The Role of Regulatory T Cells and Interleukin-2 in the Pathogenesis and Treatment of Systemic Lupus Erythematosus

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List of Abbreviations

\( \gamma_c \) common gamma chain
Ab antibody
ADP adenosine diphosphate
AICD activation-induced cell death
AMP adenosine monophosphate
APC antigen presenting cell
ATP adenosine triphosphate
Blimp-1 B lymphocyte-induced maturation protein 1
BSA bovine serum albumin
BV Brilliant Violet™
CCL CC chemokine ligand
CCR chemokine (C-C motif) receptor
CD25hi Tcon CD4+Foxp3- Tcon with high CD25 expression levels
CD25hi Treg CD4+Foxp3+CD127lo Treg with high CD25 and Foxp3 expression levels
CD25int Treg CD4+Foxp3+CD127lo Treg with intermediate CD25 expression levels
CFSE carboxyfluorescein succinimidyl ester
CRE cAMP-response element
CREB CRE-binding protein
CREM CRE-modulator
CXCR chemokine (C-X-C motif) receptor
DN CD4-CD8- double negative
DRFZ German Rheumatism Research Center
dsDNA double-strand DNA
ELISA enzyme-linked immunosorbent assay
FACS fluorescence-activated cell sorting
FCS fetal calf serum
FCS forward scatter
FDA US Food and Drug Association
Foxp3 Forkhead Box protein P3
GM-CSF granulocyte-macrophage colony-stimulating factor
GvHD Graft-versus-host disease
HC healthy control
HCV hepatitis-C virus
HIV human immunodeficiency virus
IFN interferon
Ig immunoglobulin
IL interleukin
IL-2R IL-2 receptor
IQR inter-quartile range
IU international units
KIR killer cell Ig-like receptors
lo low expression levels
MACS magnetic-activated cell sorting
MFI median fluorescence intensity
MHC major histocompatibility complex
NK natural killer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NZB</td>
<td>New Zealand black mouse</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white mouse</td>
</tr>
<tr>
<td>p</td>
<td>phosphorylated</td>
</tr>
<tr>
<td>PB</td>
<td>pacific blue™</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Pe</td>
<td>phycoerythin</td>
</tr>
<tr>
<td>PI3</td>
<td>phosphoinositide-3</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RTE</td>
<td>recent thymic emigrants</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>SLE disease activity index</td>
</tr>
<tr>
<td>SSC</td>
<td>sideward scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T-bet</td>
<td>Th1-specific T box transcription factor</td>
</tr>
<tr>
<td>Tcm</td>
<td>central memory T cell (CCR7+CD45RO+)</td>
</tr>
<tr>
<td>Tcon</td>
<td>CD4+Foxp3- conventional T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tem</td>
<td>effector memory T cell (CCR7-CD45RO+)</td>
</tr>
<tr>
<td>Term</td>
<td>terminally differentiated T cell (CCR7-CD45RO-)</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>CD4+Foxp3+(CD127lo) regulatory T cells</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylation region</td>
</tr>
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CD4+Foxp3+ regulatory T cells (Treg) are essential mediators for the preservation of peripheral immunologic self-tolerance by limiting the activation and expansion of auto-reactive cells. The peripheral homeostatic maintenance of the Treg population is dependent on signals provided by the cytokine Interleukin-2 (IL-2). The chronic autoimmune disease systemic lupus erythematosus (SLE) has been associated with a deficient production of IL-2 and with abnormalities in the Treg population. However, a possible causal relationship between these two features and SLE pathogenesis has not been adequately investigated so far. Therefore, it was the aim of this thesis to examine whether IL-2 deficiency is causative for defects in the Treg population in SLE, and whether an IL-2-based therapy could be a possible approach to reverse such defects and to specifically target pathogenetic mechanisms in SLE.

Flow-cytometric analyses of peripheral blood mononuclear cells (PBMCs) from SLE patients and healthy individuals revealed that Treg from SLE patients exhibit reduced expression of the IL-2 receptor subunit CD25 at the cell surface. In addition, the homeostatic balance between Treg and CD4+Foxp3- conventional T cells is shifted in favor of an excessive proliferation of conventional T cells. These two features are known hallmarks of IL-2 deficiency, and the low CD25 expression in Treg from SLE patients was associated with a defective IL-2 production by CD4+ T cells in vitro in this study. Low levels of CD25+ Treg and the disturbed homeostatic balance between Treg and conventional T cells were also associated with an increased SLE disease activity, indicating that IL-2 deficiency might be of importance for disease pathogenesis in SLE. Further, high CD25 expression in Treg was associated with a high degree of proliferation and expression of molecules that are indicative for the activation and suppressive function of this subset. Consequently, the lack of Treg with high CD25 expression levels is linked to a deficit of functionally and metabolically active Treg in SLE.

Stimulation of PBMCs from SLE patients with IL-2 in vitro revealed that the CD25 expression in Treg could be restored by IL-2. In addition, Treg survival was selectively increased by IL-2 stimulation. Comparison with other lymphocyte subsets showed that low IL-2 concentrations specifically target the Treg population, while only marginally affecting conventional CD4+ T cells, CD8+ T cells, natural killer T cells or B cells in terms of proliferation, survival or CD25 expression. Only CD3-CD56+ natural killer (NK) cells, which also exhibit a disturbed homeostasis in SLE patients, showed a dose-dependent response to in vitro IL-2 stimulation in terms of increased proliferation.

Based on the results of these experiments, a clinical trial with low-dose IL-2 was implemented for the treatment of patients with refractory SLE. Low-dose IL-2 treatment of SLE patients caused a selective peripheral expansion of thymic-derived and suppressive Treg with strongly increased CD25 expression levels, and improved the homeostatic balance between Treg and conventional T cells. In parallel, an expansion of immature NK cells was observed under IL-2 therapy. These immunologic effects were accompanied by clinical remission in three of five SLE patients treated with low-dose IL-2 during the course of this study.

In summary, this work demonstrates the impact of IL-2 deficiency for Treg abnormalities and disease pathogenesis in SLE and proposes an IL-2-based therapy as a safe and efficient approach to re-establish endogenous mechanisms of tolerance in SLE.
Zusammenfassung


In vitro Experimente, in denen PBMCs von SLE Patienten mit IL-2 stimuliert wurden, zeigten, dass die CD25 Expression der Treg wieder hergestellt werden kann. Zusätzlich wurde durch Stimulation mit IL-2 auch das Überleben der Treg erhöht. Der Vergleich mit anderen Lymphozyten Populationen zeigte, dass vor allem niedrige IL-2 Konzentrationen selektiv auf die Treg Population wirken, während die Proliferation, das Überleben und die CD25 Expression der konventionellen CD4+ T Zellen, CD8+ T Zellen, B Zellen und natürlichen Killer T Zellen nur wenig beeinflusst wird. Nur CD3-CD56+ natürliche Killerzellen (NK Zellen), die ebenfalls eine gestörte Homöostase beim SLE aufweisen, zeigten einen dosisabhängigen Anstieg der Proliferation als Antwort auf die Stimulation mit IL-2 in vitro.

Zusammenfassung

Diese immunologische Effekte wurden von einer klinischen Remission in drei der fünf mit IL-2 behandelten SLE Patienten begleitet.
Zusammenfassend machen die Ergebnisse der vorliegenden Arbeit die Bedeutung des IL-2 Defizits für die Veränderungen in der Treg Population und die Pathogenese des SLE deutlich und zeigen, dass eine IL-2-basierte Therapie eine sichere und effiziente Möglichkeit ist, um die endogenen Toleranzmechanismen beim SLE wieder herzustellen.
1 Introduction

1.1 Cells of the mammalian immune system

Mammals have developed a sophisticated immune system, which protects their body from infections by pathogens including viruses, bacteria, parasites and fungi. The mechanisms of the mammalian immune system can be subdivided into innate and adaptive immunity. The innate immune system is specialized in the early detection and defense of pathogens. Mediators of this type of immunity are macrophages, dendritic cells, granulocytes and natural killer (NK) cells, which recognize pathogens via unspecific non-self structures. Their defense mechanisms include phagocytosis and/or killing of pathogens and infected cells through the release of cytotoxic molecules. In addition, they play an important role in antigen-presentation and instructing the cells of the adaptive immunity to appropriately respond to an infection.

Cells of the adaptive immune system, which include B and T lymphocytes, differ from the cells of the innate immunity in that they are highly specialized and recognize antigens via antigen-specific receptors. Somatic recombination and mutation of the gene segments encoding these receptors enables the generation of a great diversity of antigen-specific receptors. Both, T and B cells develop an immunologic memory, which allows for a rapid and more efficient response upon re-infection with the same pathogen [1].

B cells, which develop in the bone marrow, recognize specific antigens via their B cell receptor. Upon antigen recognition and appropriate activation in the germinal centers of secondary lymphoid organs B cells differentiate into either memory B cells or antibody-producing plasma blasts and plasma cells. Secreted antibodies (immunoglobulins, Ig) are the soluble form of the B cell receptor and specifically recognize the same antigen. They are part of the humoral immunity and contribute to pathogen defense by binding the antigen and thereby trigger several mechanisms of pathogen clearance [1].

In contrast to B cells and antibodies, T cells cannot recognize soluble antigens, but require the presentation of processed antigens on the major histocompatibility complex (MHC) of antigen presenting cells (APC) [2][3]. T cells that express a T cell receptor (TCR) associated with the glycoprotein CD3 and the co-receptor CD4 (CD4+ T cells) require antigen-presentation on MHC class II molecules. These are expressed on dendritic cells and B cells, which function as professional APCs [4], and present peptides that are mainly derived from extracellular proteins. CD4+ T cells exert their function by activating and modulating the differentiation of B cells and other immune cells primarily through the release of cytokines and are therefore called helper T (Th) cells [5].

Immunity against virus-infected, malignant or otherwise abnormally altered cells is conferred by cytotoxic cells. Cytotoxic CD8+ T cells are characterized by TCR expression in association with CD3 and the co-receptor CD8. They recognize foreign peptides that are presented on MHC
class I molecules, which are expressed by nearly all nucleated cells and present peptides derived predominantly from cytosolic proteins. Upon stimulation CD8+ T cells are able to kill the infected cells through the release of cytolytic molecules, such as granzymes and perforin, or by surface receptor-mediated induction of apoptosis [6, 7]. In addition, they modulate immune responses through the secretion of cytokines [8].

NK cells, defined by the expression of CD56 and lack of CD3, represent another group of cytotoxic cells [9]. Their activation is regulated by the balance of inhibitory and activating receptors, including killer cell Ig-like receptors (KIRs) and C-type lectin receptors (NKG2), which interact with MHC class I molecules, and natural cytotoxicity receptors [10–12]. NK cell cytotoxicity is conferred via cytolytic molecules, such as perforin and granzymes, and receptor-mediated induction of apoptosis [13]. A subset of NK cells, associated with a less mature phenotype and expression of high levels of CD56 (CD56bright NK cells) are less cytotoxic compared to the CD56dim counterpart. Upon stimulation, CD56bright NK cells produce high amounts of cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-10, which are involved in the modulation of T and B cell mediated immune responses [14–16].

1.2 CD4+ T helper cells

1.2.1 Activation and memory differentiation of T helper cells

In addition to the recognition of their cognate antigen via the TCR, naïve CD4+ T cells require a second stimulatory signal through co-stimulatory receptors, such as CD28, for their primary activation [17]. Upon engagement of the TCR and CD28, naïve T cells produce high amounts of the cytokine IL-2, undergo clonal expansion and differentiate into specialized effector T cells. After resolution of an infection, most of the activated T cells undergo apoptosis while a subpopulation survives as memory T cells [18]. Re-activation of these antigen-primed memory T cells requires lower levels of antigen and co-stimulation, allowing for a rapid response against secondary infections with the same antigen. Naïve and memory T cells can be phenotypically distinguished according to the differential expression of the lymph-node homing chemokine receptor CCR7 and different isoforms of CD45. Naïve CD4+ T cells express high levels of CCR7 and CD45RA, but no CD45RO. In contrast, antigen-experienced CD4+ T cells express CD45RO, but lack CD45RA and can be subdivided into CCR7 positive central memory (Tcm) and CCR7 negative effector memory (Tem) populations [19, 20]. Accordingly, CD4+ CD45RO+CCR7+ Tcm travel to the T cell zone of secondary lymphoid organs. Upon secondary antigen challenge they are highly proliferative, stimulate dendritic cells and B cells and differentiate into effector cells. On the other hand, CD4+ CD45RO+CCR7- Tem provide immediate effector functions through cytokine secretion in the peripheral tissues upon secondary antigen challenge [21]. A third class of memory T cells, which express neither CCR7 nor CD45RO, but re-express CD45RA are described as terminally differentiated effector memory cells (Term, or TEMRA) [19, 22, 23]. These cells represent only a low percentage of CD4+ T cells in the peripheral blood of healthy individuals, but are increased in situations of chronic infections and inflammation [24, 25]. CD4+ Term cells...
are associated with chronic antigen-exposure, loss of co-stimulatory receptor expression and independence of TCR and co-stimulation, while they retain the ability to secrete IFN-γ [23, 26].

1.2.2 Differentiation of T helper cell subsets

Depending on the type of infecting pathogen and the corresponding cytokines secreted by the cells of the innate immune system, CD4+ Th cells acquire different effector functions and can be further subdivided into Th1, Th2 and Th17 differentiated effector T cells [5]. Th1 cell differentiation requires the cytokines IL-12 and IFN-γ. These cells are characterized by the expression of the master transcription factor T-bet and production of IFN-γ, which stimulates macrophages and a variety other immune mechanisms and mediates protection against intracellular pathogens [27–29]. Th1 polarized cells preferentially express the chemokine receptors CXCR3 and CCR5 [30], which guide them to the site of inflammation through binding to their ligands.

IL-4 drives the differentiation of Th2 cells, which express the transcription factor GATA-3 and produce the cytokines IL-4, IL-5, IL-6, IL-10, IL-13 as well as transforming growth factor (TGF)-β [29, 31]. Th2 cells mediate humoral immunity against extracellular parasites through triggering of eosinophils, basophils, mast cells and B cells, and are involved in allergic responses. Further, Th2 cells preferentially express the chemokine receptor CCR4, which is associated with skin-homing properties through interaction with chemokine ligands CCL17 and CCL22 [30, 32]. Th17 cells represent a third subclass of Th cells. These cells differentiate in response to cytokines such as IL-6, TGF-β, IL-23 and IL-21 and express the transcription factor RORγt [33–35]. Th17 cells produce IL-17, IL-6, IL-22 and TNF-α and are involved in the protection against extracellular bacteria and fungi and are mainly found at mucosal sites. Th17 cells are characterized by the expression of chemokine receptor CCR6, which mediates migration to inflammatory sites through interaction with CCL20 [36].

