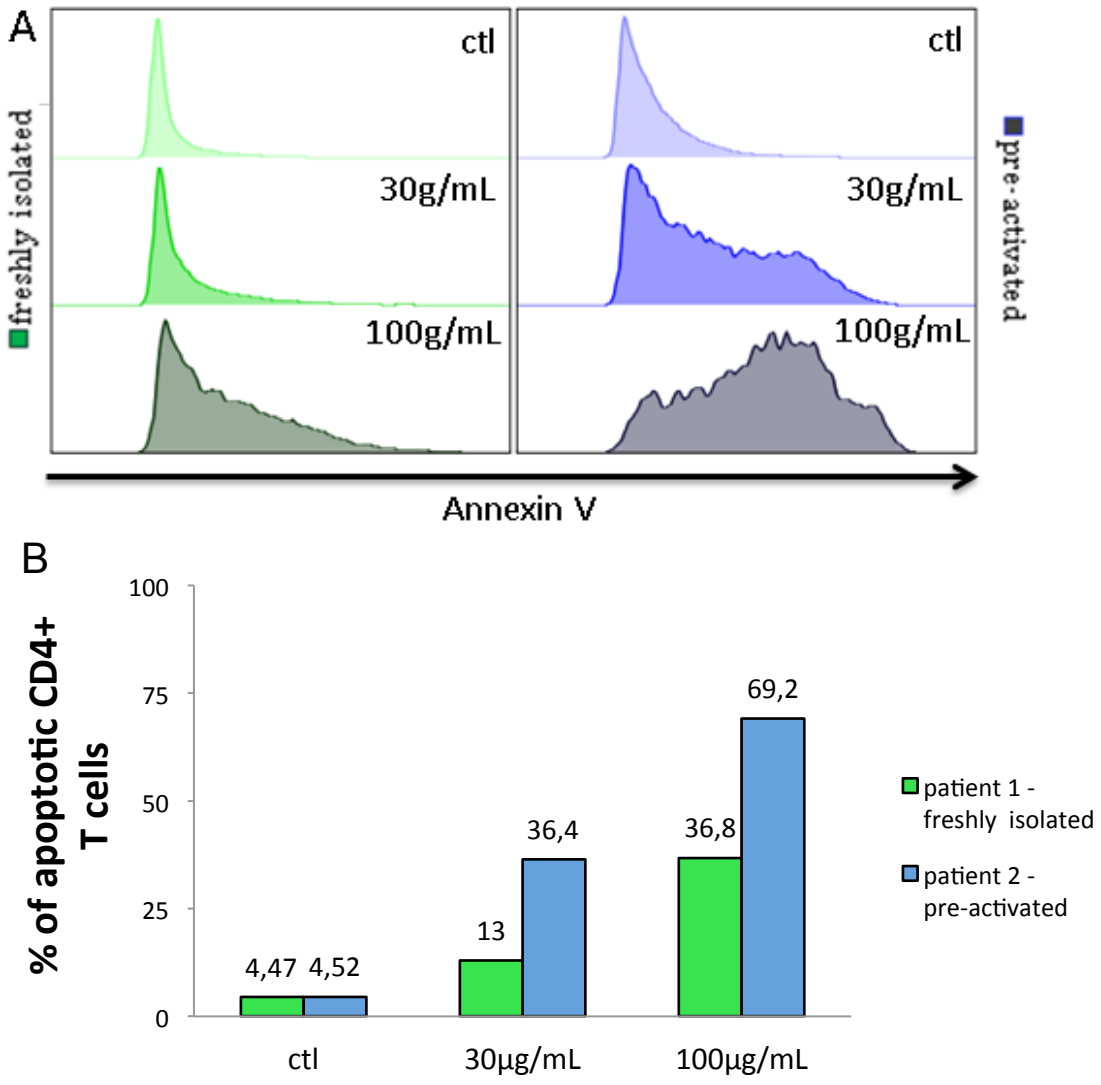

Figure 4: ATG-induced apoptosis is influenced by T cell pre-activation.

A displays the fluorescence intensity in the FITC-annexin V channel, while **B** depicts the percentage of viability dye negative (non-necrotic), annexin V positive (apoptotic) CD4+ T cells from either freshly isolated PBMCs (patient 1: green) or pre-activated PBMCs (patient 2: blue) after 18h of treatment with ATG (30µg/mL, 100µg/mL) or unspecific rabbit IgG (100µg/mL) as control treatment (ctl).

While treatment with ATG at a concentration of 100µg/mL yielded in 69.2% of apoptotic CD4+ T cells in pre-activated PBMCs, the corresponding dose of ATG only resulted in 36.8% of apoptotic CD4+ T cells in freshly isolated PBMCs. This percentage roughly equals the CD4+ T cell apoptosis rate after treatment with 30µg/mL ATG in pre-activated PBMCs (36.4%).

3.3. ATG-induced CD4+ T cell apoptosis is independent of other PBMCs

To test whether induction of CD4+ T cell apoptosis by ATG does require the immediate presence of other immune cells of the peripheral blood compartment CD4+ T cells were isolated from BCs of healthy blood donors in advance to treatment with ATG.

As displayed in figure 5, elevated numbers of apoptotic cells among the isolated CD4+ T cells are already evident in control-treated cells compared to CD4+ T cells as part of total PBMCs [11.5% (8.3-15.4) vs. 5.0% (4.5-5.4)] and might thus reflect a side-effect of the isolation process. Accordingly, treatment with 30µg/mL ATG results in moderately elevated apoptotic CD4+ T cell rates when comparing isolated CD4+ T cells and CD4+ T cells as part of total PBMCs [42.2% (37.9-52.1) vs. 34.3% (27.4-36.4)]. Analogue data were obtained for the treatment with 100µg/mL ATG [69.9% (55.4-88.3) vs. 66.5% (58.5-69.2)].

To allow analysis of ATG-induced apoptosis independent from apoptotic cells as a result of the isolation process, apoptotic rates of control-treated cells were subtracted from the rates seen after ATG-treatment. After this adjustment, apoptotic rates of isolated CD4+ T cells and CD4+ T cells as part of total PBMCs were fairly equal, with apoptotic rates of 32.2% (24.4-42.6) vs. 29.0% (22.4-31.9) at 30µg/mL ATG-treatment and 61.5% (41.9-74.6) vs. 61.5% (53.2-64.6) at 100µg/mL ATG-treatment.

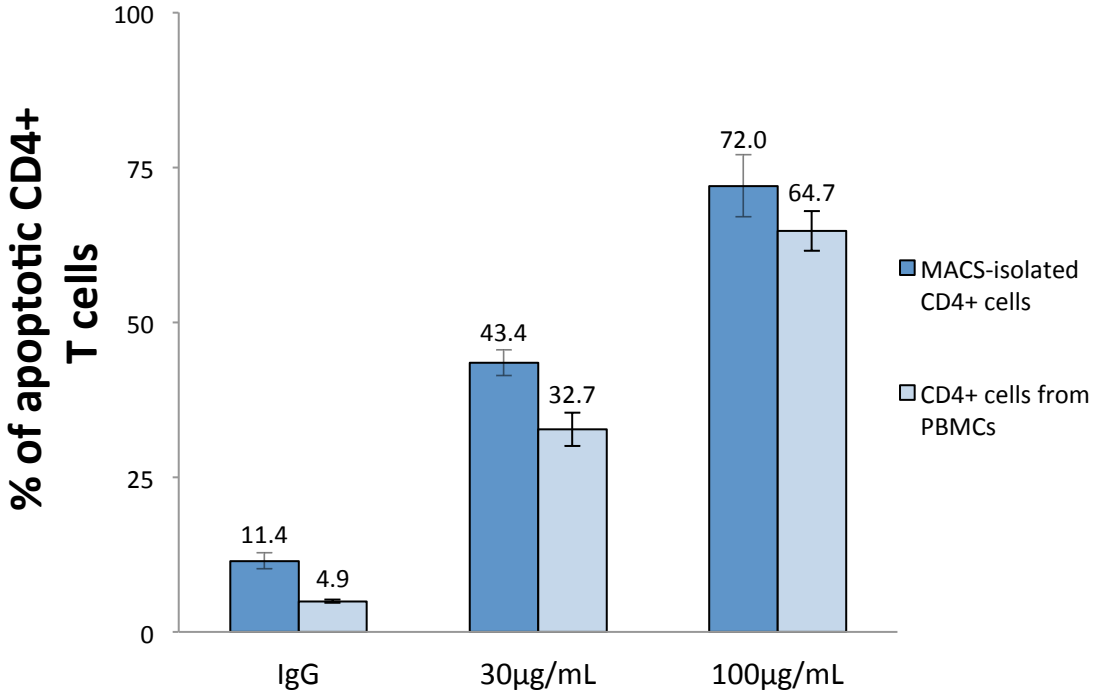

Figure 5: ATG-induced apoptosis in CD4+ T cells is independent from other immune cells. Displayed are apoptotic cell rates of MACS-isolated CD4+ T cells from healthy blood donors (dark blue, n=6) compared to CD4+ T cells from RA-patients cultured in presence of other PBMCs (light blue, n=3) in response to ATG-treatment (30 or 100µg/mL) for 18h. Unspecific rabbit IgG (100µg/mL) served as control treatment (ctl). Error bars indicate SEM.