Another subset of Th cells is found in the B cell follicles of secondary lymphoid organs. These follicular helper T (Tfh) cells express the transcription factor Bcl-6, the co-stimulatory molecule ICOS, and the B cell follicle-homing receptor CXCR5 [37, 38]. Tfh cells are important for the formation of germinal centers, where they interact with the cognate follicular B cells and drive their selection and differentiation into antibody-producing memory B cells and plasma cells [39].

1.3 Immunologic tolerance and autoimmunity

1.3.1 Central and peripheral tolerance

The highly specialized and efficient system, which protects the human body against infectious agents and cancer, also bears the risk of unwanted immune reactions against harmless environmental antigens (e.g. allergens or commensal bacteria), or immune reactivity against self-antigens (autoimmunity). Therefore, a tight regulation of the immune system is necessary to maintain the balance between immune activation and self-tolerance. Dysregulation of this balance leads to pathogenic immune activation and autoimmunity with an activation of self-reactive B and T cells and production of autoantibodies. The consequence of such aberrant self-reactive immunity is severe tissue damage due immune complex deposition, antibo-
mediated cell destruction, cytokine-mediated inflammation, or alteration of cell functions due to functional receptor-directed antibodies. To avoid such reactions, the immune system has developed two main mechanisms of immunologic self-tolerance.

Central tolerance is achieved by deleting self-reactive lymphocytes before they develop into fully immunocompetent cells. During the so-called negative selection process in the thymus and bone marrow for T and B cells, respectively, maturing lymphocytes are exposed to self-antigens and those lymphocytes that recognize these antigens with high affinity are either depleted by apoptosis or enter an anergic state [40, 41]. Although this process is very efficient, some self-reactive T cells can escape the negative selection and mature into competent lymphocytes.

Therefore, also peripheral mechanisms are necessary to maintain self-tolerance. Peripheral tolerance is attained through several overlapping means including depletion, anergy or self-ignorance as well as suppression of self-reactive T cells through immunosuppressive cells [42]. Imunosuppressive functions have been asserted to a variety of cells including subsets of T cells, NK cells, B cells and dendritic cells [43–48]. CD4+ regulatory T cells (Treg), expressing the lineage marker Foxp3, represent a crucial population of these suppressive cells, and are essential for the preservation of peripheral tolerance [49].

### 1.3.2 CD4+ Foxp3+ regulatory T cells

CD4+ regulatory T cells were first identified as cells with high surface expression levels of the IL-2 receptor \(\alpha\)-chain, CD25 [49]. These CD4+CD25+ T cells can suppress proliferation and effector functions of other T cells after antigen-specific or polyclonal activation in vitro and inhibit autoimmunity in vivo [49, 50]. In contrast, depletion of CD4+CD25+ T cells in mice causes the development of severe autoimmune syndromes [49, 51]. Also human CD4+CD25+ T cells were shown to suppress T cell proliferation and cytokine production in vitro [52–54]. In 2003, the Forkhead Box protein P3 (Foxp3) was identified as a master transcription factor for the development and suppressive function of CD4+CD25+ regulatory T cells [55, 56]. A deleting mutation in the Foxp3 gene causes a lack of CD4+Foxp3+ Treg and the development of severe autoimmune disease in the ‘scurfy’ mouse model [57, 58]. Similarly, in humans the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) is associated with deleterious mutations in the Foxp3 gene [59, 60]. In addition, numeric or functional defects of the CD4+Foxp3+ Treg population have been associated with a variety of autoimmune-related diseases [61].

In healthy individuals CD4+Foxp3+ Treg represent approximately 5-10% of the circulating CD4+ T cell population. The majority of CD4+Foxp3+ Treg develop in the thymus from T cells with TCRs of intermediate affinity to foreign and also self antigens, which escaped the negative selection process [62, 63]. In mice, Foxp3-expressing regulatory T cells develop also in the periphery from naïve T cells as a mechanism to provide tolerance against neoantigens, allergens or commensal microbiota [64, 65]. Such peripheral induction of Foxp3+ Treg from naïve T cells has been proposed to take place also in man [54, 66]. In addition, TGF-β signaling together with TCR stimulation can induce the expression of Foxp3 and the generation of suppressive Treg from murine naïve CD4+ T cells in vitro [67]. However, data about the suppressive ca-
pacity and stability of human in vitro-induced CD4+Foxp3+ T cells are controversial [68–70]. Moreover, non-suppressive, effector cytokine-producing CD4+ T cells that transiently express Foxp3 upon activation have been identified in human peripheral blood [71]. Thus, besides the thymic-derived, suppressive bona fide CD4+Foxp3+ Treg population, peripherally-induced Foxp3 expressing cells with unclear suppressive function and stability exist in humans. While the exact functional role of these different Foxp3+ T cell subsets is not clear, some effort has been invested to identify phenotypic markers that can distinguish genuine CD4+Foxp3+ Treg from peripherally-induced Treg or from effector T cells with induced Foxp3 expression in humans.

Treg suppressive function is linked to high surface expression levels of CD25 [53]. However, also not all CD4+CD25+ T cells can be allocated to the Treg population, since also conventional CD4+Foxp3- T cells (Tcon) express CD25 at intermediate levels [72] and transiently upregulate CD25 expression upon activation [73]. The expression of the IL-7 receptor, CD127, has been shown to negatively correlate with suppressive function and Foxp3 expression of Treg [74, 75] and is therefore used in combination with Foxp3 and/or CD25 to identify bona-fide Treg. In addition, the expression of the Ikaros-family transcription factor Helios has been associated with thymic origin of CD4+Foxp3+ Treg and lack of effector cytokine production, and may thus help to distinguish between the different subsets of Foxp3 expressing cells [76]. Finally, epigenetic demethylation of CpG islands in the Foxp3 conserved non-coding region 2 (Treg-specific demethylation region or TSDR) is described to reflect stable, constitutive Foxp3 expression and is a hallmark feature of suppressive, thymic-derived bona fide Treg [77]. In contrast, cells with induced Foxp3 expression and those co-expressing effector cytokines and/or lack the expression of Helios show partial methylation of the TSDR [79–81].

1.3.3 Treg mechanisms of action

Multiple mechanisms of CD4+Foxp3+ Treg-mediated immunosuppression have been identified [82, 83]. One strategy is the delivery of negative signals to responder cells through (i) the induction of cyclic adenosine monophosphate (AMP) levels in the responder cells [84]; (ii) hydrolysis of extracellular ATP to ADP and AMP by the ectonucleases CD39 and CD73 [85, 86]; (iii) CTLA-4-dependent interaction with CD28 - B7 co-stimulatory signaling in responder cells [87]; (iv) secretion of immunoregulatory cytokines IL-10, TGF-β1 and IL-35 [88–90]; and (v) cell-contact dependent inhibition of proliferation through membrane-bound TGF-β signaling [91]. Moreover, Treg modify the function of APCs through signaling via the co-stimulatory molecule CTLA-4 and thereby inhibit APC-dependent T cell activation [92], or they inhibit Tcon expansion through cytokine-deprivation [93, 94]. In addition, Treg can directly mediate target-cell lysis through the release of cytolytic molecules, such as perforin and granzyme [95, 96]. CD4+Foxp3+ Treg can, similarly to their CD4+Foxp3- Tcon counterpart, be phenotypically sub-divided into naïve, central memory and effector memory cells [97–99]. The effector memory phenotype has been associated with enhanced suppressive function [98, 100]. Further, Treg express chemokine receptors and transcription factors that are associated with CD4+ Th subset differentiation and are thus believed to specifically suppress the corresponding effector T cells at the site of inflammation [101, 102]. In line with this, about 10% of total dermal T cells
are CD4+Foxp3+ Treg and express high levels of the skin-homing chemokine receptors CCR6, CCR4 and cutaneous leukocyte-associated antigen [103].

1.4 Interleukin-2

1.4.1 Dual role of Interleukin-2 in immune activation and tolerance

The 15kDa α-helical cytokine IL-2 was originally identified as a T cell growth factor that is crucial for the induction of T cell expansion upon antigen-dependent activation [104, 105]. In T cells, activation through the TCR and appropriate co-stimulation via CD28 leads to production of IL-2 and concomitant up-regulation of high-affinity IL-2 receptor (IL-2R) expression [106]. IL-2–IL-2R interaction in turn drives the expansion and differentiation of effector T cells [107]. Conversely, experiments with mice deficient for IL-2 or one of its receptor subunits did not cause immunodeficiency but resulted in severe lymphoproliferative and autoimmune disorders [108–110]. Later studies showed that autoimmunity in IL-2-deficient mice was caused by hyperactivity of cells of the adaptive immune system due to the lack of CD4+Foxp3+ Treg [111, 112]. Thus, IL-2 is in fact essential for the survival and suppressive function of CD4+Foxp3+ Treg and is therefore a fundamental player in the maintenance of peripheral tolerance and prevention of autoimmunity [113].

1.4.2 IL-2 receptor signaling

The IL-2R consists of three subunits, IL-2Rα (CD25), IL-2Rβ (CD122), and the common gamma chain (γc, CD132). CD122 together with the γc subunit forms an intermediate-affinity IL-2R, which is constitutively expressed on NK cells, natural killer T cells (NKT cells) and memory CD8+ T cells [114–116]. The third subunit, CD25, is necessary to form a high-affinity IL-2R complex [117–119]. This high affinity IL-2R (γc, CD122 and CD25) is constitutively expressed by CD4+Foxp3+ Treg and transiently by activated conventional CD4+ and CD8+ T cells [107]. In addition, its expression has been shown on subsets of B cells, NK cells, NKT cells and also on dendritic cells, fibroblasts and endothelial cells [106, 107]. The formation of the IL-2–IL-2R complex leads to intracellular signal transduction, activating the mitogen-activated protein kinase and phosphoinositide-3 (PI3) kinase pathways, phosphorylation of the signal transducer and activator of transcription 5 (STAT5) and STAT5-dependent gene regulation [120]. While STAT5 is similarly activated after IL-2 binding in all T cell subsets, the PI3 kinase pathway is differentially activated in CD4+ and CD8+ T cells and is not induced in CD4+Foxp3+ Treg due to high expression of the phosphatase PTEN in these cells [113]. Consequently, IL-2 signaling has context-dependent functions depending on the respective responsive cell type.

In general, IL-2-mediated signaling regulates the expression of genes required for cell cycle progression and survival, promoting proliferation and activation of the responsive cell subset. In T cells, IL-2 signaling induces CD25 expression through a STAT5-dependent positive feedback loop, allowing for increased IL-2 binding and signaling [107]. At the same time IL-2 signaling inhibits its own production in a negative feedback loop through the induction of the transcription repressor B lymphocyte-induced maturation protein 1 (Blimp-1) [121–123].
In Treg, IL-2 signaling also induces the expression of Foxp3 \cite{111}, underscoring the importance of IL-2 as the critical cytokine for Treg development, homeostasis and stability. Accordingly, abrogation of IL-2 signaling in mice deficient for IL-2 or IL-2R components leads to a substantially reduced number of CD4+Foxp3+ Treg \cite{111}. The remaining CD4+Foxp3+ Treg in these animals are characterized by low CD25 expression levels as well as an impaired proliferative capacity, resulting in a disturbed homeostatic maintenance of the Treg population. Further studies have demonstrated that IL-2 signaling is also involved in the development and the suppressive function of CD4+Foxp3+ Treg \cite{93, 124, 125}.

In CD8+ T cell responses, suboptimal IL-2 signaling results in impaired CD8+ T cell expansion during primary and secondary antigen encounter and affects the memory formation of activated CD8+ T cells \cite{126}. In NK cells IL-2 stimulation induces proliferation, as well as the expression of cytokines and cytolytic molecules without additional stimulation \cite{114, 127, 128}. In summary, IL-2 signaling has pleiotropic effects on a variety of immune cells, depending on the duration and available concentration of IL-2 and the expression levels of high- or intermediate-affinity IL-2R complexes on the respective cell subset.

1.4.3 IL-2 production and availability in vivo

IL-2 is produced predominantly by activated conventional CD4+ T cells and to a lesser extent by CD8+ T cells, NK cells and NKT cells \cite{112, 129}. In addition, activated dendritic cells and mast cells have been found to express IL-2 under certain conditions \cite{130, 131}. The induction of IL-2 expression in T cells depends on TCR engagement and co-stimulation, resulting in NF-κB pathway activation and transient induction of transcription of the IL-2 gene \cite{107}. In addition to enhancing IL-2 transcription in synergy with TCR stimulation \cite{132}, co-stimulatory signaling via CD28 also leads to IL-2 mRNA stabilization in T cells \cite{133}. IL-2 transcription is repressed by the transcription factors T-bet and Blimp-1 \cite{123, 134}, the latter being especially up-regulated in chronically activated T cells \cite{135}. Thus, activated naïve T cells represent the main source of IL-2 \cite{129}. In contrast, chronically activated and terminally differentiated T cells lose the ability to produce IL-2.

In addition, IL-2 expression is repressed by Foxp3 \cite{136, 137}. Therefore, while IL-2 is essential for the homeostatic maintenance of CD4+Foxp3+ Treg, these cells are highly dependent on the availability of exogenous IL-2. IL-2 has a short in vivo half-life and is used by cells in close proximity in an autocrine and paracrine manner \cite{138}. The essential dependency of CD4+Foxp3+ Treg on IL-2 availability, indicates that, besides activation-induced IL-2 production, a homeostatic production of IL-2 is likely to be taking place in secondary lymphoid organs and the periphery in order to maintain peripheral Treg homeostasis. In fact, IL-2-producing cells of T cell origin are found independently of direct immunologic stimulation in the thymus, the epidermis of the skin and in the intestine of embryonic and neonatal mice \cite{139}. And a resident population of IL-2-producing T cells, expressing an effector memory phenotype, is present in murine peripheral lymphoid tissues \cite{140}. It remains however to be determined what factors drive such potential spontaneous IL-2 production.
1.4.4 Therapeutic use of IL-2

Due to its immuno-stimulatory functions, IL-2 has been implemented for the treatment of malignancies. High-dose IL-2 therapy with intravenous bolus administration of 0.6 million international units (IU)/kg over 15 minutes every 8 hours for a maximum of 14 doses per cycle (FDA), or continuous infusion of 18 million IU/m2/day for two cycles of 4-5 days (Europe) is approved for the treatment of renal cell carcinoma and metastatic melanoma [141]. High-dose IL-2 treatment induces a response-rate of only 15-20%, but leads to durable regression of disease in the responsive patients [141, 142]. However, it is also associated with significant toxicity including vascular capillary leak syndrome and infections [143]. The mechanism of action of IL-2 in the responders is not completely understood, however, the beneficial effects are linked to an expansion and activation of cytotoxic CD8+ T cells and NK cells which help to eliminate tumor cells. High-dose IL-2 therapy has also been used to boost CD4+ T cell-mediated immunity in HIV patients. Here, IL-2 caused an expansion of CD4+ naïve and memory T cells, however the clinical effects were limited [144].

With the discovery of the high dependence of CD4+Foxp3+ Treg homeostasis on IL-2 signaling, it became apparent that IL-2 treatment in fact causes an expansion of CD4+Foxp3+ Treg, which might contribute to the limited beneficial effects of high-dose IL-2 therapy in the majority of treated patients [145, 146]. At the same time these findings implicated a potentially beneficial effect of IL-2 for the treatment of diseases caused by chronic immune activation, including autoimmunity, where an expansion of the Treg population could be advantageous.

As described above, CD4+Foxp3+Treg constitutively express the high-affinity IL-2 receptor, consisting of γc, CD122 and CD25. Therefore, lower IL-2 concentrations are presumably sufficient to stimulate Treg, in comparison to CD4+ T cells, CD8+ T cells and NK cells that express the intermediate-affinity IL-2 receptor (γc and CD122). However, the approach to use IL-2 as a therapeutic agent to treat autoimmune diseases or diseases caused by undesirable chronic immune responses has been treated restrainedly for some time, due to the risk of stimulating immune effector cells and potentially exacerbating disease. Nonetheless, pre-clinical investigations in mice and non-human primates revealed that treatment with low doses of IL-2 was able to expand the CD4+Foxp3+ Treg population without stimulating an effector T cell response [147–149]. Further studies in autoimmune mouse models also demonstrated that IL-2 had beneficial effects in the treatment of diabetes, experimental autoimmune encephalomyelitis, myasthenia gravis and lupus [147, 148, 150–152].

In 2011, two clinical studies demonstrated the successful implementation of a low-dose IL-2 therapy for the treatment of patients with graft-versus-host-disease (GvHD) [153] and Hepatitis C virus (HCV)-induced vasculitis [154]. Both studies, using different treatment regimens but similarly low doses of 0.3-3.0 million IU IL-2 per m² body surface area per day, or 1.3 - 3.0 million IU IL-2 per patient per day, respectively, showed amelioration of disease manifestations and in parallel a significant expansion of the CD4+Foxp3+ Treg population. Further, in these studies low-dose IL-2 treatment was accompanied by only mild and transient adverse events and no evidence for effector T cell activation was observed. Together, these two studies demonstrated the safety and efficacy of low-dose IL-2 treatment for the expansion of CD4+Foxp3+ Treg in diseases associated with chronic (auto-) immune activation. Accordingly, they paved the way
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for the initiation of several clinical studies investigating the potential of low-dose IL-2 for the treatment of autoimmune diseases [155–158].

1.5 Systemic Lupus Erythematosus

1.5.1 General aspects of systemic lupus erythematosus

As described above, several mechanisms exist that are responsible for the maintenance of immune tolerance to self-antigens. If one or more of these mechanisms fail, loss of self-tolerance and uncontrolled immune activation leads to the development of autoimmune diseases. Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease, which is characterized by the loss of self-tolerance predominantly towards nuclear antigens. SLE is a chronically flaring disease with a prevalence ranging from 20 to 150 cases per 100,000 persons, depending on ethnicity and geographic location, and affects mainly young females (9 to 1 female to male ratio) [159, 160]. The disease etiology is not fully understood, but multiple factors including genetic, hormonal and environmental aspects appear to be involved in the development of SLE [161, 162]. Also the clinical manifestations of SLE are diverse, ranging from skin and joint involvement to severe disease activity affecting vital organs such as the kidneys, lungs, heart or the central nervous system. The most common clinical symptoms include fever, arthritis, cutaneous lesions, polyserositis, lupus nephritis and hematologic abnormalities, including leucopenia and thrombocytopenia, and are accompanied by characteristic serological abnormalities, including high levels of anti-nuclear autoantibodies and low complement levels [162]. Several scoring systems have been established, which take these common manifestations into account in order to assess disease severity and changes of disease activity in individual SLE patients [163]. One of these indices is the modified SLE disease activity index (SELENA-SLEDAI), which is used as a measure for SLE disease activity also in the present study [164].

1.5.2 Immunologic characteristics of SLE

The pathogenesis of SLE cannot be explained by a single mechanism, but involves the dysregulation of diverse aspects of the innate and adaptive immune system.

Dysregulated innate immunity

SLE is associated with a high rate of necrosis and apoptosis and with defective clearance of apoptotic material by phagocytes, resulting in the abnormal release of nuclear self-antigens [165]. The normally tolerogenic presentation of such self-antigens by dendritic cells, macrophages and monocytes, is disturbed in SLE. The release of DNA- or RNA-associated proteins activates Toll-like receptor (TLR) signaling and activation of dendritic cells to release pro-inflammatory IFN-α [166]. In addition, external factors like UV-irradiation or proteolytic cleavage can cause the modification of auto-antigens to neo-antigens, and molecular mimicry of microorganisms results in the activation of innate cells and consequent presentation of self-antigens as foreign. In summary, these mechanisms lead to a shift from tolerogenic to immunogenic antigen presentation and cytokine secretion and consequent activation of cells of the adaptive immune response [167].
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The role of B cells and autoantibodies in the pathogenesis of SLE

A major role in the pathogenesis of SLE is attributed to auto-reactive B and plasma cells, which produce auto-antibodies directed against a variety of mainly nuclear antigens. These predominantly include antibodies against double-strand DNA (anti-dsDNA Ab) and the ribonuclear protein SmD1 which are highly specific for SLE and are associated with disease activity [168-170]. These autoantibodies appear to be pathogenic since the deposition of immune complexes in tissues such as the kidneys and the skin causes inflammation and consequently tissue destruction [171]. Although the exact pathogenic mechanism is not clear, several factors, which most likely work in concert, may explain the loss of tolerance and the activation of autoreactive B cells and respective autoantibody production.

As described above, defects in apoptotic cell clearance and activation of the innate immune system lead to the immunogenic presentation of autoantigens and subsequent activation of T and B cells. In addition, B cells themselves are activated through TLR signaling. While mechanisms of tolerance prevent an immune reaction against self-antigens in healthy individuals, hyper-activity of B cells allows for the escape from tolerogenic checkpoints in SLE and leads to pathogenic immune activation [172]. Such B cell hyper-activity is attributed to intrinsic molecular abnormalities, causing for example a lower activation threshold or lower susceptibility to apoptosis, and to increased help by auto-reactive CD4+ T cells [173, 174]. Once autoantibodies are present, they also trigger a self-perpetuating cycle of immune activation through the activation of complement-induced cell lysis and formation of immune complexes.

Contribution of CD4+ T cells to SLE development

Besides auto-reactivity of B cells and the presence of autoantibodies, abnormalities in the T cell compartment play a major role in the pathogenesis of SLE. Experiments with T cell-deficient mouse models or T cell depletion have demonstrated the importance of CD4+ T cells for the development of lupus [175, 176]. Further, CD4+ T cells have been shown to be present in cellular infiltrates of inflamed kidneys and in the urine of SLE patients with renal involvement [177-179]. Furthermore, circulating T cells reactive to the SLE-specific autoantigen SmD1 have been detected in SLE patients and lupus mouse models [180, 181]. In addition, increased numbers of circulating Tfh cells were associated with SLE [182]. In healthy individuals, autoreactive T cells are prone for anergy or apoptosis. However, aberrations in intrinsic signal transduction, a high prevalence of external stimulatory factors, and insufficient silencing through immunosuppressive mechanisms allows for their activation and persistence in SLE [183]. This aberrant activation of CD4+ T cells and the consequent production of pro-inflammatory cytokines leads to dysregulation of the immune system by initiating and enhancing the activation of autoreactive B cells and macrophages, and also directly promotes tissue inflammation.

Unclear impact of cytotoxic cells for SLE pathogenesis

Apart from B cells and CD4+ T cells, CD8+ T cells have also been detected in cellular infiltrates in SLE kidney biopsies, suggesting that they are also involved in tissue injury [184]. Conversely, other studies suggest that an impaired cytolytic activity of CD8+ T cells could be a risk factor for
SLE pathogenesis due to an impaired lysis of hyper-reactive B cells [185]. Similarly, numerical deficits of NK cells and an impaired cytotoxic response have been associated with SLE disease activity [186, 187]. However, the contribution of these cell subsets to the pathogenesis of SLE has not been conclusively elucidated [187, 188].

CD4+Foxp3+ Treg in SLE

The CD4+Foxp3+ Treg population is essential for the maintenance of self-tolerance and the prevention of autoimmunity. A number of studies have therefore analyzed aspects of Treg biology in SLE. Most investigators have found reduced numbers or frequencies of circulating Treg in SLE patients, while others reported normal or increased levels of Treg or Treg subsets (reviewed in [61]). Similarly, associations of Treg numbers with disease activity were inconsistent [189–193], and investigations of the suppressive function of Treg from SLE patients have also provided conflicting results [189, 190, 194]. Interestingly, studies defining CD4+ Treg on the basis of CD25 expression alone or in combination with Foxp3 detected lower Treg levels, while those using expression of Foxp3 alone as the Treg-defining marker mostly observed normal or increased numbers of Treg in SLE [195]. Thus, a major reason for the controversies concerning Treg in SLE lies most likely in their imprecise phenotypic designation. Nonetheless, despite the conflicting results concerning qualitative and quantitative characteristics of Treg, the mentioned studies provide increasing evidence suggesting that abnormalities in the CD4+Foxp3+ Treg compartment may play a role in the pathogenesis of SLE. However, the origins of such Treg defects are poorly understood.

Investigations from the group of G. Riemekasten and others have demonstrated a low prevalence of Treg in mouse models that develop lupus-like disease [148, 196]. In addition, experimental depletion of CD4+CD25+ Treg in such animals results in acceleration of disease with expansion of autoreactive T cells, production of anti-dsDNA autoantibodies and organ inflammation [148, 197]. In contrast, increasing the pool size of peripheral Treg through adoptive transfer of isolated CD4+CD25+ Treg delays disease progression [148, 197, 198] and can prolong disease remission induced by cyclophosphamide treatment in lupus-prone mice [199].

IL-2 and SLE

Recent analyses demonstrated that an acquired deficiency of IL-2 is responsible for defects in the Treg biology of lupus-prone (NZBxNZW)F1 mice and thus contributes significantly to disease development [148]. In parallel to a loss of IL-2-producing cells, Treg from these lupus-prone mice acquire a phenotype that is very similar to that of IL-2 knock-out mice [111]. Accordingly, CD4+Foxp3+ Treg from diseased mice have a significantly reduced CD25 expression, but increased percentages of CD69+ and CD44+ activated cells [148]. Moreover, analysis of cell proliferation revealed a progressive homeostatic imbalance between CD4+Foxp3+ Treg and CD4+Foxp3- Tcon resulting in uncontrolled Tcon proliferation and activation [148]. In vivo neutralization of IL-2 in young, clinically healthy (NZBxNZW)F1 mice accelerated the occurrence of these phenotypic characteristics and the development of disease. In contrast, treatment of (NZBxNZW)F1 mice with recombinant IL-2 expanded the CD4+Foxp3+ Treg population and strongly increased their CD25 expression levels as well as their proliferation. In parallel, IL-2
treatment impeded disease progression and prolonged the survival of diseased (NZBxNZW)F1 mice [148]. These findings were in accordance with early studies describing a deficiency of IL-2 production in lupus-prone mouse strains [200–202] and provide a causal relationship between IL-2 deficiency, Treg abnormalities and disease pathogenesis in murine lupus.

A defect in IL-2 production has also been described for human SLE. Already in the early 1980ies it was shown by J. Alcocer-Varela and M. Linker-Israeli and colleagues that supernatants of polyclonally stimulated T cells or peripheral blood mononuclear cells (PBMCs) from SLE patients were unable to stimulate IL-2-dependent proliferation in responder cells, indicating a lack of IL-2 production [203, 204]. More recently, the IL-2 defect in SLE has been attributed signaling abnormalities in CD4+ T cells. A dysbalance between the transcription-activating phosphorylated CRE-binding protein (pCREB) and the repressing CRE-modulator CREMα has been proposed to be responsible for diminished IL-2 expression in CD4+ T cells from SLE patients [205].

While the IL-2 deficiency in lupus-prone mice was clearly linked to an impaired Treg homeostasis and lupus pathogenesis [148], the impact of IL-2 deficiency for human SLE pathogenesis is less well defined and several IL-2-dependent mechanisms contributing to SLE pathogenesis are being debated [206]:

(i) IL-2 deficiency could be responsible for defects in Treg function and homeostasis also in human SLE and thus contribute to an imbalance between immune-activating and regulatory mechanisms.  
(ii) IL-2 limits the production of IL-17 and low levels of IL-2 might thus favor the occurrence of highly pro-inflammatory Th17 cells in SLE [207, 208].  
(iii) IL-2 is involved in activation-induced cell death (AICD), a controlled apoptotic mechanism, which is proposed to be disturbed in SLE and might be responsible for insufficient regulation of excessive T cell activation in SLE [209, 210].  
(iv) IL-2 is involved in the development of CD8+ T cell and NK cell cytotoxicity [211, 212] and a lack of IL-2 might therefore be responsible for the observed defects in cytotoxic activity of these cells in SLE.

In summary, there is growing evidence that IL-2 deficiency might impact various mechanisms of immunoregulation in SLE, and thus presents a potential therapeutic target for the treatment of SLE.

Together, the available immunological data from SLE patients and animal models suggests a model in which intrinsic and extrinsic factors cause a hyper-activity of T and B cells on the one side and a deficiency of regulatory mechanisms on the other side, ultimately resulting in an imbalance between immune-activation and immune-regulation (Fig. [1]). Since both defects potentiate each other, they result in a vicious cycle of self-perpetuating immune activation and loss of self-tolerance in SLE.

1.5.3 Therapeutic strategies for the treatment of SLE

Current SLE treatment is mainly aimed at controlling symptoms and is largely based on nonsteroidal anti-inflammatory drugs, glucocorticoids and non-selective immunosuppression. Such agents include antimetabolites, like azathioprine and methotrexate, as well as cytostatic agents,
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Plasma cell, Treg, CD8+ T cell, NK cell, environmental triggers, genetic predisposition, defective clearance, APC, pro-inflammatory cytokines, CD4+ T cell, autoreactive B cell, IL-2, Treg, NK cell, CD8+ T cell, apoptosis, necrosis, tissue injury + inflammation, immune complex formation, autoantibody production, defective activation of regulatory mechanisms?