Additionally, the observed difference in susceptibility to the apoptotic stimulus, comparing pre-activated cells with their freshly isolated counterparts, was now confirmed in isolated CD4+ T cells.

ATG-treatment (30µg/mL & 100µg/mL) of freshly isolated CD4+ T cells resulted in similar levels of apoptotic cells [13.9% (2.9-7.8) & 30.9% (19.3-52.1); data not shown] as seen in freshly isolated PBMCs gated on CD4+ T cells (13.8% & 36.8%, figure 4B in green). Compared to that, enhanced apoptosis was observed in pre-activated isolated CD4+ T cells [42.1% (37.9-52.1) & 69.8% (55.4-88.3), data not shown] similar to what was seen in pre-activated PBMCs gated on CD4+ T cells (36.4% & 69.2%, figure 4B in blue).

3.4. Down-regulation of specific cell surface markers upon ATG-treatment

In addition to the induction of apoptosis we analyzed the influence of ATG on the T cell phenotype. Therefore, PBMCs were treated with ATG for 18h and the expression of typical T cell markers was studied. We observed down-regulation of surface CD3, CD8 and CD28 in a dose-dependent manner (figure 6A-D). To exemplify the depicted data, median fluorescence intensity (MFI) for CD3 decreases from 7414 in control treated cells to 5688 and 3693 in cells treated with 30µg/mL and 100µg/mL ATG. A similar effect was seen in CD8 (MFI 6689 - 5016 - 3113) and CD28 (MFI 11248 - 6516 - 4398). In contrast, CD4 remained largely unaffected by ATG at any of the applied doses (MFI 4944 - 4721 - 4585).

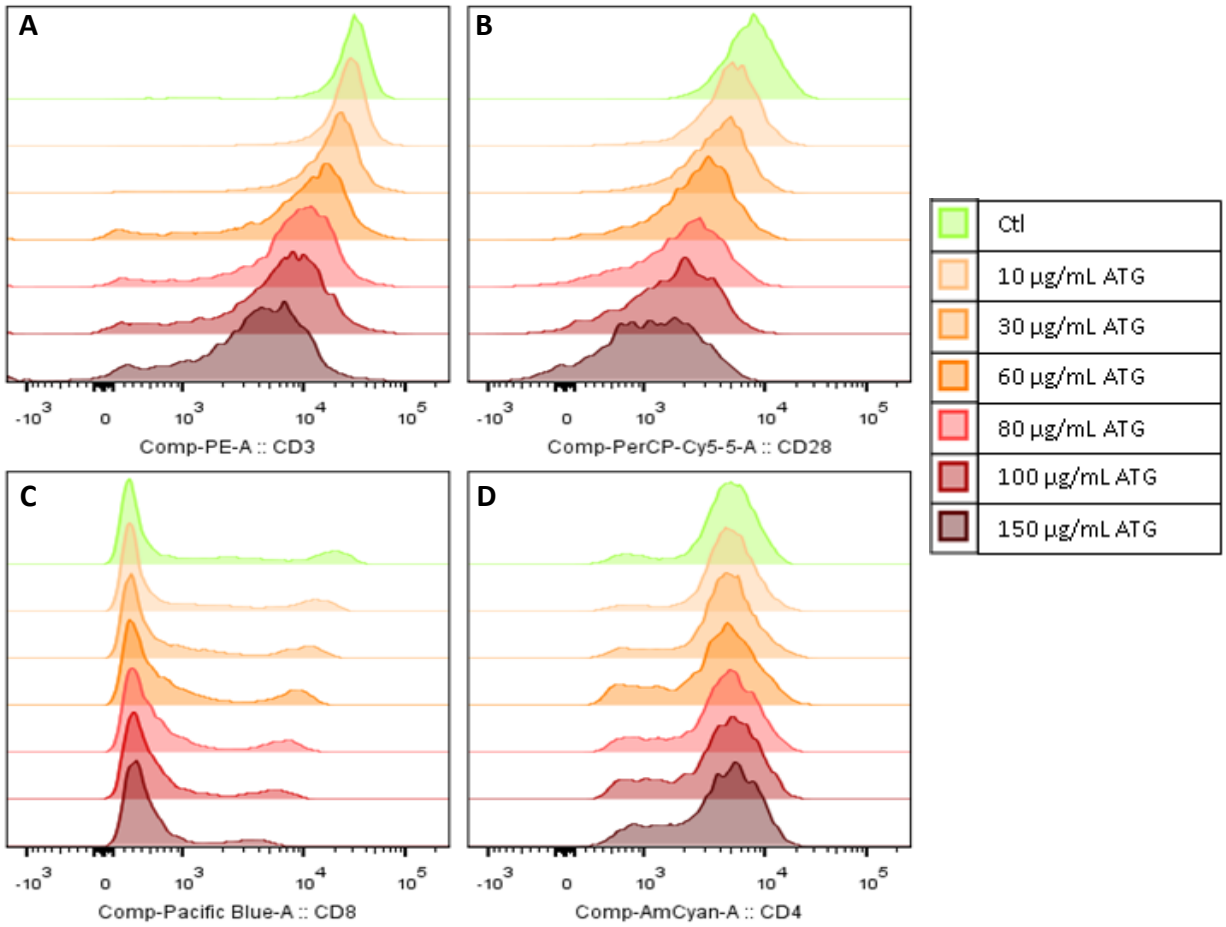

Figure 6: Decrease in signal intensity of certain surface markers.
Representative example of how signal intensity in the fluorescence-channels for CD3 (**A**), CD28 (**B**) and CD8 (**C**) is inversely proportional to increasing concentrations of ATG while signal intensity in the CD4 fluorescence-channel (**D**) is not affected at any dose of ATG-treatment.

To confirm an actual down-regulation of these certain cell surface molecules we first had to eliminate the possibility of a secondary effect due to changes in cell-size as a response to the treatment. As can be seen in figure 7A, the size of the analyzed cells remained largely unaffected. Moreover, a possible binding competition of the staining-antibodies with the polyclonal antibodies from the ATG seemed plausible. Therefore, ATG was added 30 minutes prior to staining (a time interval where no downregulation of surface markers can be expected) to preclude a blockage of binding sites of cell surface proteins. Figure 7B indicates that ATG does not affect the binding capacity of the staining antibodies we used in our experiments. Both findings support the hypothesis of a decrease in the expression of certain surface molecules on T cells in response to ATG treatment.

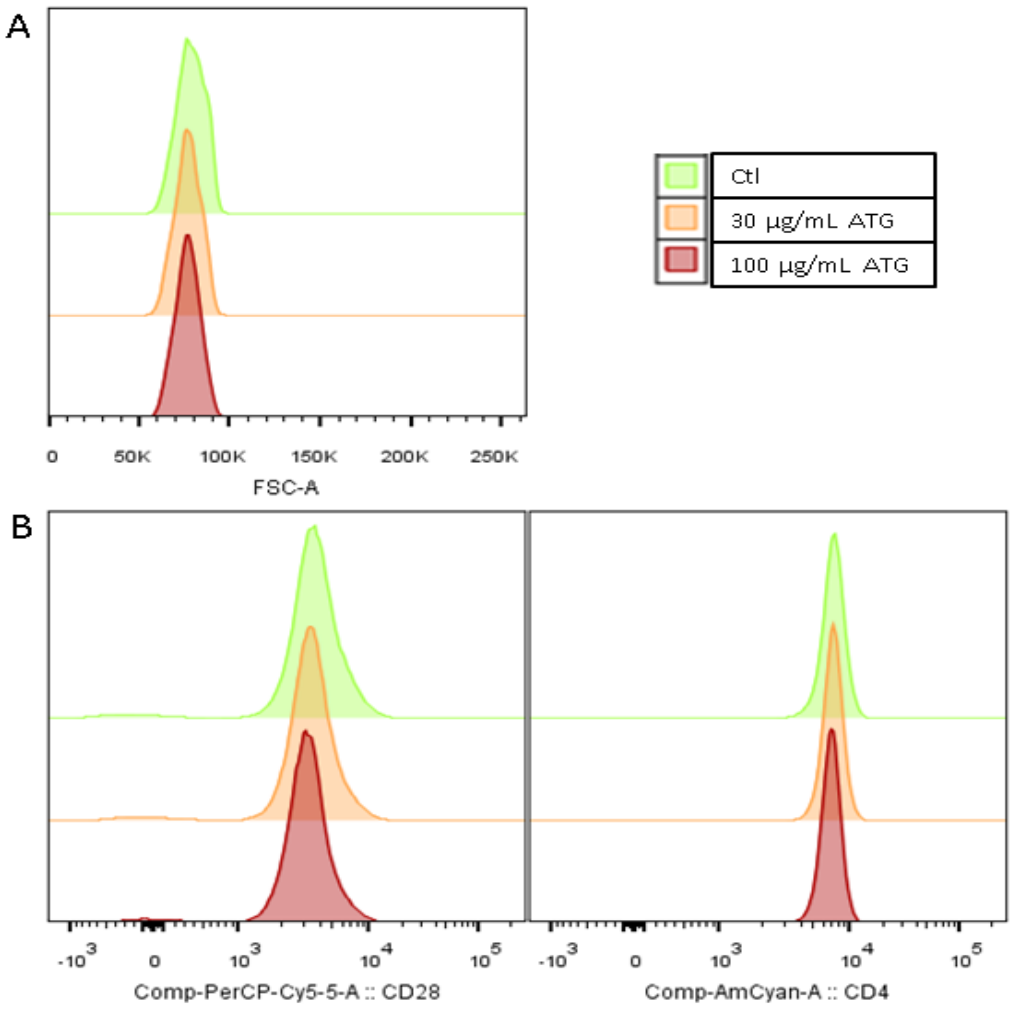

Figure 7: Effects on signal intensity not a result of antibody binding-competition or decreased cell size. A: No changes in cell size (depicted by FSC-A) after ATG-treatment for 18h. **B:** Binding capacity of anti-CD28- and anti-CD4-staining-antibodies on the surface of the cells remains unaffected after 30 min. of pre-incubation with ATG. Unspecific rabbit IgG (green) was used as control treatment.

3.5. ATG alters expression-levels of certain genes related to apoptosis

Duftner et al. reported that CD4+CD28- T-cells are more susceptible to apoptosis following treatment with ATG.[140] To clarify the mechanisms of this phenomenon the following experiments aimed to investigate changes in the expression levels of a selection of genes that are known to play a role in different apoptotic pathways, exerting either pro-apoptotic or anti-apoptotic functions (as indicated in table 9). mRNA-levels of pro-apoptotic BAD, BID and HRK were also analyzed but could not be detected in any of the samples and the genes were therefore excluded from table 9.
For these experiments, CD4+ T cells were sorted into a memory (CD45RO+CD28+) and a senescent (CD45RO+CD28-) subset in advance to ATG-treatment, allowing for the assessment of possible differences in gene expression alterations between these distinct subsets.

Comparing basal expression of memory as well as senescent CD4+ T cells NR4A1 (2.2-fold), NR4A2 (3.1-fold) and FAS-L (3.3-fold) for example tend to be slightly elevated in the CD28- subset without induction of apoptosis by ATG. At the same time, there is evidence that TRAIL basal expression (0.85-fold) might be decreased in CD28- T cells.
In both, memory and senescent CD4+ T cells, a massive increase in mRNA-levels of NR4A1 (235.5-fold vs. 190.2-fold), NR4A2 (117.9-fold vs. 58.5-fold) as well as NR4A3 (32.8-fold vs. 77.5-fold) after ATG-treatment was the most prominent finding. A definite increase in mRNA-levels was furthermore observed for BCL-XL (9.9-fold vs. 12.4-fold) and FAS-L (14.7-fold vs. 17.8-fold), while moderately elevated levels could be shown for BCL-2 (3.6-fold vs. 3.9-fold) and NOXA (2.5-fold vs. 2.9-fold).
Furthermore, FAS (0.85-fold vs. 0.85-fold) and PUMA (0.44-fold vs. 0.38-fold) would rather decrease upon ATG-treatment.

However, no significant differences were observed comparing gene expression levels of senescent and memory CD4+ T cells.

Table 9: Changes in mRNA-levels of apoptosis-related genes.

Mean-fold change in gene expression of IgG-treated CD28- cells, ATG-treated memory cells and ATG-treated CD28- cells compared to expression of the same genes in IgG-treated memory cells and ATG-treated CD28- cells (values set to 1), as indicated below. n=3.

Anti-apoptotic genes are displayed in green, pro-apoptotic genes in red. Blue highlights genes for which both, pro- and anti-apoptotic functions, are described in the literature.

FOLDCHANGES	BCL-2	BCL-XL	MCL-1	NR4A1	NR4A2	NR4A3
CD28-/IgG *(memory/IgG = 1)*	1.30	1.02	0.97	2.21	3.13	1.19
memory/ATG *(memory/IgG = 1)*	3.56	9.85	0.82	235.46	117.94	32.81
CD28-/ATG *(CD28-/IgG = 1)*	3.92	12.37	1.09	190.21	58.52	77.54

FOLDCHANGES	FAS	FAS-L	NOXA	*PUMA*	*TRAIL*
CD28-/IgG *(memory/IgG = 1)*	1.32	3.26	1.18	1.12	0.85
memory/ATG *(memory/IgG = 1)*	0.85	14.72	2.50	0.44	1.12
CD28-/ATG *(CD28-/IgG = 1)*	0.85	17.79	2.89	0.38	1.52

Figure 8 graphically depicts the massive increase of NR4A1, NR4A2 and NR4A3 mRNA levels described in table 9.

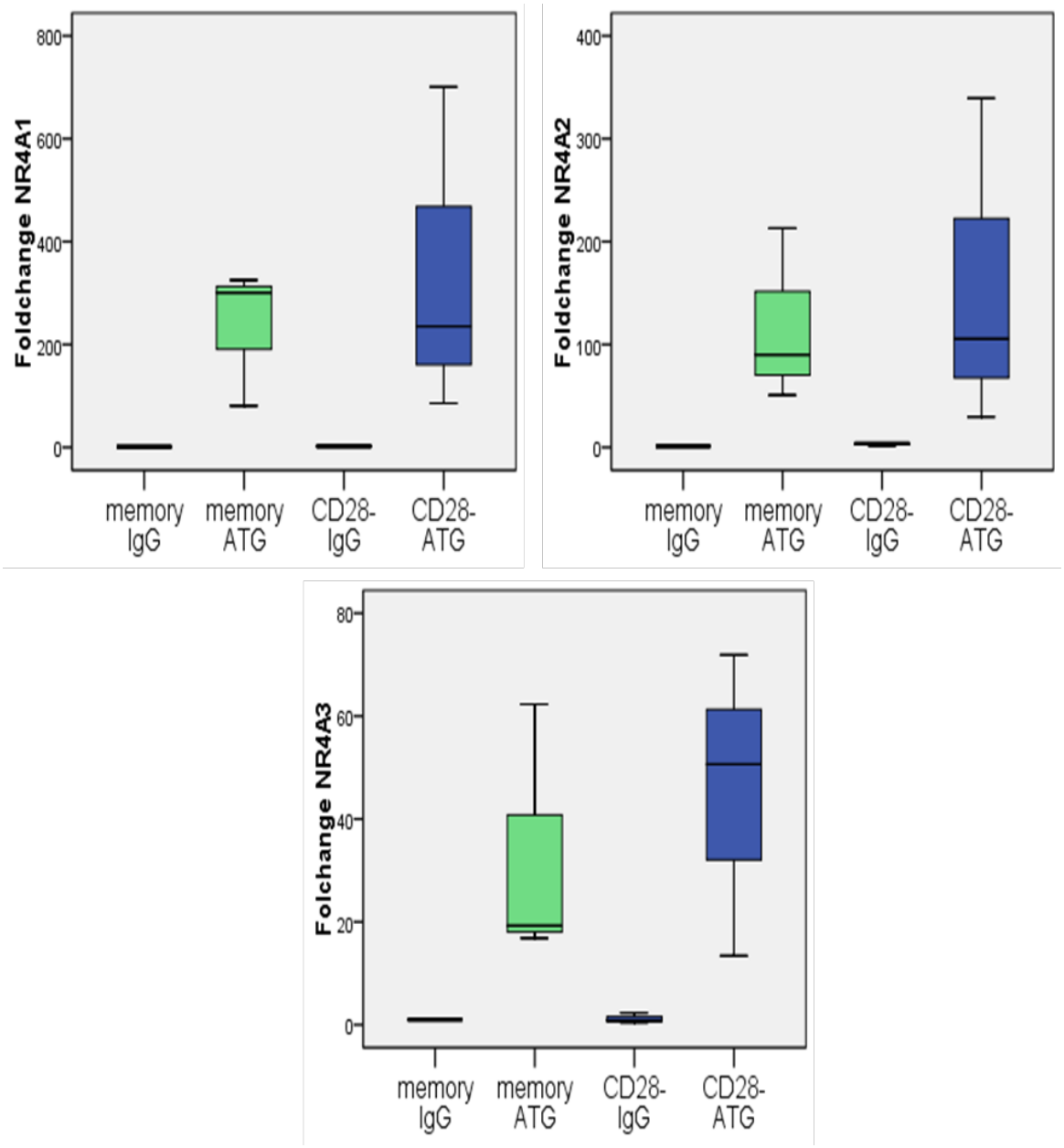

Figure 8: Induction of NR4A1, NR4A2 and NR4A3 expression.