Figure 1: Simplified schematic overview of immunologic factors contributing to SLE pathogenesis. A high degree of apoptosis and necrosis, as well as defective clearance of intracellular material leads to activation of innate immune cells, immunogenic presentation of autoantigens by antigen presenting cells (APC), and activation of autoreactive T and B cells. Secretion of pro-inflammatory cytokines results in further activation of these immune processes. Abberant differentiation of autoreactive B cells into plasma cells results in the production of autoantibodies, which through the formation of immune complexes lead to tissue injury and amplification of inflammatory processes. The role of NK cells and CD8+ T cells in the inflammatory process is still ambiguous. Tolerogenic and immunoregulatory mechanisms, such as Foxp3+ Treg-mediated suppression are possibly defective and thus contribute the abberant activation of autoreactive T and B cells. Deficient IL-2 production may add to the imbalance between immuno-regulating and -activating mechanisms. Both, genetic predisposition and environmental factors, may trigger or contribute to the described processes, which result in a vicious cycle of self-perpetuating immune activation.

such as cyclophosphamide, and immunosuppressant drugs, including mycophenolate mofetil. With these drugs the management of lupus has improved dramatically over the past 50 years with a 10-year survival rate of 90% today [162]. Nonetheless, most of these drugs do not specifically interfere with the pathophysiologic mechanisms in SLE but generally suppress immune activation mainly through the inhibition of cell division and cytokine production. Significant side-effects include opportunistic infections and organ toxicity. In addition, the long-term use of glucocorticoids is associated with a high risk for osteoporosis and cardiovascular diseases. Monoclonal antibodies interfering with specific immune pathways represent a promising approach for more targeted therapies in autoimmune diseases. Many of such biologics have been approved for the treatment of rheumatoid arthritis in recent years. However, several of these agents, including the B cell-depleting anti-CD20 antibody Rituximab and the inhibitor of CTLA-4-dependent co-stimulation Abatacept, have so far failed to prove efficacy in clinical studies with SLE patients [213]. Results from a clinical study with the B cell-modifying anti-CD22 antibody Epratuzumab are still pending [214]. Only Belimumab, which inhibits B cells through the blockade of B-lymphocyte stimulator BLyS, has recently been approved for use in seropos-
itive, active SLE [215], and the efficacy of further BLyS-directed agents for the treatment of SLE is now assessed in Phase II/III studies [216]. Thus, there is still a high demand for more effective targeted therapeutic approaches to specifically interfere with the pathophysiology of SLE and avoid broad immunosuppression and toxicity.

1.6 Aims of this thesis

The prototypic autoimmune disease SLE is characterized by a loss of immunologic self-tolerance, resulting in severe organ damage. CD4+Foxp3+ Treg and the cytokine IL-2 are essential for the maintenance of peripheral tolerance. Defects or abnormalities in either of these two factors have been associated with SLE. However, a causal link between IL-2 deficiency, Treg abnormalities, and SLE pathogenesis has not been conclusively shown thus far. It was therefore the aim of this work to investigate whether IL-2 deficiency had an impact on Treg biology in human SLE and whether this was clinically relevant. For this purpose, the phenotype of circulating CD4+Foxp3+ Treg from SLE patients with varying disease activity was characterized in detail, and the IL-2 production by lymphocytes from SLE patients was analyzed ex vivo and in vitro. These data revealed a relationship between IL-2 deficiency, Treg defects and SLE pathogenesis. Therefore, it was next aimed to investigate whether the Treg defects were reversible and could thus provide a target for therapeutic intervention in SLE. Thus, in view of a potential clinical application, effects of IL-2 stimulation on the Treg population, as well as on other lymphocyte subsets, were analyzed in in vitro experiments. Finally, based on the results of this work, low-dose IL-2 treatment was translated to clinical application with the aim to restore a functional Treg population and thereby to re-establish tolerance in SLE. Thus, the in vivo effects of a low-dose IL-2-based immunotherapy were investigated in five patients with refractory SLE.
2 Material and Methods

2.1 Materials

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<td>Oligo-dT Primers</td>
<td>Quiagen, Germany</td>
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<td>Gibco®, Life Technologies, USA</td>
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<td>Sodium chloride (NaCl)</td>
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<td>Tris (Tris-(hydroxymethyl)-aminomethan)</td>
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<td>Trypan Blue</td>
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<td>Tween-20</td>
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<td>2-Mercaptoethanol</td>
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Table 2: Buffers and solutions

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| Phosphate buffered saline (PBS)* | 2.7mM KCl (Roth)  
1.5mM KH₂PO₄ (Roth)  
137mM NaCl (Roth)  
8.1mM Na₂HPO₄ (Roth)  
in ddH₂O |
| PBS/BSA* | 0.2% BSA (PAA) in PBS |
| PBS/BSA/Azid* | 0.01% Sodium-azide (Roth) in PBS/BSA |
| PBS/BSA/EDTA | 2mM EDTA (Sigma) in PBS/BSA |
| Erylysis buffer (pH7.5) | 0.01M KHCO₃ (Roth)  
0.155M NH₄Cl (Roth)  
0.1M EDTA (Sigma) |
| Phosphate-Citrate buffer (pH5.0) | 25.7ml 0.2M Na₂HPO₄ (Roth)  
24.3ml 0.1M Citric Acid (monohydrate, Sigma)  
50ml ddH₂O |
| Tris-buffered saline (TBS, 20mM, pH7.2) | 20mM Tris (Roth)  
150mM NaCl (Roth)  
in ddH₂O |
| ELISA diluent buffer | 0.1% BSA (Sigma)  
0.05% Tween-20 (Merck)  
in TBS(20mM) |

*kindly prepared by the labmanagers of the DRFZ, Berlin

Table 3: Instruments

<table>
<thead>
<tr>
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<td>Casy® cell counter</td>
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<td>CO₂ Incubator</td>
<td>Binder, Tuttingen, Germany</td>
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<td>Centrifuge 5804 R</td>
<td>Eppendorf, Hamburg, Germany</td>
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<td>FACSARia™ cell sorter</td>
<td>BD, Heidelberg, Germany</td>
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<tr>
<td>Heraeus® Biofuge fresco</td>
<td>Thermo Scientific, USA</td>
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<tr>
<td>Heraeus® Multifuge 3r</td>
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<td>Heraase safety cabinet</td>
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<td>LSRFortessa™ cell analyzer</td>
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<td>MACSQuant® Analyzer</td>
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<td>MACS LS columns &amp; magnets</td>
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<tr>
<td>Mastercyler gradient</td>
<td>Eppendorf, Hamburg, Germany</td>
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<tr>
<td>Neubauer chamber</td>
<td>Marienfeld GmbH, Lauda-Königshofen, Germany</td>
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<tr>
<td>SpectraMax Plus Microplate Reader</td>
<td>Molecular Devices, USA</td>
</tr>
<tr>
<td>Stratagene Mx3000P</td>
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<tr>
<td>Vacuum Concentrator 5301</td>
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Table 4: Software

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<td>GraphPad Software, La Jolla, USA</td>
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### Table 5: Antibodies for flow cytometry

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</tbody>
</table>

BV=Brilliant Violet™, PB=Pacific Blue™, *kind gift from Prof. Romagnani, DRFZ, Berlin
2 Material and Methods

2.2 Enzyme-linked immunosorbent assay (ELISA) and bead-based cytokine detection assays

2.2.1 ELISA

A sandwich-ELISA was used to detect IL-2 in the serum and in cell culture supernatants of PBMCs from healthy donors and SLE patients. For a sandwich-ELISA polystyrene microtiter plates are coated with a capture-antibody that is specific for the antigen to be detected. Upon addition of the sample, the antigen present in the sample is immobilized by specific binding to the capture-antibody. Subsequently, the immobilized antigen is detected by a biotinylated antibody (detection-antibody), which specifically binds a different epitope of the antigen. Addition of enzyme-labeled streptavidin, which specifically binds biotin with high affinity, allows for quantitation of the analyte after addition of a substrate that is converted by the enzyme in a fluorogenic reaction. The amount of the chromatic product of the enzyme reaction is detected using a spectrometer, and is proportional to the quantity of the antigen in the analyzed sample. The amount of IL-2 in serum and supernatants was detected using the IL-2 Duo Set ELISA (R&D Systems) based on the described Sandwich ELISA method. IL-2 standards were diluted in ELISA diluent in a two-fold dilution series ranging from 1000pg/ml to 3.9pg/ml and prepared in duplicates. Serum samples were used undiluted and in 1:2 to 1:8 dilutions in ELISA diluent. Cell culture supernatants were analyzed undiluted. Between all incubation steps, wells were washed three times using PBS containing 0.05% Tween20 in order to remove unbound proteins. 96-well microtiter ELISA plates (Corning) were coated with 100µl/well mouse-anti-human IL-2 antibody (MAB602, diluted to 4µg/ml in PBS) and incubated over night at room temperature (RT). After washing, non-specific binding sites were blocked by addition of 300µl of PBS containing 1% BSA for a minimum of 2h. Standards and samples were added at 100µl/well and incubated for 2h at RT, before 100µl of biotin-labeled goat-anti-human IL-2 detection antibody were added (400ng/ml in ELISA diluent). After incubation for 2h at RT, 100µl streptavidin-coupled horseradish-peroxidase (1:180 in ELISA diluent) was added for 20min at RT. The enzyme reaction was started by addition of 100µl TMB substrate (Sigma, 1 TMB tablet resolved in 10ml phosphate-citrate-buffer and 2µl 30% H$_2$O$_2$) and stopped by addition of 50µl 12.5% H$_2$SO$_4$. The optical density was photometrically read with a SpectraMax Elisa reader (Molecular Devices) at 450nm.

2.2.2 Bead-based cytokine detection

For some serum samples a bead-based assay was used to detect cytokine levels. Such bead-based assays are based on the same principles as a Sandwich-ELISA, but the capture antibody is bound to soluble beads instead of being immobilized on a well-plate, and the detection mechanism is based on fluorescent molecules (phycoerythin, PE) instead of enzyme-based changes in optical density, allowing for the detection via flow-cytometry. The use of differently sized or fluorescently colored beads also allows for the detection of several analytes within a single sample. Here, an enhanced-sensitivity cytometric bead array (CBA$^\text{TM}$, BD) flex-set and a LEGENDplex$^\text{TM}$ (Biolegend) were used according to the manufacturers’ instructions using the reagents provided with the kit. Serum samples were used undiluted or in 1:2 or 1:3 dilutions.
using the provided Assay Diluent and measured with a MACSQuant® Analyzer (Miltenyi).

2.3 PBMC isolation

Whole-blood was collected in Heparin-coated Vacutainer® tubes (BD) from healthy donors and SLE patients after informed consent. PBMCs were isolated by gradient centrifugation with the hydrophilic polysaccharide Ficoll-Paque (d=1.077g/ml), which allows for the separation of PBMCs from the more dense granulocytes and erythrocytes. 50ml conical tubes were prepared with 15ml Ficoll-Paque and the blood (2:1 diluted with PBS/BSA) was carefully layered on top. After centrifugation at 400g and RT for 17min without break, the cell layer containing PBMCs was transferred to a fresh 50mL tube, washed with PBS/BSA and centrifuged for 8min at 300g and 4°C. The cell pellet was resuspended in 5-10ml erythrocyte lysis buffer for 5min at 4°C and washed again with PBS/BSA. A second wash-step with PBS/BSA and centrifugation for 15min at 250g and 4°C was included to remove platelets. Subsequently, cells were counted and re-suspended in an appropriate amount of buffer or medium depending on following experiments.

2.3.1 Cell number determination

Cells were counted electronically using a CASY® cell counter (Schärfe System), which discriminates viable cells and their size based on the integrity of their polarized membrane and the according resistance when passing through an electric field. Cells were diluted 1:1000 in Casy-Flow buffer and subjected to measurement. Viable PBMCs were defined as cells with a size between 5 and 12.5µm.

Alternatively, cells were counted using a Neubauer-Chamber. Cells were diluted 1:2 with trypan blue, which selectively stains dead cells due to their low membrane integrity, and unstained cells were counted using a light-microscope. The cell number was calculated according to the following formular: cell concentration /ml = (cell count/number of squares)x2x10^4/ml.

2.4 Flow cytometry

Cell surface and intracellular protein expression can be analyzed by the use of specific antibodies that are conjugated with a fluorescent dye in order to visualize the presence and the quantity of a specific protein in a cell. Fluorescent dyes are excited by light of specific wavelengths and emit light of higher wavelength. Flow cytometry allows for multiparametric characterization of cells based on such immunofluorescent labeling. The pressure of the fluidics system in a flow cytometer causes the suspension of labeled cells to be aligned and the cells to pass through laser beams one by one, allowing for the analysis of single cells and up to several thousand cells per second. When passing the light of a laser beam, a cell causes the light to be scattered in a forward direction (forward scatter, FSC), which is proportional to the cell size, and sideward (SSC), which is dependent on the granularity of a cell. At the same time, fluorochromes, with which the cell is labeled, are excited and emit light of a specific wavelength range. The scattered and emitted light is detected by photomultiplier tubes (PMTs) that convert the light into an electrical pulse which is then converted into a digital signal. The signal intensity depends on the amount of fluorescent molecules per cell and permits the relative quantitation of the amount of
2 Material and Methods

a specific antigen per cell, which is presented as the fluorescence intensity. The use of lasers emitting monochromatic light of different wavelengths allows for the excitation of different kinds of fluorescent dyes. In addition, the combination of different optical filters, which reflect light of lower wavelength to the next detector (longpass filter) and transmit only light of a specific spectrum (bandpass filter) to pass through to the PMT, allows for the detection of a variety of fluorescent signals within one sample. Most phenotypical cell analyses in this work were performed with a LSRFortessa™ (BD), using four lasers and up to ten fluorescent parameters, which are listed in Table 6. CS&T Beads (BD) were used according to the manufacturer’s instructions in order to track cytometer performance during the clinical study. For some analyses with fewer parameters, a MACSQuant® Analyzer (Miltenyi) was used.

Table 6: LSRFortessa™ configuration

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<td>Pe-Dazzle594</td>
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<td>-</td>
<td>585/15</td>
<td></td>
<td>Pe</td>
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<tr>
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<td>750LP</td>
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</tr>
<tr>
<td>-</td>
<td>670/30</td>
<td></td>
<td>APC/Cy5</td>
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2.4.1 Fluorescence-activated cell sorting

Specifically labeled cell subsets can be isolated from a cell suspension on the basis of flow-cytometry using fluorescence-activated cell sorting (FACS). In specialized flow-cytometers, after passing the laser beam, the stream of cells is separated into droplets containing no more than one cell each. Depending on the emitted light the cell-containing droplet is electrically charged and subsequently deflected into different directions for collection in separate tubes. A FACSArta™ sorter (BD) was used at and with the help of the flow-cytometry core facility of the DRFZ, Berlin to isolate subsets of CD4+ T cells.

2.4.2 Staining of cell surface molecules

Most cell staining procedures were performed in 96-well round-bottom plates and up to $1 \times 10^6$ cells per well at 4°C in the dark. Washing steps were performed using 100µl of the respective buffer and centrifugation of plates for 3min at 300g and 4°C. For labeling of proteins expressed on the cell surface, cells were first incubated for 10min in 100µl PBS/BSA containing 0.5% human immunoglobulin in order to block unspecific binding of antibodies to Fc receptors. After washing with PBS/BSA, cells were resuspended in 100µl PBS/BSA containing the mixture of
specific surface antibodies and incubated for 30min. Subsequently, cells were washed with PBS/BSA and either processed for secondary and/or intracellular staining, or resuspended in PBS/BSA for direct analysis by flow-cytometry or FACS. In a few cases, surface antibodies were not directly conjugated with a fluorochrome, but were coupled to biotin. In such cases, a secondary staining step with streptavidin coupled to a fluorescent dye was performed, following the same principle as the primary surface staining.