Displayed is a massive induction of NR4A1, NR4A2 and NR4A3 mRNA levels upon ATG-treatment (100µg/mL) in both, CD28- and memory CD4+ T cells. n=3. No statistical significance was tested for differences between treatments or subsets.

3.6. *No inhibition of ATG-induced apoptosis by the potential NR4A1-inhibitor C-DIM-8*

Since the most prominent increase in gene expression was observed in the members of the NR4A receptor group, we then tested for changes in the apoptotic rates of cells that were treated with ATG and C-DIM-8, a potential inhibitor of NR4A1.

As shown in figure 9, treatment with 6.25µM, 12.5µM and 25µM C-DIM-8 did not show an effect in control-treated cells while treatment with the highest concentration (50µM) resulted in a marked increase in CD4+ T cell-apoptosis already without ATG-stimulation [19.1% (10.1-72.0)].
ATG-treatment at a dose of 30µg/mL led to an apoptotic rate of 34.3% (16.7-55.1), whereas treatment with C-DIM-8 at concentrations of 6.25µM, 12.5µM and 25µM in addition to ATG could not reduce the number of apoptotic cells. As already indicated in control cells, 50µM of C-DIM-8 further increased ATG-induced apoptosis [82.1% (50.4-96.9)].

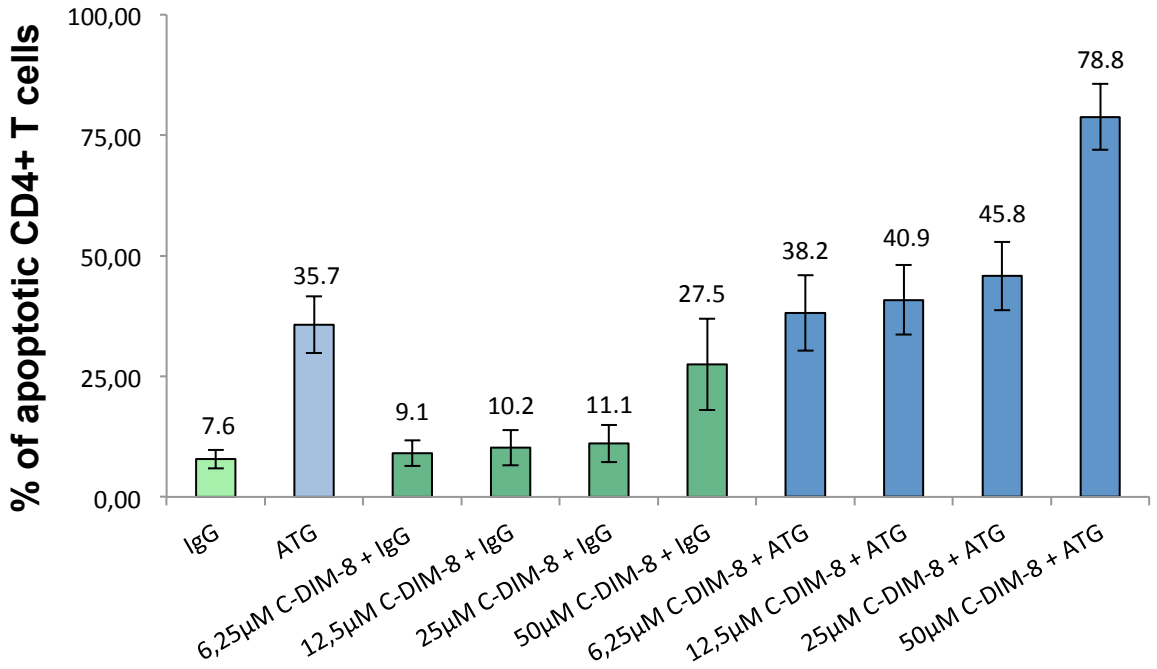

Figure 9: No reduction of ATG-induced CD4+ T cell-apoptosis by NR4A1-inhibitor C-DIM-8. Displayed is the mean value of apoptotic cell rates from 6 different experiments. Error bars indicate SEM.

Additionally, when real-time PCR was performed in these experiments ATG-enhanced gene expression of NR4A1 was unaffected by C-DIM-8 at any of the applied concentrations.

Figure 10A shows no effect of the applied C-DIM-8 concentrations on basal levels of NR4A1 mRNA levels in IgG-treated control cells. ATG-induced increase in NR4A1 gene expression can be seen in figure10B (135.5-fold). Addition of the potential NR4A1 inhibitor C-DIM-8 could not abrogate this induction at any of the applied doses. The fold-change decrease seen in cells treated with ATG and 50µM C-DIM-8 (92.1-fold) may result from the high numbers of apoptotic cells observed in these samples (see figure 9).

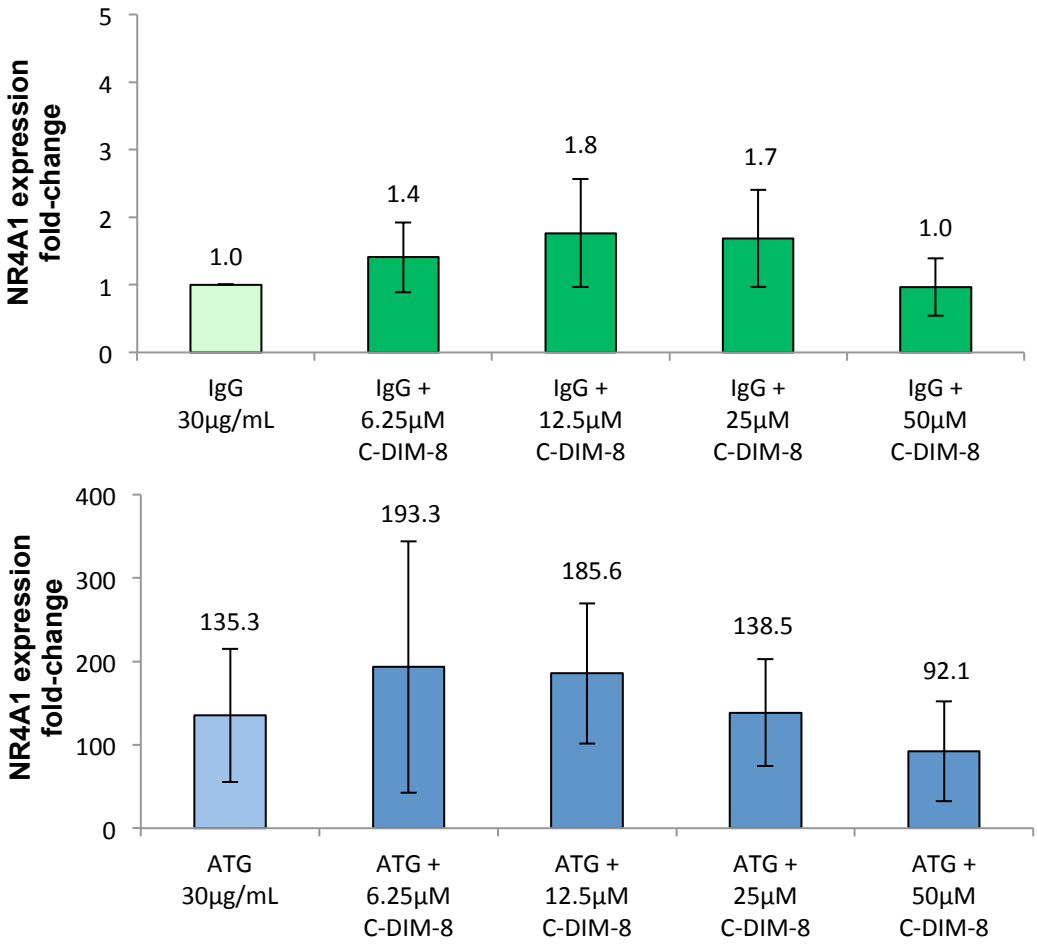

Figure 10: C-DIM-8 does not affect NR4A1 gene-expression.

A Cells were treated with IgG (30µg/mL, light green) combined with C-DIM-8 (indicated concentrations, dark green). **B** Cells were treated with ATG only (30µg/mL, light blue) or ATG combined with C-DIM-8 (indicated concentrations, dark blue). Fold-change was calculated by normalization of to control cells treated with IgG only (30µg/mL).

4. Discussion

In this work, we confirmed that ATG provokes depletion of CD4+ T cells in PBMCs from RA patients *ex vivo* via induction of apoptosis. As expected, apoptotic rates positively correlated with the applied ATG concentrations. Notably, our results suggest that T cell activation confers greater susceptibility to the apoptotic stimulus since freshly isolated cells showed markedly decreased apoptotic rates compared to cells that were cultured under activating conditions for several days. In addition, ATG induce a vigorous increase in NR4A family mRNA levels suggesting an involvement in this apoptotic process.