### 2.4.3 Staining of intra-cellular proteins

The labeling of intracellular proteins requires fixation and permeabilization of cells. Fixation and permeabilization buffers, which are specifically designed for the labeling of the intranuclear transcription factors like Foxp3 (Foxp3/Transcription Factor Staining Buffer Set, eBioscience) were used for all intracellular stainings. After surface staining the cells were fixed by incubation for 40min in 100µl fixation/permeabilization working solution. After washing with permeabilization buffer, cells were incubated for 30min in 50µl of the same buffer containing the intracellular antibodies. Afterwards, cells were washed once with permeabilization buffer and once with PBS/BSA/Azid before they were resuspended in PBS/BSA/Azid and subjected to flow-cytometric analysis or FACS.

### 2.4.4 Staining of phosphorylated STAT5

For the analysis of STAT5 phosphorylation, freshly isolated PBMCs were transferred to FACS tubes immediately after gradient centrifugation, washed and stained for 20min at 4°C with 100µl surface antibodies in PBS/BSA. Subsequently, cells were fixed in 100µl fixation/permeabilization solution for 30min, washed with 1ml ice-cold PBS and permeabilized by drop-wise addition of 100% methanol at -20°C for 10min. Tubes were filled up with ice-cold PBS to remove the methanol before washing and intracellular staining in permeabilization buffer for 30min at 4°C.

### 2.4.5 Analysis of absolute lymphocyte numbers

During IL-2 therapy, patients’ absolute lymphocyte numbers per µl whole blood were determined using BD Trucount™ tubes according to the manufacturer’s instructions. Briefly, 50µl whole blood was added to Trucount™ tubes, which contain a defined number of fluorescently labeled beads. After incubation with 30µl surface antibody mixture for 15min at RT, 450µl BD FACSTM Lysing Solution was added for another 15min to lyse erythrocytes and samples were subjected to flow-cytometry without washing.

### 2.4.6 Labeling of cells with CFSE

*In vitro* proliferation of cells can be analyzed using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), which upon passive cell entry is cleaved to the fluorescent carboxyfluorescein succinimidyl ester (CFSE) by cytosolic esterases. CFSE is less cell-permeable and trapped in the cell by covalent binding to proteins. Upon cell division, CFSE is equally distributed in the daughter cells, allowing for the flow-cytometric detection of different cell generations, since the CFSE 2-fold diluted and consequently fluorescence intensity is sequentially halved in every
new cell generation. For CFSE-labeling cells were resuspended in PBS containing 5% FCS at a concentration of 1x10^6/ml in a 15ml tube. CFDA-SE was pre-diluted to 10µM in PBS and rapidly mixed with the cells at a 1:1 ratio for 5min at RT (final concentration 5µM). Labeled cells were washed three times with RPMI containing 10% FCS by centrifugation for 8min at 300g and 4°C before further processing for in vitro assays.

### 2.5 Magnetic-activated cell sorting of CD4+ T cells

Cell enrichment and separation using magnetic-activated cell sorting (MACS) is based on the detection of specific cell populations according to their expression of characteristic surface antigens. Specific antibodies against these surface antigens are either directly or indirectly coupled to magnetic beads, allowing for the isolation of the antibody-bound cells by temporary immobilization in a strong magnetic field (positive fraction), while unlabeled cells can pass through and are collected as negative fraction.

For the analysis of IL-2 mRNA expression in CD4+ T cells, these cells were MACS-isolated from PBMCs using the CD4+ T cell isolation kit II (Miltenyi). According to the manufacturer’s instructions 1x10^7 cells were resuspended in 40µl PBS/BSA/EDTA and incubated for 10min at 4°C with 10µl of biotin-labeled antibody cocktail, containing antibodies against surface molecules specific for non-CD4+ T cells (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and glycophorin A). Subsequently, 30µl PBS/BSA/EDTA and 20µl of anti-biotin conjugated microbeads were added and incubated for 15min at 4°C. After washing, cells were resuspended in PBS/BSA/EDTA and loaded on a LS column that had been rinsed with PBS/BSA/EDTA and placed in the magnetic field of a MACS Separator. The negative fraction containing the CD4+ T cell population was collected and processed for further experiments. An aliquot of this sample was subjected to flow-cytometric analysis in order to determine the purity of the isolated cell subset, which was usually between 90 and 99%.

### 2.6 Cell culture

For assays involving cell culture, all procedures were performed under sterile conditions using a laminar flow safety cabinet. If not otherwise stated cells were cultured in RPMI-1640 medium containing GlutaMAX™ (Gibco), 100U/ml penicillin, 100µg/ml streptomycin and 10% fetal calf serum (RPMI/FCS) in a humidified incubator at 37°C and 5% CO_2.

#### 2.6.1 Analysis of spontaneous IL-2 production

For the analysis of IL-2 production by resting PBMCs, freshly isolated PBMCs were cultured in RPMI/FCS in 96-well flat-bottom plates at a concentration of 1x10^7/ml and 150µl/well. Complete supernatants were collected at the respective time-points, transferred to 0.5µl tubes and stored at -80°C until analysis by ELISA.

To address spontaneous IL-2 mRNA expression levels, CD4+ T cells were isolated as described (section 2.5) and cultured in RPMI/FCS at a concentration of 1x10^7/ml and 150µl/well for 15h or 24h. Supernatants were collected before cells were washed with PBS, resuspended in 350µl RA1 buffer (Macherey-Nagel) supplemented with 1% beta-Mercaptoethanol and stored
2.6.2 Analysis of the effects of IL-2 in vitro

To detect effects of endogenously produced IL-2, PBMCs were cultured in 96-well round-bottom plates for 48h at a concentration of 2x10^6/ml and 100µl/well either in RPMI/FCS alone or in the presence of 5µg/ml neutralizing anti-IL-2 antibody (eBiocience), before they were subjected to flow-cytometric analyses. Alternatively, PBMCs from SLE patients were cultured in supernatants of CD4+ T cells from healthy controls (section 2.6.1) in the presence or absence of anti-IL-2 antibody.

To determine effects of exogenously added IL-2, PBMCs or FACS-isolated CD4+CD127lo CD25- cells were cultured in RPMI/(1%)FCS and stimulated with 0, 1.0, 2.5, 4.0 or 10.0 ng/ml recombinant human IL-2 (Proleukin®, Novartis) immediately and then every 24h. Cells were harvested either 24h after the first or 24h after the fifth stimulation with IL-2 and analyzed by flow cytometry.

2.7 In vitro suppression assay

The in vitro suppressive capacity of Treg to inhibit Tcon proliferation was determined in a mixed lymphocyte reaction with CD4+CD127+CD25- Tcon and CD4+CD127loCD25+ Treg. FACS-isolated Tcon were labeled with CFSE (section 2.4.6) and cultured in RPMI/FCS at 1x10^4 cells per well in a 96-well round-bottom plate either alone or together with varying amounts of unlabeled FACS-isolated Treg at Treg:Tcon ratios of 1:1 to 1:16. The cells were polyclonally stimulated by addition of anti-CD3, anti-CD2 and anti-CD28 antibodies conjugated to microbeads (bead-to-cell ratio of 3:1, Treg Suppression Inspector, Miltenyi). After stimulation for 4 days, cells were fixed and stained for Foxp3 in order to discriminate Treg from Tcon. Tcon proliferation was assessed by flow-cytometric analysis of CFSE dilution, where each CFSE-peak represents a cell generation. A division index (DI) was calculated based on the percentage of the original cell population that have divided at least once (PD) and the average number of divisions of the responding cell subset (proliferation index, PI) [217]. Percent suppression was calculated as (100-(DI_{Tcon+Treg}/DI_{Tcon alone})*100).

\[
PD = \frac{\sum_{i=1}^{n} N_i}{N_0} \\
PI = \frac{\sum_{i=1}^{n} i \times N_i}{\sum_{i=1}^{n} N_i^2} \\
DI = \frac{PD}{PI} \quad i = \text{generation number} (0 = \text{undivided population}), \ N_i = \text{number of events in generation } i.
\]

2.8 RNA expression analyses

For the analysis of IL-2 and Blimp-1 expression, RNA was isolated from MACS-isolated CD4+ T cells either ex vivo or after in vitro cultures, transcribed to cDNA and analyzed by quantitative
2 Material and Methods

2.8.1 RNA isolation and reverse transcription

RNA was isolated from CD4+ T cells using the NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturers’ instructions. At the final step RNA from 1-1.5x10^6 cells was eluted from the column three times with 30µl RNAse free water and further concentrated in a Vacuum concentrator (xyz) for 20-30 minutes at 30°C to reach a volume of 10-15µl.

For reverse transcription, the freshly isolated RNA was transferred to 0.2µl tubes and if necessary filled up with H₂O to reach a volume of 13.5µl. OligodT Primers (1µl, 400µg/ml, Quiagen) and dNTPs (1µl, 10mM mix) were allowed to anneal by heating at 65°C for 10min in a PCR cycler. After addition of 6µl 5x RT-buffer (Promega) for 2min at 42°C, the reaction mixture was cooled down to 4°C before 0.5µl M-MLV Reverse Transcriptase (Promega) were added. cDNA synthesis followed at 42°C for 50min before the reaction was stopped by heating up to 70°C for 15min. cDNA was stored at -20°C until further use.

2.8.2 Quantitative real-time PCR

SYBR® Green-based qPCR was performed using a MX3000P cycler (Stratagene) in order to amplify IL-2 and Blimp-1 cDNA and to quantify them in relation to the house-keeping gene EEF1A1 (eukaryotic translation elongation factor 1-α1).

A 20µl qPCR reaction contained 5µl cDNA (1:5 diluted in H₂O), 0.25mM of each dNTP (Bioline), 0.5nmol/ml forward and reverse primers (TIB MOLBIOL, Table 7), 1x SYBR Green-I (Molecular Probes), 12µg/ml BSA, 500mM Tris (pH8.8), 6mM MgCl₂, 1.5% DMSO and 1U Immolase (Bioline). The thermal profile of the PCR reaction is shown in table 8. Data was analyzed using the MxPro Software (Stratagene). RNA expression of the gene of interest (goi) was calculated in relation to the expression of the house-keeping gene (hkg) according to the following formular:

\[ 2^{(-\Delta\Delta CT_{\text{goi}} - \Delta\Delta CT_{\text{hkg}})} \]

Table 7: Primer used for qPCR

<table>
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</tr>
<tr>
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<td>reverse</td>
<td>5'-TCC-TGG-TGA-GTT-TGG-GAT-TC-3'</td>
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<tr>
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<td>EEF1A1</td>
<td>forward</td>
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</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5'-GCC-AGC-TCC-AGC-AGC-CTT-C-3'</td>
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2.9 TSDR methylation analysis

For the investigation of the methylation status of the TSDR region, CD4+Foxp3+CD127lo CD25+ and CD25-, and CD4+CD127+Foxp3- cells were isolated by FACS and stored in PBS at -80°C until further analysis. Methylation analysis was performed by the group of Prof. B.
Table 8: qPCR thermal profile

<table>
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<td>95</td>
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<td>95</td>
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Sawitzki (Institute for Medical Immunology, Charité, Berlin) based on bisulfite treatment of isolated DNA and sequence-specific qPCR for the methylated and non-methylated version. Bisulfite treatment of genomic DNA results in the deamination of unmethylated cytosine to uracil, which will be replicated as thymidine in subsequent PCR amplification. In contrast, methylated cytosine will remain unchanged, allowing for the differentiation between methylated and unmethylated copies using respective primers and probes.

2.10 Study subjects and approval

For the ex vivo and in vitro studies, ethical approval was obtained by the institutional review board of the Charité - University Medicine Berlin (EA 1/342/12 and EA 1/098/07). Peripheral blood samples were obtained from a total of 61 SLE patients (21-70 years, mean 40.1, 89% females) and 52 healthy donors (22-63 years, mean 35.8, 79% females) after written informed consent. Age, disease duration, disease manifestations, serum-levels of anti-dsDNA antibodies, disease activity according to the SLEDAI, as well as immunosuppressive treatments at the time of blood drawing were retrieved from the patients’ medical records by authorized medical staff and pseudonymized for further analyses. These data are summarized in table A1 of the appendix.

Prior to the initiation of the off-label treatment or the clinical study with IL-2 written informed consent was obtained from the patients in accordance to the Declaration of Helsinki in the revised version of 1996, and the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP). Treatments of patient 1 and 2 were announced as ‘off-label’ therapy (‘Individuelle Heilversuche’) to the institutional ethics committee of the Charité - University Medicine Berlin. Patients 3, 4 and 5 were treated within a phase I/IIa clinical trial, addressing the safety, tolerability, efficacy and immunological responses of a low-dose therapy with recombinant human IL-2 (aldesleukin) in the treatment of SLE (PRO-IMMUN; EudraCT-Number: 2013-001599-40 [218]). This clinical study was approved by the responsible Ethics Committee (Ethik-Kommission des Landes Berlin, Germany; 13/0449 - EK10) and authorized by the competent governmental authority (Bundesinstitut für Arzneimittel und Medizinprodukte - BfArM, Germany; 61-3910-4039436).
2.11 IL-2 therapy

The therapeutic regimen for the five SLE patients treated with low-dose IL-2 consisted of four 5-day treatment cycles that were separated by wash-out periods of 9 days after the first cycle and 16 days after the following cycles. Each 5-day treatment cycle consisted of daily subcutaneous IL-2 injections, starting with single doses of 1.5 million IU IL-2 (Proleukin®, aldesleukin) per day and the option to increase the dose to 3.0 million or 4.5 million IU per day during the following treatment cycles. Dose-escalation was realized if the previous dose was well tolerated without adverse events and the percentage of Foxp3+CD127lo Treg among CD4+ T cells did not exceed 50%. Dose reduction criteria included, among others, an expansion of Foxp3+CD127lo Treg to more than 50% among CD4+ T cells, and resulted in reduction of the dose by half for the following cycle.

Blood was collected before IL-2 administration on the first day of each treatment cycle, and one day after the last IL-2 injection of each treatment cycle. During the first treatment cycle blood was collected daily approximately 20h after IL-2 administration. Blood samples were processed immediately after blood collection according to the described methods.