ATG has successfully been applied as immunosuppressant in the field of transplantation, as well as for the treatment of aplastic anemia for decades already. The main immunosuppressive property described for ATG is a broad depletion of T cells and especially prolonged reduction in effector CD4+ T cells eventually resulting in a CD4/CD8 T cell ratio inversion.[142]

Genestier et al. have described antibody-dependent cell-mediated cytotoxicity (ADCC) as a possible mechanism for ATG-induced elimination of activated T cells, while resting T cells remained resistant.[130] ADCC is classically mediated by a variety of cells (e.g. neutrophils, eosinophils, NK cells and macrophages) that eliminate immune cells expressing surface molecules that have been bound by specific antibodies.[143] In this study we noted that apoptotic cell rates of isolated CD4+ T cells were similar compared to those of CD4+ T cells in the presence of total PBMCs. This finding suggests that ATG-induced apoptosis is independent of indirect impact via other immune cells and ADCC.

Additionally, elevated apoptotic rates were observed in isolated CD4+ T cells pre-activated in culture, compared to CD4+ T cells that were freshly isolated from RA patients' blood before ATG-treatment, pointing to an involvement of the cells' activation status.

Signs of premature immunosenescence have been found in several autoimmune disorders, including RA.[144] Moreover, elevated levels of the senescent CD4+CD28- T cells are reported in patients with RA and are correlated with disease activity in these patients.[145,146] These cells are suggested to be involved in the pathogenesis of RA due to the acquisition of pro-inflammatory features that are atypical for conventional CD4+ T cells and thus favor the so-called senescence-associated secretory phenotype.[66]

More recently, Duftner et al. have reported increased susceptibility of CD4+CD28- T cells to ATG-triggered apoptosis,[140] an observation contrasting the fact that this cell subset is generally described to exhibit greater resistance to apoptotic stimuli than conventional T cells.[88,85] Schirmer et al. have observed increased levels of Bcl-2 protein in CD4+CD28- T cells analyzed by flow cytometry, proposing that a dysregulation of this pro-survival protein may enable the clonal outgrowth of autoreactive T cells and thus contribute to the pathogenesis of rheumatoid arthritis.[88]

To elucidate the mechanisms of this phenomenon, we isolated CD4+CD28- T cells in order to analyze ATG-induced expression changes in a selection of genes known to be involved in diverse apoptotic pathways. Overall, we could not detect significant differences in expression levels of any of the selected genes after ATG-treatment when comparing CD4+CD28- T cells with conventional CD4+ T cells.

Our data could not confirm a remarkable up-regulation of Bcl-2 when mRNA levels of untreated CD4+CD28- cells (1.3-fold increase) were compared to untreated memory CD4+ T cells. This might indicate that the expression difference reported by Schirmer et al. was due to naïve cells within the CD4+CD28- T cell population, a subset we excluded from our experiments.[88] However, we observed a moderate increase in Bcl-2 gene expression in both - memory and senescent T cells - after ATG-treatment.

Another proposed mechanism for ATG-induced apoptosis is activation induced cell death, a pathway mainly depending on the binding of surface receptor Fas to its ligand Fas-L expressed on another cell.[130] This mechanism of apoptosis not only requires the presence of Fas receptors on the cell surface, it additionally involves cell activation by IL-2 to enhance AICD.[147]

The increased apoptotic-susceptibility observed in pre-activated CD4+ T cells points to AICD as mechanism of action for ATG. Indeed, FAS-L expression was upregulated in CD4+ T cells following ATG-treatment in this study. On the contrary, FAS expression was rather decreased in both, CD28- and conventional memory CD4+ T cells comparing ATG-treated to control treated cells. This trend, however, might result from an increase of apoptotic cells in response to ATG-treatment.

Another interesting observation was the massive increase in gene expression of the NR4A receptor family, with the highest elevations detected in NR4A1 expression. A pro-survival effect of NR4A1 has been suggested for several cancer cell lines.[148] In contrast, other studies have described a pro-apoptotic mechanism of action for NR4A1 upon nuclear export of the receptor to the mitochondria and a subsequent complex-formation with Bcl-2, eventually resulting in cytochrome C release and apoptosis.[23,20] Our findings in combination with the described pro-apoptotic mechanism of action led us to the hypothesis that NR4A1 might also be a potential player in the pathway of ATG-induced apoptosis of T cells.

Therefore, we tested if addition of the 1,1-Bis(3-indolyl)-1-(*p*-substitutedphenyl)-methane analogue C-DIM-8 (described as an inhibitor of NR4A1[149]) would abrogate or reduce ATG-induced apoptosis. However, in our experiments C-DIM-8 could neither lower the apoptotic rate of ATG-treated cells, nor did it show an effect on the levels of NR4A1 gene expression. Thus, we cannot evaluate if NR4A1 does actually play a role in ATG-induced apoptosis of CD4+ T cells since the NR4A1-inhibitor we used might have been ineffective.

Apart from the induction of apoptosis, another effect of ATG was observed in our experiments. ATG-treatment resulted in a decreased expression of T cell-specific surface markers CD3, CD8 and CD28 in a concentration dependent manner when analyzed by flow cytometry. This finding might reflect a protective mechanism to countervail immoderate activation. Interestingly, surface expression of CD4 molecules remained largely unaffected. Upon further analysis we could preclude a possible secondary effect due to changes in cell size.

Additionally, we performed binding-competition experiments to show that this decrease does not result from ATG-antibodies blocking the binding sites of the staining antibodies we use for flow cytometric analyses. These findings indicate an actual downregulation of certain surface molecules in response to ATG.

4.1. Conclusion

ATG is a potent inducer of apoptosis in CD4+ T cells, especially when cells are pre-activated. This leads to the assumption that ATG might induce apoptosis via AICD, which is dependent on interaction of Fas with Fas-L. This hypothesis is supported by the observed up-regulation of FAS-L gene expression, although at the same time it is contradicted by the down-regulation of FAS gene expression in response to ATG. Alternatively, NR4A1 might be involved in the apoptotic mechanism induced by ATG. However, this hypothesis needs further confirmation. Additionally, our data did not support the recently reported elevated susceptibility of the pro-inflammatory CD4+CD28- T cell subset to undergo ATG-induced apoptosis. Further investigations will be required in this course to clarify if ATG might be indicated in future therapy of RA and other disease where involvement of CD4+CD28- T cells is described.

5. References

1. Parkin, J. & Cohen, B. An overview of the immune system. *Lancet* **357,** 1777–1789 (2001).
2. Turvey, S. E. & Broide, D. H. Innate immunity. *J. Allergy Clin. Immunol.* **125,** 24–S32 (2010).
3. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* **125,** 33–S40 (2010).
4. Gallegos, A. M. & Bevan, M. J. Central tolerance: good but imperfect. *Immunol. Rev.* **209,** 290–296 (2006).
5. Basson, M. A. & Zamoyska, R. The CD4/CD8 lineage decision: integration of signalling pathways. *Immunol. Today* **21,** 509–514 (2000).
6. Annunziato, F. & Romagnani, S. Heterogeneity of human effector CD4 + T cells. *Arthritis Res. Ther.* **11,** 1–8 (2009).
7. Raphael, I., Nalawade, S., Eagar, T. N. & Forsthuber, T. G. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74,** 5–17 (2015).
8. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance. *Annu. Rev. Immunol.* **22,** 745–763 (2004).
9. Mathis, D. & Benoist, C. Back to Central Tolerance. *Immunity* **20,** 509–516 (2004).
10. Halverson, R., Torres, R. M. & Pelanda, R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. **5,** 645–650 (2004).
11. Li, L. & Boussiotis, V. A. Physiologic regulation of central and peripheral T cell tolerance: lessons for therapeutic applications. *J. Mol. Med.* **84,** 887–899 (2006).
12. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* **133,** 775–787 (2008).
13. Gershon, R. & Kondo, K. Cell Interactions in the Induction of Tolerance: The Role of Thymic Lymphocytes. *Immunology* **18,** 723–737 (1970).
14. Smith, T. R. F. & Kumar, V. Revival of CD8+ Treg–mediated suppression. *Trends Immunol.* **29,** 337–342 (2008).
15. Hodgkin, P. D. & Basten, A. B cell activation , tolerance and antigen-presenting function. *Curr. Opin. Immunol.* **7,** 121–129 (1995).
16. Rathmell, J. C. & Thompson, C. B. Pathways of Apoptosis in Lymphocyte Development, Homeostasis, and Disease. *Cell* **109,** 97–107 (2002).
17. O'Flaherty, E. *et al.* Regulation of T-cell apoptosis: a mixed lymphocyte reaction model. *Immunology* **100,** 289–299 (2000).
18. Wong, B., Arron, J. & Choi, Y. T Cell Receptor Signals Enhance Susceptibility to Fas-mediated Apoptosis. *J. Exp. Med.* **186,** 1939–1944 (1997).
19. Waring, P. & Müllbacher, A. Cell death induced by the Fas / Fas ligand pathway and its role in pathology. *Immunol. Cell Biol.* **77,** 312–317 (1999).
20. Li, H. *et al.* Cytochrome c Release and Apoptosis Induced by Mitochondrial Targeting of Nuclear Orphan Receptor TR3. *Science (80-.).* **289,** 1159–1165 (2000).

21. Renault, T., Elkholi, R., Bharti, A. & Chipuk, J. B Cell Lymphoma-2 (BCL-2) Homology Domain 3 (BH3) Mimetics Demonstrate Differential Activities Dependent upon the Functional Repertoire of Pro- and Anti-apoptotic BCL-2 Family Proteins. *J. Biol. Chem.* **289,** 26481–26491 (2014).
22. Karim, C. B. *et al.* Structural Mechanism for Regulation of Bcl-2 protein Noxa by phosphorylation. *Sci. Rep.* **5,** 1–9 (2015).
23. Lin, B. *et al.* Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3. *Cell* **116,** 527–540 (2004).
24. Smith, D. A. & Germolec, D. R. Introduction to Immunology and Autoimmunity. *Environ. Health Perspect.* **107,** 661–665 (1999).
25. Eaton, W. W., Rose, N. R., Kalaydjian, A., Pedersen, M. G. & Bo, P. Epidemiology of autoimmune diseases in Denmark. *J. Autoimmun.* **29,** 1–9 (2007).
26. Jacobson, D. L., Gange, S. J., Rose, N. R. & Graham, N. M. H. Epidemiology and Estimated Population Burden of Selected Autoimmune Diseases in the United States. *Clin. Immunol. Immunopathol.* **84,** 223–243 (1997).
27. Cooper, G. S. & Stroehla, B. C. The epidemiology of autoimmune diseases. *Autoimmun. Rev.* **2,** 119–125 (2003).
28. Wang, L., Wang, F. & Gershwin, M. E. Human autoimmune diseases: a comprehensive update. *J. Intern. Med.* **278,** 369–395 (2015).
29. Moudgil, K. D. & Sercarz, E. E. Crypticity of self antigenic determinants is the cornerstone of a theory of autoimmunity. *Discov. Med.* **5,** 378–382 (2005).
30. Ellis, N. M. J. *et al.* T Cell Mimicry and Epitope Specificity of Cross-Reactive T Cell Clones from Rheumatic Heart Disease. *J. Immunol.* **175,** 5448–5456 (2005).
31. Fernando, M. M. A. *et al.* Defining the Role of the MHC in Autoimmunity: A Review and Pooled Analysis. *PLoS Genet.* **4,** e1000024 (2008).
32. Goronzy, J. J. & Weyand, C. M. Rheumatoid arthritis. *Immunol. Rev.* **204,** 55–73 (2005).
33. Tobón, G. J., Youinou, P. & Saraux, A. The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. *J. Autoimmun.* **35,** 10–14 (2010).
34. Guillemin, F. *et al.* Prevalence of rheumatoid arthritis in France : 2001. *Ann. Rheum. Dis.* **64,** 1427–1431 (2005).
35. Symmons, D. *et al.* The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century. *Rheumatology* **41,** 793–800 (2002).
36. Carbonell, J., Cobo, T., Balsa, A., Descalzo, M. A. & Carmona, L. The incidence of rheumatoid arthritis in Spain: results from a nationwide primary care registry. *Rheumatol* **47,** 1088–1092 (2008).
37. Carmona, L. *et al.* The prevalence of rheumatoid arthritis in the general population of Spain. *Rheumatology* **41,** 88–95 (2002).
38. Scott, D. L., Wolfe, F. & Huizinga, T. W. J. Rheumatoid arthritis. *Lancet* **376,** 1094–108 (2010).
39. van der Woude, D. *et al.* Quantitative Heritability of Anti – Citrullinated Protein Antibody – Positive and Anti – Citrullinated Protein Antibody – Negative Rheumatoid Arthritis. *Arthritis Rheum.* **60,** 916–923 (2009).
40. Skeoch, S. & Bruce, I. N. Atherosclerosis in rheumatoid arthritis: is it all about inflammation? *Nat Rev Rheumatol* **11,** 390–400 (2015).

41. Holmqvist, M. E. *et al.* Rapid increase in myocardial infarction risk following diagnosis of rheumatoid arthritis amongst patients diagnosed between 1995 and 2006. *J. Intern. Med.* **268,** 578–585 (2010).
42. Mcinnes, I. B. & Schett, G. The Pathogenesis of Rheumatoid Arthritis. *New Engl. J. Med.* **365,** 2205–2219 (2012).
43. Simon, T. A., Thompson, A., Gandhi, K. K., Hochberg, M. C. & Suissa, S. Incidence of malignancy in adult patients with rheumatoid arthritis : a meta-analysis. *Arthritis Res. Ther.* **17,** 1–10 (2015).
44. Wasserman, A. M. Y. M. Diagnosis and Management of Rheumatoid Arthritis. *Am Fam Physician* **84,** 1245–1252 (2011).
45. Aletaha, D. *et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann. Rheum. Dis.* **69,** 1580–1588 (2010).
46. Kerlan-Candon, S. *et al.* HLA-DRB1 gene transcripts in rheumatoid arthritis. *Clin. Exp. Immunol.* **124,** 142–149 (2001).
47. Choy, E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology* **51,** 3–11 (2012).
48. Mellado, M. *et al.* T cell migration in rheumatoid arthritis. *Front. Immunol.* **6,** 1–12 (2015).
49. Goldring, S. R. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. *Rheumatology* **42,** 11–16 (2003).
50. Mauri, C. & Ehrenstein, M. R. Cells of the synovium in rheumatoid arthritis. B cells. *Arthritis Rheumatol.* **9,** (2007).
51. Kinne, R. W., Stuhlmüller, B. & Burmester, G. Cells of the synovium in rheumatoid arthritis. Macrophages. *Arthritis Res. Ther.* **9,** (2007).
52. Lundy, S. K., Sarkar, S., Tesmer, L. A. & Fox, D. A. Cells of the synovium in rheumatoid arthritis. T lymphocytes. *Arthritis Res. Ther.* **9,** (2007).
53. Thomas, R., McIlraith, M., Davis, L. S. & Lipsky, P. E. Rheumatoid synovium is enriched in CD45RBdim mature memory T cells that are potent helpers for B cell differentiation. *Arthritis Rheum.* **35,** 1455–1465 (1992).
54. Schulze-Koops, H. & Kalden, J. R. The balance of Th1/Th2 cytokines in rheumatoid arthritis. *Best Pract. Reseach Clin. Rheumatol.* **15,** 677–691 (2001).
55. Steinman, L. A brief history of Th17, the first major revision in the Th1/Th2 hypothesis of T cell–mediated tissue damage. *Nat. Med.* **13,** 139–146 (2007).
56. Boissier, M., Assier, E., Falgarone, G. & Bessis, N. Shifting the imbalance from Th1/Th2 to Th17/treg: The changing rheumatoid arthritis paradigm. *Jt. Bone Spine* **75,** 373–375 (2008).
57. Onishi, R. M. & Gaffen, S. L. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* **129,** 311–321 (2010).
58. Ziegler, S. F. & Buckner, J. H. FOXP3 and the Regulation of Treg/Th17 Differentiation. *Microbes Infect.* **11,** 594–598 (2009).
59. Nakae, S., Nambu, A., Sudo, K. & Alerts, E. Suppression of Immune Induction of Collagen-Induced Arthritis in IL-17-Deficient Mice. *J. Immunol.* 6173–6177 (2003). doi:10.4049/jimmunol.171.11.6173
60. Zwerina, K. *et al.* Anti IL-17A therapy inhibits bone loss in TNF-a-mediated murine arthritis by modulation of the T-cell balance. *Eur. J. Immunol.* **42,** 413–423 (2012).