2.12 Statistics

Statistical analyses were performed with GraphPad Prism (GraphPad Software Inc., LaJolla, USA). The Mann-Whitney test was used to compare samples from SLE patients to those from healthy donors. Two-tailed Wilcoxon signed-rank tests were used for paired comparisons. Correlation analyses were performed by nonparametric correlation using Spearman’s rank correlation coefficients. The data are expressed as median and interquartile range (IQR), unless stated differently. Differences were considered significant if p-values were smaller than 0.05 (*), 0.01 (**) or 0.001 (***).
3 Results

3.1 Characterization of IL-2 production in SLE

A deficient IL-2 production in SLE has been shown already in the 1980ies by experiments demonstrating that the supernatants of exogenously stimulated lymphocytes or T cells from SLE patients were not able to induce IL-2-dependent proliferation in responder cells, leading to the assumption that they produced less IL-2 compared to lymphocytes from healthy donors [203, 204]. More recently, possible molecular mechanisms responsible for a transcriptional repression of the IL-2 gene in SLE have been identified [205, 219]. Nonetheless, it should be borne in mind that a strong exogenous stimulus was used in those initial experiments, and the use of different stimulation methods have yielded conflicting results [220, 221]. Therefore, it was aimed at to investigate the IL-2 availability in SLE patients and healthy controls under more physiological conditions, without exogenous stimulation.

3.1.1 IL-2 protein is not detectable in serum of healthy controls and SLE patients

In a first approach to investigate the endogenous IL-2 availability, the protein levels of IL-2 in the sera of SLE patients and healthy controls were analyzed by ELISA. For this, different dilutions of serum samples were analyzed, however no considerable amounts of IL-2 could be detected in the sera of both SLE patients and healthy controls, since values of most samples were below the detection limit of 3pg/ml. Alternatively, an Enhanced Sensitivity BD™ Cytometric Bead Array was performed, which follows the same principles as a Sandwich-ELISA, but the analytes are captured on antibody-coated beads, detected with biotin-labeled antibodies and the signal is enhanced by subsequent labeling with PE. PE is a bright fluorescent molecule, which allows for flow-cytometric detection of the bead-bound analyte. This system has a theoretical detection limit for IL-2 of 2.6pg/ml. Also with this method IL-2 protein could only be detected in the sera of 20% of both SLE patients and healthy donors. Thus, it was not possible to reliably and directly determine IL-2 protein levels in the serum of either healthy controls or SLE patients.

3.1.2 Deficient spontaneous IL-2 production by CD4+ T cells from SLE patients

As IL-2 levels in the serum were too low to be reliably detected by the available methods, it was next tested whether IL-2 could be detected in the supernatants of resting lymphocytes in vitro. For this, PBMCs were isolated from peripheral blood and cultured in resting conditions, i.e. in RPMI medium containing 10% FCS but no additional stimulus, for up to 96h before collecting the supernatant for analysis by ELISA. A kinetic study performed with PBMCs from healthy donors showed that IL-2 could be detected in the supernatant and a peak concentration was reached after 48h (Fig. 2A). Comparison of 25 SLE and 17 healthy control samples showed significantly lower levels of IL-2 in the supernatants of 44h rested PBMCs from SLE patients (median 0.0 pg/ml) compared to those from healthy donors (14.0 pg/ml) (Fig. 2B).
3 Results

Figure 2: Low IL-2 production by PBMCs from SLE patients. IL-2 protein levels were analyzed by ELISA in supernatants of 1.5x10^6 PBMCs cultured in RPMI/FCS. (A) IL-2 protein levels in supernatants of healthy control PBMCs (n=6) during a time-course of up to 96h. (B) Comparison of IL-2 protein levels in supernatants of PBMCs from healthy controls (HC, n=17) and SLE patients (n=25) after 44h culture (Mann-Whitney Test, *=p<0.05).

Since CD4+ T cells are the main producers of IL-2, the frequencies of CD4+ T cells among total PBMCs were compared between the SLE patient and healthy control samples, to investigate whether differences in CD4+ T cell frequencies could be the reason for the low IL-2 production in SLE samples. Although the frequencies of CD4+ T cells among PBMCs were significantly lower in SLE patient samples compared to healthy controls (median 20.5 vs 41.0%, Fig. 3A), no significant correlation between the CD4+ T cell frequencies and IL-2 protein levels in SLE patients was observed (Fig. 3B). Thus, a numeric deficit of CD4+ T cells seemed not to be the sole reason for the low IL-2 production in cultures of SLE PBMCs.

Therefore, IL-2 mRNA expression in CD4+ T cells was analyzed by qPCR in order to determine whether the reduced IL-2 protein levels could be attributed to lower IL-2 expression levels in the CD4+ T cell population in SLE. For this, CD4+ T cells were isolated from PBMCs by MACS-technology and RNA was isolated ex vivo and after 15h or 24h resting cultures. The ex vivo IL-2
mRNA levels in CD4+ T cells were low, but did not differ between cells from SLE patients and healthy controls. *In vitro* cultures in resting conditions, however, showed that IL-2 mRNA levels increased in CD4+ T cells from healthy donors over time without the addition of an exogenous stimulus, whereas no increase of IL-2 expression was observed in CD4+ T cells from SLE patients. Thus, after 24 h resting cultures CD4+ T cells from SLE patients showed significantly lower IL-2 expression levels compared to those from healthy donors (Fig. 4).

In summary, although the *in vivo* IL-2 availability could not be directly addressed, these data demonstrate that CD4+ T cells from SLE patients produce reduced amounts of IL-2 without exogenous stimulation *in vitro*, suggesting that an IL-2 deficiency may also be present in SLE patients *in vivo*.

**Figure 4: No increase of IL-2 expression in resting CD4+ T cells from SLE patients.** IL-2 mRNA expression levels were analyzed by qPCR from MACS-isolated CD4+ T cells either *ex vivo* or after 15 h and 24 h resting cultures in RPMI/FCS. Relative IL-2 expression levels were calculated in relation to the expression levels of the housekeeping gene EEF1A1 as $2^{-\Delta\Delta CT}$ and compared between the different time-points (Wilcoxon signed-rank test), or between healthy controls (HC) and SLE patient samples (Mann-Whitney test, **=p<0.01, ***=p<0.001).

### 3.1.3 Chronic activation of CD4+ T cells may account for the IL-2 deficiency in SLE

CD4+ T cells are the main source of IL-2 in healthy individuals [129] and the reduced IL-2 mRNA expression in CD4+ T cells of SLE patients indicates a defect in the CD4+ T cell compartment in SLE. IL-2 is mainly produced by naïve and memory T cells upon TCR stimulation and co-stimulation via CD28 [107]. In contrast, terminally differentiated, chronically activated memory T cells lose the ability to produce IL-2. This is due to the repression of the IL-2 promoter by the transcription factors Blimp-1 and T-bet [123, 134], as well as the loss of CD28 expression and the consequent absence of co-stimulation [222]. In order to analyze whether the reduced IL-2 expression by CD4+ T cells from SLE patients could be attributed to chronic activation and increased differentiation of these cells, the differentiation of CD4+ Foxp3- Tcon into memory cells was analyzed according to the expression of CCR7 and CD45RO, as well as CD28 expression by flow-cytometry. The gating strategy for CD4+ T cells, the CD4+Foxp3- Tcon population and its memory subsets is shown in Figure 5A and B.

SLE patients exhibited similar frequencies of CCR7+CD45RO- naïve, CCR7+CD45RO+ Tcm, CCR7-CD45RO+ Tem and CCR7-CD45RO- Term cells among CD4+ Foxp3- Tcon when compared with healthy donors (Fig. 5C). However, an increased percentage of Tcon that lacked CD28 expression was observed in SLE patients compared to healthy controls in whom such
3 Results

Figure 5: Equal distribution of naïve and memory cells among Tcon in SLE patients and healthy donors. Gating-strategy for the flow-cytometric detection of CD4+ T cells (A), and of CD4+ Foxp3-Tcon and CCR7+CD45RO- naïve, CCR7+CD45RO+ central memory (CM), CCR7-CD45RO+ effector memory (EM), and CCR7-CD45RO- terminally differentiated memory (Term) cells among Tcon (B). (C) Frequencies of naive, CM, EM and Term among CD4+Foxp3- Tcon were compared between healthy controls (HC, circles, n=36) and SLE patients (squares, n=46, Mann-Whitney test).

Cells were mostly absent (Fig. 5A). In contrast to their CD28+ counterparts, these CD28- Tcon expressed T-bet (51.2 vs 2.3%), and had a CCR7-CD45RO+ Tem (61.3 vs 7.5%) or CCR7-CD45RO- Term (14.6 vs 0.7%) phenotype (Fig. 5B). In addition, analysis of the mRNA expression of the transcription factor Blimp-1 revealed significantly higher levels of Blimp-1 expression in CD4+ T cells from SLE patients when compared to CD4+ T cells from healthy controls ex vivo (Fig. 5C). Thus, CD4+Foxp3- Tcon from SLE patients exhibit a phenotype that is associated with chronic activation and a low capability to produce IL-2. In summary, these data indicate that an enhanced chronic activation of CD4+ T cells in SLE might contribute to the low production of IL-2.
Results

Figure 6: SLE Tcon show signs of chronic activation. (A) Representative histogram showing the expression of CD28 among CD4+Foxp3- Tcon (upper panel) and percentages of CD28- cells among CD4+Foxp3- Tcon from healthy controls (HC, n=38) in comparison to SLE patients (n=48). (B) Representative histograms and dot-plots showing the expression of T-bet and the distribution of CCR7+CD45RO- naïve, CCR7+CD45RO+ central memory (CM), CCR7-CD45RO+ effector memory (EM), and CCR7-CD45RO- terminally differentiated memory (Term) cells among CD28- and CD28+ CD4+Foxp3- Tcon from SLE patients. (C) qPCR-based analysis of Blimp-1 expression relative to the housekeeping gene EEFA1A ($2^{-\Delta CT}$) in MACS-isolated CD4+ T cells from SLE patients (n=11) and HC (n=10) ex vivo (Mann-Whitney test, *=p<0.05).
3 Results

3.2 Phenotypic characterization of regulatory T cells in SLE

3.2.1 Low CD25 expression in Treg from SLE patients

Having confirmed a deficient IL-2 production by CD4+ T cells from SLE patients in vitro, it was next aimed to investigate whether this had any relevance for the biology of the CD4+Foxp3+ Treg population in SLE patients in vivo. In mice lacking IL-2, loss of CD25 expressing Treg was identified as a hallmark of IL-2 deficiency [111, 148]. Thus, in order to determine whether Treg biology was affected by IL-2 deficiency in SLE, ex vivo flow-cytometric analyses of Treg were performed with emphasis on the surface expression of CD25. For this, PBMCs from 61 SLE patients were analyzed and compared to those from 52 healthy donors. In addition, the patients' disease activity was scored according to the SLEDAI and correlated with the phenotypic parameters, which were determined by flow cytometry. The epidemiological data, SLEDAI, clinical manifestations and current treatment of the included patients are listed in table A1 of the appendix.

Treg were defined as cells expressing Foxp3 and low levels of CD127 (Foxp3+CD127lo) among CD3+CD4+ T cells. The gate for CD25+ cells was set according to a 'full-minus-one plus isotype' (FMO+I) control in which the same antibodies were used for staining as in the full stain, except for the anti-CD25 antibody, which was substituted with an isotype control antibody labeled with the same fluorochrom. Within the Foxp3+CD127lo Treg population, CD25+ cells were further subdivided into two populations according to high expression levels of Foxp3 and CD25 (CD25hi) or intermediate levels Foxp3 and CD25 expression (CD25int). Among Tcon, a population expressing high levels of CD25 could not be clearly distinguished in these ex vivo analyses. The gating strategy for the Treg and Tcon subsets is depicted in Figure 7A, showing dot-plots from a representative healthy donor and SLE patient.

The total frequency of Foxp3+CD127lo Treg among CD4+ T cells was found to be significantly higher in SLE patients compared to healthy controls (11.2 vs. 6.4%) (Fig. 7A,B). However, expression of CD25 within the Foxp3+CD127lo Treg population was reduced in SLE patients, resulting in significantly lower frequencies of CD25hi (6.8 vs. 15.7%) and CD25int (58.8 vs. 67.1%) cells among Foxp3+CD127lo Treg and the appearance of a substantial CD25 negative (CD25neg) Treg population in SLE patients (Fig. 7A,C). This loss of CD25 expression was associated with SLE disease activity as the frequencies of CD25+ cells among Treg correlated inversely with the patients’ SLEDAI and serum levels of anti-dsDNA antibodies (Fig. 7D).

In contrast, no significant difference in the CD25 expression of CD4+Foxp3- Tcon was observed between healthy controls and SLE patients (Fig. 7A+E).

Thus, Treg from SLE patients present a hallmark feature of IL-2 deficiency - low CD25 expression - indicating that an IL-2 deficiency affects Treg biology in vivo and that it may play a role in SLE disease pathogenesis.

3.2.2 Low CD25 expression in Treg can be linked to deficient IL-2 production in SLE

Next, it was aimed to determine whether the low CD25 expression in Treg from SLE patients could indeed be attributed to the defective IL-2 production in SLE. As shown in section 3.1.2 PBMCs from SLE patients spontaneously produce lower amounts of IL-2 in vitro as compared
**Figure 7: Low CD25 expression in SLE Treg.** Purified PBMCs from SLE patients (n=61) and healthy controls (HC, n=52) were analyzed *ex vivo* by flow cytometry. (A) Representative HC and SLE sample dot plots showing the gating strategy for Foxp3+CD127lo Treg and Foxp3- Tcon among CD4+ T cells (upper row) and of CD25+, CD25hi, CD25int and CD25neg subsets among Treg and Tcon (mid row), using a ‘full-minus-one plus isotype’ (FMO+I) control for CD25 (lower row). (B) Frequencies of Foxp3+CD127lo Treg among CD4+ T cells, and (C) of CD25hi, CD25int and CD25neg cells among Foxp3+Cd127lo Treg compared between SLE patients and HC. (D) Inverse correlation of CD25+ cell frequencies among Treg with SLE patients’ SLEDAI (left) and serum levels of anti-dsDNA antibodies (right). (E) Percentage of CD25+ cells among Foxp3- Tcon from HC and SLE patients. (Mann-Whitney tests and Spearman’s rank correlation coefficient, **=p<0.01, ***=p<0.001).
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to healthy control PBMCs. Thus, PBMCs were cultured in resting conditions for 48h with or without the addition of neutralizing anti-IL-2 antibodies in order to assess the effect of the differing amounts of IL-2 produced on the expression of CD25 in Treg.

In healthy control samples, the CD25 expression among Foxp3+CD127lo Treg increased significantly during 48h resting cultures of PBMCs (Fig. 8A). Addition of neutralizing anti-IL-2 antibody inhibited this effect (Fig. 8A), indicating that spontaneously produced IL-2 was responsible for the induction of CD25 expression in healthy control Treg in vitro.

In contrast, when performing the same experiments with PBMCs from SLE patients, no increase of CD25 expression was observed among Treg after 48h resting cultures (Fig. 8B left); and accordingly, IL-2 neutralization had no effect.