61. Kellner, H. Targeting interleukin-17 in patients with active rheumatoid arthritis: rationale and clinical potential. *Ther. Adv. Musculoskelet. Dis.* **5,** 141–152 (2013).
62. Joller, N. *et al.* Treg cells expressing the co-inhibitory molecule TIGIT selectively inhibit pro-inflammatory Th1 and Th17 cell responses. *Immunity* **40,** 569–581 (2014).
63. van Amelsfort, J. M. R. *et al.* Proinflammatory Mediator–Induced Reversal of CD4+,CD25+ Regulatory T Cell–Mediated Suppression in Rheumatoid Arthritis. *Arthritis Rheum.* **56,** 732–742 (2007).
64. Boisser, M., Assier, E., Biton, J. & Denys, A. Regulatory T cells (Treg) in rheumatoid arthritis. *Jt. Bone Spine* **76,** 10–14 (2009).
65. Lawson, C. A. *et al.* Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. *Rheumatology* **45,** 1210–1217 (2006).
66. Weyand, C. M., Yang, Z. & Goronzy, J. J. T Cell Aging in Rheumatoid Arthritis. *Curr Opin Rheumatol* **26,** 93–100 (2014).
67. Chalan, P., Van Den Berg, A., Kroesen, B.-J., Brouwer, L. & Boots, A. Rheumatoid Arthritis, Immunosenescence and the Hallmarks of Aging. *Curr. Aging Sci.* **8,** 131–146 (2015).
68. Chou, J. P. & Effros, R. B. T Cell Replicative Senescence in Human Aging. *Curr. Pharm. Des.* **19,** 1680–1698 (2013).
69. Vallejo, A. N. *et al.* Modulation of CD28 Expression: Distinct Regulatory Pathways During Activation and Replicative Senescence. *J. Immunol.* **162,** 6572–6579 (1999).
70. Vallejo, A. N. *et al.* Molecular Basis for the Loss of CD28 Expression in Senescent T Cells*. *J. Biol. Chem.* **277,** 46940–46949 (2002).
71. Schmidt, D., Goronzy, J. J. & Weyand, C. M. CD4+ CD7- CD28- T Cells Are Expanded in Rheumatoid Arthritis and Are Characterized by Autoreactivity. **97,** 2027–2037 (1996).
72. Thewissen, M. *et al.* Premature Immunosenescence in Rheumatoid Arthritis and Multiple Sclerosis Patients. *Ann. N. Y. Acad. Sci.* **1051,** 255–262 (2005).
73. Schönland, S. *et al.* Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. *PNAS* **100,** 13471–13476 (2003).
74. Schmidt, D., Martens, P. B., Weyand, C. M. & Goronzy, J. J. The Repertoire of CD4+ CD28- T Cells in Rheumatoid Arthritis. *Mol. Med.* **2,** 608–618 (1996).
75. Wagner, U. *et al.* Clonally expanded CD4+CD28null T cells in rheumatoid arthritis use distinct combinations of T cell receptor BV and BJ elements. *Eur. J. Immunol.* **33,** 79–84 (2003).
76. Fasth, A. E. R., Bjo, N. K., Malmberg, K. & Malmstro, V. Activating NK-cell receptors co-stimulate CD4+CD28- T cells in patients with rheumatoid arthritis. *Eur. J. Immunol.* **40,** 378–387 (2010).
77. van Bergen, J., Thompson, A., van der Slik, A. & Ottenhoff, T. H. M. Phenotypic and Functional Characterization of CD4 T Cells Expressing Killer Ig-Like Receptors. *J. Immunol.* **173,** 6719–6726 (2004).
78. Groh, V., Brühl, A., El-Gabalawy, H., Nelson, J. L. & Spies, T. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. *PNAS* **100,** 9452–9457 (2003).

79. Goronzy, J., Henel, G., Sawai, H., Singh, K. & Weyand, C. M. Costimulatory Pathways in Rheumatoid Synovitis and T-Cell Senescence. *Ann. Rheum. Dis.* **1062,** 182–194 (2005).
80. Warrington, K. J., Takemura, S., Goronzy, J. J. & Weyand, C. M. CD4+, CD28- T Cells in Rheumatoid Arthritis Patients Combine Features of the Innate and Adaptive Immune Systems. *Arthritis Rheum.* **44,** 13–20 (2001).
81. Nakajima, T. *et al.* T-Cell–Mediated Lysis of Endothelial Cells in Acute Coronary Syndromes. *Circulation* **105,** 570–575 (2002).
82. Coppé, J., Desprez, P., Krtolica, A. & Campisi, J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu Rev Pathol* **5,** 99–118 (2010).
83. Hohensinner, P. J., Goronzy, J. J. & Weyand, C. M. Targets of Immune Regeneration in Rheumatoid Arthritis. *Mayo. Clin. Proc.* **89,** 563–575 (2014).
84. Brenner, D., Krammer, P. H. & Arnold, R. Concepts of activated T cell death. *Crit. Rev. Oncol. Hematol.* **66,** 52–64 (2008).
85. Vallejo, A. N., Schirmer, M., Weyand, C. M. & Goronzy, J. J. Clonality and Longevity of CD4+CD28null T Cells Are Associated with Defects in Apoptotic Pathways. *J. Immunol.* **165,** 6301–6307 (2000).
86. Hoff, H. *et al.* Surface CD152 (CTLA-4) Expression and Signaling Dictates Longevity of CD28null T Cells. *J. Immunol.* **182,** 5342–5351 (2009).
87. Cory, S. & Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2,** 647–656 (2002).
88. Schirmer, M. *et al.* Resistance to Apoptosis and Elevated Expression of Bcl-2 in Clonally Expanded CD4+CD28− T Cells from Rheumatoid Arthritis Patients. *J. Immunol.* **161,** 1018–1025 (1998).
89. Wienecke, T. & Gøtzsche, P. Paracetamol versus nonsteroidal anti-inflammatory drugs for rheumatoid arthritis. *Cochrane Database Syst. Rev.* CD003789 (2004). doi:10.1002/14651858.CD003789.pub2.www.cochranelibrary.com
90. Scott, P., Kinglsey, G., Smith, C., CHoy, E. & Scott, D. Non-steroidal anti-inflammatory drugs and myocardial infarctions: comparative systematic review of evidence from observational studies and randomised controlled trials. *Ann. Rheum. Dis.* **66,** 1296–1305 (2007).
91. Schaffer, D. *et al.* Risk of serious NSAID-related gastrointestinal events during long-term exposure: a systematic review. *Med. J. Aust.* **185,** 501–506 (2006).
92. Chen, Y. *et al.* Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs (etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib) for osteoarthritis and rheumatoid arthritis: a systematic review and economic evaluation. *Health Technol. Assess. (Rockv).* **12,** 1–278 (2008).
93. Kirwan, J., Bijlsma, J., Boers, M. & Shea, B. Effects of glucocorticoids on radiological progression in rheumatoid arthritis. *Cochrane Database Syst. Rev.* CD006356 (2009). doi:10.1002/14651858.CD006356.www.cochranelibrary.com
94. Goossens, P. H. *et al.* Reliability and sensitivity to change of various measures of hand function in relation to treatment of synovitis of the metacarpophalangeal joint in rheumatoid arthritis. *Rheumatology* **39,** 909–913 (2000).

95. Ravindran, V., Rachapalli, S. & Choy, E. H. Safety of medium- to long-term glucocorticoid therapy in rheumatoid arthritis: a meta-analysis. *Rheumatology* **48,** 807–811 (2009).
96. Goodman, S., Cronstein, B. & Bykerk, V. Outcomes Related to Methotrexate Dose and Route of Administration in Patients with Rheumatoid Arthritis: A Systematic Literature Review. *Clin. Exp. Rheumatol.* **33,** 272–278 (2015).
97. Cipriani, P., Ruscitti, P., Carubbi, F., Liakouli, V. & Giacomelli, R. Methotrexate in Rheumatoid Arthritis: Optimizing Therapy Among Different Formulations. Current and Emerging Paradigms. *Clin. Ther.* **36,** 427–435 (2014).
98. Dale, J., Alcorn, N., Capell, H. & Madhok, R. Combination therapy for rheumatoid arthritis: methotrexate and sulfasalazine together or with other DMARDs. *Nat. Clin. Pract.* **3,** 450–458 (2007).
99. Capell, H. A. *et al.* Combination therapy with sulfasalazine and methotrexate is more effective than either drug alone in patients with rheumatoid arthritis with a suboptimal response to sulfasalazine: results from the double-blind placebo-controlled MASCOT study. *Ann. Rheum. Dis.* **66,** 235–241 (2007).
100. Li, E. K., Tam, L., Uk, M. & Tomlinson, B. Leflunomide in the Treatment of Rheumatoid Arthritis. *Clin. Ther.* **26,** 447–459 (2004).
101. Golicki, D. *et al.* Leflunomide in monotherapy of rheumatoid arthritis: meta-analysis of randomized trials. *Pol. Arch. Med.* **122,** 22–32 (2012).
102. Munster, T. *et al.* Hydroxychloroquine Concentration – Response Relationships in Patients With Rheumatoid Arthritis. *Arthritis Rheum.* **46,** 1460–1469 (2002).
103. Negrei, C. *et al.* Management of rheumatoid arthritis: Impact and risks of various therapeutic approaches. *Exp. Ther. Med.* **11,** 1177–1183 (2016).
104. Choy, E. H. S., Smith, C., Dore, C. J. & Scott, D. L. A meta-analysis of the efficacy and toxicity of combining disease-modifying anti-rheumatic drugs in rheumatoid arthritis based on patient withdrawal. *Rheumatology* **44,** 1414–1421 (2005).
105. Salliot, C. & Heijde, D. Van Der. Long-term safety of methotrexate monotherapy in patients with rheumatoid arthritis: a systematic literature research. *Ann. Rheum. Dis.* **68,** 1100–1104 (2009).
106. Alcorn, N., Saunders, S. & Madhok, R. Benefit-Risk Assessment of Leflunomide. An Appraisal of Leflunomide in Rheumatoid Arthritis 10 Years After Licensing. *Drug Saf.* **32,** 1123–1134 (2009).
107. Strangfeld, A. *et al.* Comparative effectiveness of tumour necrosis factor a inhibitors in combination with either methotrexate or leflunomide. *Ann. Rheum. Dis.* **68,** 1856–1862 (2009).
108. Choy, E. H. S. & Panayi, G. S. Cytokine Pathways and Joint Inflammation in Rheumatoid Arthritis. *N. Engl. J. Med.* **344,** 907–916 (2001).
109. Ramiro, S. *et al.* Safety of synthetic and biological DMARDs: a systematic literature review informing the 2013 update of the EULAR recommendations for management of rheumatoid arthritis. *Ann. Rheum. Dis.* **73,** 529–535 (2014).
110. Leombruno, J. P., Einarson, T. R. & Keystone, E. C. The safety of anti-tumour necrosis factor treatments in rheumatoid arthritis: meta and exposure-adjusted pooled analyses of serious adverse events. *Ann. Rheum. Dis.* **68,** 1136–1145 (2009).