To clarify whether the absence of CD25 up-regulation in SLE Treg was indeed due to the low IL-2 availability or whether it originated from a Treg-intrinsic defect to express CD25 at normal levels, PBMCs from SLE patients were next cultured with supernatants from unstimulated healthy control CD4+ T cells. These experiments showed that healthy control supernatants were able to induce an up-regulation of CD25 expression in Treg from SLE patients. This effect was also inhibited by the addition of neutralizing anti-IL-2 antibody (Fig. 8B right). Thus, SLE Treg are generally able to respond to exogenous IL-2 by up-regulation of surface CD25. Accordingly, the lack of CD25 up-regulation in unmodified SLE samples in vitro could be attributable to the deficient IL-2 production by SLE lymphocytes.

In line with this, the ex vivo frequencies of CD25+ Treg weakly correlated with the ex vivo IL-2 mRNA expression levels of CD4+ T cells from SLE patients (r=0.636, p=0.054) (Fig. 8C).

Together, these ex vivo and in vitro observation indicate that the low CD25 expression in Treg from SLE patients is likely to be linked to the defective IL-2 production in SLE.

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**Figure 8: IL-2-dependent CD25 expression by Treg.** PBMCs from healthy controls (A, n=7) and SLE patients (B, n=7) were cultured in RPMI/FCS with (gray) or without (black) addition of neutralizing anti-IL-2 antibody and the frequencies of CD25hi cells among Foxp3+CD127lo Treg were analyzed by flow-cytometry either ex vivo or after 48h culture (Wilcoxon signed-rank test, *=p<0.05). Some SLE samples (n=3) were cultured in supernatants of 24h resting (unstimulated) CD4+ T cell cultures (HC supernatant) in stead of RPMI/FCS. (C) Correlative analysis between ex vivo CD25+ cell frequencies among Treg and relative IL-2 mRNA expression in CD4+ T cells ex vivo (Spearman’s rank correlation coefficient).
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3.2.3 The Foxp3+CD25neg cell subset in SLE comprises bona fide Treg

The above-presented results support the hypothesis that the large proportion of Foxp3+CD127lo CD25neg T cells that were observed in SLE patients originate from Treg that lack CD25 expression due to IL-2 deprivation. The appearance of CD4+ Foxp3+CD25neg cells [223, 224], and an expanded population of cells with low Foxp3 and low CD25 expression levels [71] have been reported for SLE patients. Yang and colleagues have proposed that these Foxp3+CD25- T cells in SLE are not comprised of natural Treg, but contain cytokine-producing conventional T cells [224]. In addition, Miyara and colleagues have described Foxp3low CD45RAlow T cells, which exhibit also low CD25 expression, as cytokine-producing non-Treg cells in healthy individuals [71]. Therefore, the origin of the Foxp3+CD25neg cell subset in our SLE patient cohort was investigated in order to determine whether they are comprised of bona-fide Treg.

Expression of the Ikaros-family transcription factor Helios has been associated with naturally occurring thymic-derived Treg [76] and in SLE patients it has been shown that Helios+Foxp3+ T cells do not produce effector cytokines, have a demethylated TSDR region and are therefore considered genuine Treg [79, 80]. Here, analysis of Helios expression by flow cytometry showed that in healthy donors Helios expression was restricted to CD25int (68.7% Helios+) and CD25hi Treg (77.9% Helios+), whereas the small population of CD25neg Treg in healthy donors contained mainly Helios- cells (62.4% Helios-), indicating an abundance of cells with presumably peripherally-induced Foxp3 expression in this subset (Fig. 9A). In contrast, in SLE patients all three Treg subsets showed high frequencies of Helios+ cells, and a significantly higher percentage of Helios+ cells was present in the CD25neg Treg subset of SLE patients (76.4%) compared to healthy controls (37.6%, Fig. 9A right).

In addition, CD25+ and CD25neg Foxp3+CD127lo Treg from one SLE patient were isolated by FACS and send for methylation analysis (AG Sawitzki) in order to assess the CpG methylation status of the TSDR region of the Foxp3 promoter. This revealed 89.1% demethylation among CD25+ Treg and 56.2% demethylation among CD25neg Treg, as opposed to complete CpG methylation of the TSDR in the Foxp3- Tcon population from this SLE patient (Fig. 9B). Together, these observations indicate that in healthy donors, the small CD4+ Foxp3+CD25-T cell subset may indeed contain a large proportion of effector T cells as suggested by Yang and colleagues [224]. However, in SLE patients this subset appears to be largely comprised of genuine Treg, which presumably lost CD25 expression due to low IL-2 availability.
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![Figure 9: All SLE Treg subsets comprise bona fide Treg.](image)

(A) Purified PBMCs from SLE patients (n=13) and healthy controls (HC, n=8) were analyzed ex vivo by flow cytometry to compare the frequencies of Helios expressing cells among CD25hi, CD25int and CD25neg Foxp3+CD127lo Treg (Wilcoxon signed-rank test) and between HC and SLE samples (Mann-Whitney test, *=p<0.05, **=p>0.01, ***p=<0.001). (B) Indicated subsets of CD3+CD4+ T cells were FACS-isolated from peripheral blood of one SLE patient and subjected to TSDR CpG methylation analysis. The percentage of demethylated copies of the TSDR are shown for each cell subset (n=1).

3.2.4 The homeostatic balance between Treg and Tcon is disturbed in SLE

Autoimmunity is associated with aberrant activation and proliferation of effector cells. Another hallmark of IL-2 deficiency is a disturbed homeostatic balance between Treg and Tcon, where excessive Tcon proliferation is not sufficiently controlled by the Treg population [112, 148]. In view of this, the proliferation of Treg and Tcon was analyzed in ex vivo peripheral blood samples from SLE patients and healthy controls. The nuclear protein Ki67 is expressed during all phases of the cell cycle, except the G0 phase [225], because of which Ki67 is a commonly used and well established marker to identify proliferating cells.

The percentage of Ki67+ (proliferating) cells in Foxp3+CD127lo Treg from SLE patients ranges from 2 to 28%, and this is similar in Treg from healthy controls (Fig. 10A). In contrast, the proportion of proliferating Foxp3- Tcon in SLE patients was significantly higher compared to that of Tcon from healthy controls reaching up to 13% Ki67+ cells (median 3.3% vs. 1.5%, Fig. 10B). In addition, the high proliferation among Tcon correlated positively with the patients’ disease activity and anti-dsDNA antibody levels (not shown).

In order to estimate the state of the homeostatic balance between Treg and Tcon, the ratio between Ki67+ cells among Treg and Ki67+ cells among Tcon was calculated. In healthy controls, this Treg/Tcon proliferation ratio has a median value of 8.4. In contrast, in SLE patients the proliferation ratio was significantly reduced to a median of 3.3 (Fig. 10C) and correlated inversely with the patients’ disease activity and serum anti-dsDNA antibody levels (Fig. 10D). Furthermore, the Treg/Tcon proliferation ratio correlated positively with the frequencies of CD25+ cells among Foxp3+CD127lo Treg in SLE patients, and again there was a tendency towards positive correlation with the ex vivo IL-2 mRNA expression levels of CD4+ T cells (r=0.575, p=0.08) (Fig. 10E).

Together, these data suggest that the homeostatic balance between Treg and Tcon is disturbed in SLE patients with a shift towards Tcon proliferation and that this may be due to the IL-2-deprivation of Treg, which in turn fail to control Tcon proliferation.
Figure 10: Disturbed homeostatic balance between Treg and Tcon in SLE. Purified PBMCs from SLE patients (n=61) and healthy controls (HC, n=52) were analyzed ex vivo by flow cytometry to compare the frequencies of Ki67+ cells among Foxp3+CD127lo Treg (A) and among Foxp3- Tcon (B). The ratio between Ki67+ cells among Treg and Ki67+ cells among Tcon (Treg:Tcon [%Ki67+]) was compared between SLE and HC samples (C), and correlated with SLE patients’ SLEDAI (D, left) and serum levels of anti-dsDNA antibodies (D, right), as well as with the frequencies of CD25+ cells among Foxp3+CD127lo Treg (E, left) and relative ex vivo IL-2 mRNA expression in CD4+ T cells (E, right) (Mann-Whitney tests and Spearman’s rank correlation coefficient, **=p<0.01, ***p<0.001).

3.2.5 Increased apoptosis of Treg and Tcon in SLE

The maintenance of a homeostatic balance is also regulated by controlled cell death (apoptosis). A tight balance of pro- and anti-apoptotic proteins is responsible for the regulation of apoptosis, which is typically triggered by multiple extrinsic or intrinsic factors, including death receptor-mediated signals and growth factor deprivation. Such signals initiate a cascade that leads to the activation of effector caspases, including Caspase-3, and ultimately results in protein cleavage, nuclear condensation and formation of apoptotic bodies [165]. It has been proposed that IL-2 deficiency is responsible for a lack of AICD in SLE and thus contributes the
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uncontrolled expansion of auto-reactive T cells in SLE [226]. The presence of the active form of Caspase-3 was thus analyzed in peripheral blood CD4+ T cells by flow-cytometry in order to investigate the frequency of apoptotic cells.

Both, Foxp3+CD127lo Treg and Foxp3- Tcon of SLE patients showed higher ex vivo frequencies of active Caspase-3 positive cells compared to healthy controls (1.3 vs 0.7% and 0.4 vs 0.1%, respectively, Fig. 11A+B, left). Similarly, although this observation was not statistically significant, flow-cytometric analysis of anti-apoptotic protein Bcl-2 expression revealed an increase of cells expressing low Bcl-2 levels (Bcl-2lo) in Treg and Tcon from SLE patients compared to healthy controls (Fig. 11A+B, middle). In Tcon from SLE patients the higher frequency of apoptotic cells (Bcl-2lo and active Caspase-3+) significantly correlated with the augmented Tcon proliferation (shown for Bcl-2lo, Fig. 11B, right). In contrast, the increased rate of apoptosis in Treg could not be associated with their proliferative activity (Fig. 11A right). In summary, these data indicate that a high level of Tcon cell death observed in SLE samples can be attributed to their hyper-activation and are a likely to be a result of physiologic homeostatic mechanisms like AICD. In contrast, the increased death of Treg in SLE appears to be independent of proliferative activity, but might instead be a result of the lack of survival factors.

Figure 11: Increased apoptosis of Treg and Tcon in SLE. Purified PBMCs from SLE patients and healthy controls (HC) were analyzed ex vivo by flow cytometry to compare the frequencies of active Caspase3+ cells (left, n=51 and 43) and of Bcl-2lo cells (middle, n=16 and 8) among Foxp3+CD127lo Treg (A) and among Foxp3- Tcon (B) (Mann-Whitney test**=p>0.01, ***p=<0.001). Frequencies of Bcl-2lo cells among Treg and Tcon were correlated with the percentage of Ki67+ cells among the respective cell subset (A+B, right, Spearman's rank correlation coefficient).
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3.2.6 Treg activation is impaired in active human SLE

Considering the apparent hyperactivity of the Tcon population in SLE patients, a strong activation of the Treg compartment would be expected as a means to counter-regulate the autoimmune reaction. In line with this, in IL-2 deficient mice, as well as in lupus-prone animals with an acquired IL-2 deficiency an increased activation and memory-differentiation of the remaining Treg population has been demonstrated [111, 148].

In the SLE patient cohort, analysis of CCR7 and CD45RO expression revealed a significantly lower percentage of naïve cells among Foxp3+CD127lo Treg, and slightly increased frequencies of central memory Treg in comparison to Treg from healthy controls. However, frequencies of Treg with a CCR7-CD45RO+ effector memory phenotype, which have been shown to be responsible for suppression at the site of inflammation [98, 100], were not significantly altered in peripheral blood from SLE patients when compared to healthy controls (Fig. 12A).

In addition, analysis of CD137, a marker for recently activated Treg [227], showed no differences between Treg activation in healthy controls and SLE patients (Fig. 12B left). Conversely, a high disease activity in SLE patients was associated with low frequencies of CD137+ Treg (r=−0.398) (Fig. 12B right). This is in contrast to the previous findings in mouse models [111, 148], and together with the impaired homeostatic balance between Treg and Tcon is indicative for a lack of sufficient Treg activation to counteract autoimmunity in human SLE.

Figure 12: Impaired activation of Treg in active SLE. Purified PBMCs from SLE patients and healthy controls (HC) were analyzed ex vivo by flow cytometry to compare the frequencies of (A) CCR7+CD45RO- naïve, CCR7+CD45RO+ central memory (CM) and CCR7-CD45RO+ effector memory (EM) cells, and of (B) CD137+ cells among Foxp3+CD127lo Treg (Mann-Whitney test). (B, right) Frequencies of CD137+ cells among Treg correlated negatively with the patients’ SLEDAI (Spearman’s rank correlation coefficient, *=p>0.05, ***p=<0.001).
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3.3 Evaluation of the impact of IL-2 deprivation for the functional activity of Treg in SLE

In the previous section it was demonstrated that abnormalities in Treg biology in SLE, which were linked to IL-2 deprivation, are also associated with an increased disease activity. It was therefore of interest to understand, whether this phenotypic alteration, i.e. the reduced CD25 expression, could also be linked to an altered functionality of the Treg population in SLE. The suppressive capacity of Treg is commonly assessed in an in vitro assay, where the proliferation of responder cells (e.g. Tcon) in response to a polyclonal stimulus is analyzed in cultures with or without Treg cells. The isolation of Treg for this kind of assay relies on the use of CD25 as a surface marker for Treg identification, since staining for Foxp3 requires cell fixation and is therefore not suitable for the isolation of living cells. In the present case, where a substantial proportion of the Treg population is devoid of CD25, such an assay would cover only the CD25+/hi Treg subset and would thus not provide a true estimate about the suppressive capacity of the whole Foxp3+ Treg compartment in SLE. Therefore, in an alternative approach, it was aimed to estimate the functional activity of the Treg population by phenotypic analyses. For this, Treg were subdivided according to their CD25 expression levels into CD25hi, CD25int and CD25neg expressing cells and their phenotype was compared among the three Treg subsets.

3.3.1 Activated effector memory cells are enriched in the CD25hi Treg compartment

This subdivision of Treg revealed a differential distribution of memory and naïve cells among CD25hi, CD25int and CD25neg Treg subsets. CCR7-CD45RO+ effector memory cells were enriched in the CD25hi Treg subset, whereas naïve Treg were mainly found in CD25int and CD25neg Treg, and central memory cells were equally present in all Treg subsets (Fig. 13A). Except for the overall lower frequencies of naïve Treg in SLE patients (compare also Fig. 12), the distribution of memory and naïve cells among the three Treg subsets was similar in SLE patients and healthy controls. Furthermore, the CD25hi Treg compartment also contained the highest frequency of CD137+ (48.4%) cells compared to CD25int and CD25neg Treg (8.3% and 4.7%, respectively) in SLE patients and healthy donors (Fig. 13B). Thus, both in SLE patients and healthy individuals, activated and effector memory Treg are found in the CD25hi Treg subset, while CD25int and CD25neg Treg are comprised mainly of Treg with a naïve or central memory phenotype.