111. Jones, G. *et al.* Comparison of tocilizumab monotherapy versus methotrexate monotherapy in patients with moderate to severe rheumatoid arthritis: the AMBITION study. *Ann. Rheum. Dis.* **69,** 88–96 (2010).
112. Kaufmann, J., Feist, E., Roske, A. & Schmidt, W. A. Monotherapy with tocilizumab or TNF-alpha inhibitors in patients with rheumatoid arthritis: efficacy, treatment satisfaction and persistence in routine clinical practice. *Clin. Rheumatol.* **32,** 1347–1355 (2013).
113. Mertens, M. & Singh, J. Anakinra for rheumatoid arthritis. *Cochrane Database Syst. Rev.* CD005121 (2009). doi:10.1002/14651858.CD005121.pub3.www.cochranelibrary.com
114. Weinblatt, M. E. *et al.* Head-to-Head Comparison of Subcutaneous Abatacept Versus Adalimumab for Rheumatoid Arthritis. *Arthritis Rheum.* **65,** 28–38 (2013).
115. Caporali, R., Bugatti, S., Cavagna, L., Antivalle, M. & Sarzi-puttini, P. Modulating the co-stimulatory signal for T cell activation in rheumatoid arthritis: Could it be the first step of the treatment? *Autoimmun. Rev.* **13,** 49–53 (2014).
116. Atzeni, F. *et al.* Long-term safety of abatacept in patients with rheumatoid arthritis. *Autoimmun. Rev.* **12,** 1115–1117 (2013).
117. Khraishi, M., Russell, A. & Olszynski, W. P. Safety Profile of Abatacept in Rheumatoid Arthritis: A Review. *Clin. Ther.* **32,** 1855–1870 (2010).
118. Emery, P. *et al.* The Efficacy and Safety of Rituximab in Patients With Active Rheumatoid Arthritis Despite Methotrexate Treatment. *Arthritis Rheum.* **54,** 1390–1400 (2006).
119. Edwards, J., Szczepanski, L., Szechinski, J. & Filipowicz-Sosnowska, A. Efficacy of B-Cell–Targeted Therapy with Rituximab in Patients with Rheumatoid Arthritis. *N. Engl. J. Med.* **350,** 2572–2581 (2004).
120. Adlowitz, D. G. *et al.* Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response. *PLoS One* **10,** e0128269 (2015).
121. Reddy, V. *et al.* Internalization of Rituximab and the Efficiency of B Cell Depletion in Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Arthritis Rheumatol.* **67,** 2046–2055 (2015).
122. Sebastiani, M. *et al.* Efficacy and safety of rituximab with and without methotrexate in the treatment of rheumatoid arthritis patients: Results from the GISEA register. *Jt. Bone Spine* **81,** 508–512 (2014).
123. Tak, P. P. *et al.* Inhibition of joint damage and improved clinical outcomes with rituximab plus methotrexate in early active rheumatoid arthritis: the IMAGE trial. *Ann. Rheum. Dis.* **70,** 39–46 (2011).
124. Smolen, J. S. *et al.* EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. *Ann. Rheum. Dis.* **73,** 492–509 (2014).
125. Mohty, M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* **21,** 1387–94 (2007).
126. Popow, I. *et al.* A comprehensive and quantitative analysis of the major specificities in rabbit antithymocyte globulin preparations. *Am. J. Transplant.* **13,** 3103–3113 (2013).
127. Bonnefoy-Bérard, N., Vincent, C. & Revillard, J. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. *Transplantation* **51,** 669–73. (1991).

128. Pistillo, M., Tazzari, P., Bonifazi, F. & Bandini, G. Detection of a Novel Specificity (CTLA-4) in ATG/TGM Globulins and Sera from ATG-treated Leukemic Patients. *Transplantation* **73,** 1295–1302 (2002).
129. Rebellato, L., Gross, U., Verbanac, K. & Thomas, J. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. *Transplantation* **57,** 685–94 (1994).
130. Genestier, L. *et al.* Induction of Fas (Apo-1, CD95)-Mediated Apoptosis of Activated Lymphocytes by Polyclonal Antithymocyte Globulins. *Blood* **91,** 2360–2368 (1998).
131. Michallet, M. C. *et al.* Cathepsin-B-dependent apoptosis triggered by antithymocyte globulins: A novel mechanism of T-cell depletion. *Blood* **102,** 3719–3726 (2003).
132. Ippoliti, G. *et al.* Immunomodulation with rabbit anti-thymocyte globulin in solid organ transplantation. *World J. Transplant.* **5,** 261–266 (2015).
133. Socie, G. *et al.* Chronic graft-versus-host disease: long-term results from a randomized trial on graft-versus-host disease prophylaxis with or without anti–T-cell globulin. *Transpl. Int.* **117,** 6375–6383 (2016).
134. Storek, J., Mohty, M. & Boelens, J. J. Rabbit Anti-T Cell Globulin in Allogeneic Hematopoietic Cell Transplantation. *Biol. Blood Marrow Transplant.* **21,** 959–970 (2015).
135. Frickhofen, N. & Rosenfeld, S. Immunosuppressive treatment of aplastic anemia with antithymocyte globulin and cyclosporine. *Semin Hematol* **37,** 56–68 (2000).
136. Schmitt, W., Hagen, E., Neumann, I., Nowack, R. & Felipe, L. Treatment of refractory Wegener's granulomatosis with antithymocyte globulin (ATG): An open study in 15 patients. *Kidney Int.* **65,** 1440–1448 (2004).
137. Stratton, R. J., Wilson, H., Black, C. M., Hospital, R. F. & Nw, L. Pilot study of anti-thymocyte globulin plus mycophenolate mofetil in recent-onset diffuse scleroderma. *Rheumatology* **40,** 84–88 (2001).
138. Tarkowski, A., Andersson-Gäre, B. & Aurell, M. Use of Anti-thymocyte Globulin in the Management of Refractory Systemic Autoimmune Diseases. *Scand J Rheumatol* **22,** 261–266 (1993).
139. Tarkowski, A. & Lindgren, I. Beneficial effects of antithymocyte globulin in severe cases of progressive systemic sclerosis. *Transplant. Proc.* **26,** 3197–3199 (1994).
140. Duftner, C. *et al.* Apoptotic effects of antilymphocyte globulins on human pro-inflammatory CD4+CD28- T-cells. *PLoS One* **7,** 1–10 (2012).
141. Mohty, M. & Gaugler, B. Mechanisms of action of antithymocyte globulin: old dogs with new tricks! *Leuk. Lymphoma* **49,** 1664–1667 (2008).
142. Crepin, T. *et al.* ATG-induced accelerated immune senescence: Clinical implications in renal transplant recipients. *Am. J. Transplant.* **15,** 1028–1038 (2015).
143. Neumann, J. *Immunbiologie - Eine Einführung*. (Springer-Verlag Berlin Heidelberg, 2008).
144. Koetz, K., Bryl, E., Spickschen, K. & Fallon, W. M. O. T cell homeostasis in patients with rheumatoid arthritis. *PNAS* **97,** 9203–9208 (2000).
145. Dejaco, C., Duftner, C., Klauser, A. & Schirmer, M. Altered T-cell subtypes in spondyloarthritis, rheumatoid arthritis and polymyalgia rheumatica. *Rheumatol Int* **30,** 297–303 (2010).
146. Maly, K. & Schirmer, M. The story of CD4+CD28- T cells revisited: Solved or still ongoing? *J. Immunol. Res.* **2015,** (2015).

147. Fournel, S., Genestier, L., Robinet, E., Flacher, M. & Revillard, J. Human T cells require IL-2 but not G1/S transition to acquire susceptibility to Fas-mediated apoptosis. *J. Immunol.* **157,** 4309–4315 (1996).
148. Li, Q., Ke, N., Sundaram, R. & Wong-Staal, F. NR4A1, 2, 3 – an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. *Histol. Histopathol.* **21,** 533–540 (2006).
149. Andey, T., Patel, A., Jackson, T., Safe, S. & Singh, M. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methane compounds inhibit lung cancer cell and tumor growth in a metastasis model. *Eur. J. Pharm. Sci.* **50,** 227–241 (2013).