3.3.2 CD25hi Treg are highly proliferative

As the homeostasis between Treg and Tcon seems to play an important role in the counter-regulation of autoimmunity, the proliferation within the different Treg subsets was analyzed. In fact, proliferating cells were highly enriched in the CD25hi Treg subset, with 30% of CD25hi Treg being Ki67+ in both SLE patients and healthy controls (Fig. 13C). In contrast, the CD25int and CD25neg Treg compartment showed lower levels of proliferation (9.8 and 15.3%, respectively).
### 3 Results

#### 3.3.3 Treg with a suppressive phenotype are enriched in the CD25hi Treg compartment

Treg confer their suppressive function by several different mechanisms. One of these mechanisms is the hydrolysis of extracellular ATP to ADP and AMP by the ectonuclease CD39 which works in concert with CD73 [85]. Analysis of the Treg subsets for expression of CD39 revealed that almost all CD25hi Treg expressed CD39 (85.6% in SLE and 90.4% in HC). In contrast, only 40 to 50% of CD25int and CD25neg Treg were CD39+ (Fig. 13C).

In summary, activated, proliferating, and suppressive effector Treg are strongly associated with high CD25 expression levels. Thus, these data show that the level of CD25 expression serves as an indicator for the activatory and functional state of the Treg population. Consequently, the loss of CD25hi expressing Treg in response to IL-2 deprivation, leads to a deficit of highly metabolically active and suppressive Treg in SLE.

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**Figure 13: Enrichment of activated and suppressive Treg in the CD25hi subset.** Foxp3+CD127lo Treg from purified PBMCs were subgated into CD25hi (dark gray), CD25int (gray) and CD25neg (light gray) Treg and the frequencies of (A) CCR7+CD45RO- naïve, CCR7+CD45RO+ central memory (CM) and CCR7-CD45RO+ effector memory (EM) cells (n=48(SLE), 43(HC)), (B) CD137+ cells (n=9(SLE), 7(HC)), (C) Ki67+ cells (n=61(SLE), 51(HC)), and (D) CD39+ (n=56(SLE), 48(HC)) cells were compared between the different Treg subsets (Wilcoxon signed-rank test) or between healthy control (HC) and SLE patient samples (Mann-Whitney test, *=p>0.05, **=p<0.01, ***p<0.001)
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3.4 Investigating the potential of low-dose IL-2 to selectively reverse Treg defects in vitro

As demonstrated in sections 3.2 and 3.3, Treg biology is affected by the IL-2 deficiency in SLE in terms of reduced CD25 expression. This was associated with a relative loss of functionally, and metabolically active Treg, and a concomitantly disturbed homeostasis between Treg and Tcon. Next, it was thus investigated whether IL-2-supplementation could be a suitable tool to restore CD25 expression and thus to reconstitute a healthy Treg phenotype.

IL-2 is a pleiotropic cytokine, which potentially also causes activation or expansion of effector lymphocytes including CD4+Foxp3- Tcon, CD8+ T cells or NK cells, bearing high- or intermediate-affinity IL-2-R variants. Therefore, besides the aim to determine the potential of IL-2 to restore a functional Treg phenotype, the goal was also to determine the Treg-directed selectivity of a prospective IL-2 treatment. Treg-selective effects of low-dosed IL-2 have been demonstrated in a clinical study with patients suffering from HCV-induced vasculitis [154]. In that study, daily doses of 1.5 and 3.0 million IU IL-2 were subcutaneously administered in 5-day treatment cycles. This treatment regimen was adopted for a pre-clinical assay to analyze the effects of IL-2 on PBMCs from SLE patients. According to Smith et al, subcutaneous administration of 16.7µg/m² IL-2 (total 0.5 million IU) results in peak serum levels of 25pmol/l (or 0.38ng/ml) IL-2 [228]. Accordingly, PBMCs were stimulated with 1.0ng/ml, 2.5ng/ml, 4ng/ml and 10ng/ml IL-2 in vitro on five consecutive days in order to reflect a prospective in vivo treatment regimen with IL-2 doses of 1.5 to 4.5 million IU. PBMCs were analyzed 24h after the first and the fifth (last) stimulation with IL-2 by flow cytometry and compared to untreated samples.

3.4.1 Low-dose IL-2 selectively induces CD25 expression in Treg in vitro

Stimulation of PBMCs with IL-2 in vitro caused an increase of Foxp3 and CD25 expression levels (MFI) in Treg of SLE patients and resulted in significantly increased frequencies of CD25hi cells among Foxp3+CD127lo Treg (Fig. 14A). Frequencies of CD25int and CD25neg cells among Treg were diminished. 24h stimulation with 2.5ng/ml IL-2 induced the most prominent effects (45.5% CD25hi), while repetitive stimulation and stimulation with increasing doses of IL-2 were less effective.

Overall CD25 expression levels (MFI) in the Foxp3- Tcon population were not augmented by IL-2 stimulation (Fig. 14B). Nevertheless, the frequency of CD25hi cells among Tcon was increased, but in contrast to Treg, this effect was dose-dependent after 24h, showing the most prominent effects with 4ng/ml (0.8%) and 10ng/ml IL-2 (0.7%). After repetitive stimulation with IL-2 for 5 days the increase of CD25hi cells among Tcon was not as strong as after 24h.

Stimulation of PBMCs from healthy controls with IL-2 had similar effects as in SLE patient samples concerning the induction of CD25 expression in Treg (Fig. A1 of the appendix). Also here, low IL-2 doses were most efficient. However, the final percentage of CD25hi cells among Treg reached higher levels (62%) as compared to SLE patient samples. In healthy control samples, IL-2 stimulation did also cause a transient dose-dependent increase of CD25hi cells among Tcon, while no overall increase of CD25 expression levels was observed in Tcon.

Thus, low concentrations of IL-2 can selectively increase CD25 expression in Treg from SLE
Figure 14: IL-2 induces CD25 expression in Treg in vitro. PBMCs from SLE patients (n=10) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) in vitro and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. Representative dot-plots show the expression of CD25 among Foxp3+CD127lo Treg (A) and Foxp3- Tcon (B) in 24h and 5d samples, which were left unstimulated or stimulated with 2.5ng/ml IL-2. Bar-diagrams show the fold-change of CD25 expression levels (MFI) of IL-2-stimulated samples compared to unstimulated samples (A+B, left), and the frequencies of CD25hi cells among Treg or Tcon (A+B, right) of differently treated samples. Bars indicate the median with interquartile range. Wilcoxon signed-rank test was used to analyze changes induced by IL-2 compared to untreated samples (asterisks above error bars) or between different IL-2 concentrations (asterisks above horizontal lines, *=p>0.05, **=p>0.01, ***p=<0.001).
patients without inducing a strong reaction in the Tcon population. And, as suggested also in section 3.2.2, Treg from SLE patients have a similar capacity to up-regulate CD25 upon IL-2 signaling as Treg from healthy donors.

### 3.4.2 IL-2 restores the CD25 expression in CD25neg Treg in vitro

The above analyses showed that CD25 expression could be increased in Treg from SLE patients by stimulation with low concentrations of IL-2. However, whether the increase of CD25 expression in Treg could be attributed only to cells that were already CD25+ before stimulation, or whether CD25 expression could actually be restored also in the CD25neg Treg compartment, remained to be resolved. Thus, in a next experiment CD4+CD127loCD25- T cells were FACS-sorted from PBMCs of SLE patients and subjected to in vitro stimulation with IL-2. As depicted in Figure 15A these CD4+ CD127loCD25- cells contained a considerable percentage of Foxp3+ cells. Stimulation with IL-2 for 24h resulted in up-regulation of CD25 expression among these Foxp3+ cells resulting in 30% CD25+ cells among the formerly CD25 negative Foxp3+ Treg population (Fig. 15B).

These experiments demonstrated that the CD25 expression in Treg from SLE patients can be restored also from the CD25neg Treg subset by stimulation with low IL-2 doses.

**Figure 15: IL-2 restores CD25 expression in CD25neg Treg.** (A) FACS-sorting strategy (left) and Foxp3 expression (right) of CD3+CD4+ CD127loCD25- T cells isolated from SLE patients’ PBMCs. (B) Dot-plots of one representative experiment (n=3) showing the expression of CD25 among Foxp3+ cells of CD127loCD25- sorted T cells after 24h cultures with and without stimulation with different concentrations of IL-2.
3.4.3 Low-dose IL-2 only marginally influences proliferation of Treg \textit{in vitro}

IL-2 is especially important for the homeostasis of Treg, and a disturbance of the homeostatic balance between Treg and Tcon has been observed in SLE patients [3.2.4]. Therefore, the effects of IL-2 stimulation on the proliferation of Treg and Tcon were addressed. Analysis of Ki67 expression revealed that the frequencies of proliferating cells were reduced by approximately 2/3 in both Treg and Tcon after 24h \textit{in vitro} cultures without IL-2 stimulation, when compared to the \textit{ex vivo} analyses, and almost no Ki67+ cells were detectable after 5 days of culture (Fig. 16 white bars, compare to Fig. 10A+B). These observations were similar in samples from healthy donors (not shown). 24h stimulation with IL-2 was able to rescue proliferation to a small degree in the Treg population. This was only significant in healthy control samples (not shown), but a tendency was also detectable in samples from SLE patients stimulated with 1 and 2.5ng/ml IL-2 (Fig. 16 left). In contrast, IL-2 did not increase the percentage of proliferating cells in the Tcon population (Fig. 16 right). In additional experiments PBMCs from SLE patients were labeled with CFSE prior to IL-2 stimulation, in order to detect proliferating cells by CFSE dilution. Also here, no or only minor CFSE dilution, was observed upon stimulation with IL-2 \textit{in vitro} (not shown). Thus, \textit{in vitro}, IL-2 if at all only marginally influenced the proliferation of Treg and Tcon in the absence of further stimuli.

\begin{figure}[h]
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\caption{IL-2 marginally influences proliferation of Treg \textit{in vitro}. PBMCs from SLE patients (n=10) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) \textit{in vitro} and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. Bar-diagrams show the percentage (median + interquartile range) of Ki67+ cells among Foxp3+CD127lo Treg (left) and among Foxp3- Tcon (right) of untreated and IL-2 stimulated samples. Wilcoxon signed-rank test was used to analyze changes induced by IL-2 compared to untreated samples.}
\end{figure}
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3.4.4 IL-2 stimulation selectively induces pro-survival signals in Treg in vitro

In addition to the proliferative activity, also the balance between survival and apoptosis determines whether an adequate homeostatic balance between Treg and Tcon is met. As shown above, increased levels of apoptosis among Treg in SLE patients were likely due to the lack of survival factors (3.2.5). The anti-apoptotic protein Bcl-2 is a known transcriptional target of IL-2 signaling [229] and is therefore expected to be influenced by low-dose IL-2 stimulation.

Indeed, flow-cytometric analyses showed that Bcl-2 expression was increased in the Treg population after both single and repetitive stimulation with any dose of IL-2 (Fig. 17A, left). In contrast, the effect of IL-2 stimulation on Bcl-2 expression in the Tcon population was more modest, and here the effect was dose-dependent (Fig. 17A, middle). Thus, the ratio between Bcl-2 expression levels in Treg and Tcon, which is typically around 0.6 both in healthy controls and SLE patients, was increased to 1.1 by 5-day repetitive IL-2 stimulation in vitro (shown for SLE samples in Fig. 17A, right).

This selective effect of IL-2 on Bcl-2 expression levels in Treg compared to Tcon was also reflected by an improved Treg recovery: While the total numbers of CD4+ T cells remained stable throughout 5-day cultures with and without IL-2 stimulation, the frequency of Foxp3+CD127lo Treg among CD4+ T cells increased with IL-2 stimulation (Fig. 17B), indicating a preferential survival of the Treg population in response to IL-2 signals.

Analysis of the Treg subsets according to their CD25 expression revealed further that the ex vivo expression of Bcl-2 was lower in the CD25hi Treg compartment compared to the CD25int and CD25neg Treg populations in both healthy controls and SLE patients (Fig. 17C, white boxes). Conversely, stimulation with IL-2, exemplarily shown for 5-day stimulation with 2.5ng/ml in Figure 17C, induced Bcl-2 expression primarily in Treg expressing high and intermediate levels of CD25 but less in the CD25neg Treg subset.

In summary, these experiments show that IL-2 stimulation in vitro selectively improves the survival of Treg. In addition, they indicate that IL-2 in the applied concentrations preferentially induces IL-2 receptor signaling in cells with intermediate to high CD25 expression levels. This is in line with the finding that IL-2 in low concentrations selectively targets Treg, while affecting the Tcon population to a lesser extent.
Figure 17: IL-2 improves Treg survival in vitro. PBMCs from SLE patients (n=10) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) in vitro and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. (A) Bar-diagrams (median + interquartile range) show the fold-change of Bcl-2 expression levels (MFI) in Treg and Tcon (left), and the ratio between Treg Bcl-2 MFI to Tcon Bcl-2 MFI (right) of IL-2-stimulated samples in comparison to unstimulated samples. (B) Absolute numbers of CD3+CD4+ T cells per 100µl culture medium (left), and frequencies of Foxp3+CD127lo Treg among CD3+CD4+ T cells (right) of differently treated samples. (C) Box-plots indicate the Bcl-2 MFI of CD25hi, CD25int and CD25neg Treg before (ex vivo) and after 5-day stimulation with 2.5ng/ml IL-2. Wilcoxon signed-rank test was used to analyze changes induced by IL-2 compared to untreated samples (asterisks above error bars) or between different IL-2 concentrations (asterisks above horizontal lines, *p>0.05, **p>0.01, ***p<0.001).
3 Results

3.5 Assessment of the effects of IL-2 stimulation on other lymphocyte subsets

in vitro

IL-2 is a pleiotropic cytokine, which confers signals to a variety of cells expressing the high- or intermediate-affinity IL-2 receptor complex. These cells include besides CD4+ T cells also B cells, NKT cells, CD8+ T cells and NK cells, which were therefore also analyzed for changes in proliferation, Bcl-2 and CD25 expression after stimulation of PBMCs with different doses of IL-2 in vitro. The gating strategies for CD3-CD56+ NK cells, CD3+CD56+ NKT cells, CD3-CD19+ B cells and CD3+CD8+ T cells are shown in Figure 18.

Figure 18: Gating strategy for lymphocyte subsets. Dot-plots representing the flow-cytometric definition of CD3-CD56+ NK cells, CD3+CD56+ NKT cells, CD3-CD19+ B cells, CD3-CD56- CD8+ T cells and CD3-CD56- CD4+ T cells.

3.5.1 B cells, CD8+ T cells and NKT cells are marginally affected by IL-2 stimulation

in vitro

24h stimulation with IL-2 had no effects on the expression of CD25, Bcl-2, and Ki67 of CD3-CD19+ B cells, CD3+CD8+ T cells and CD3+CD56+ NKT cells, and only minor effects were observed upon 5-day repetitive stimulation (Fig. 19). In CD3-CD19+ B cells a slight increase of CD25 expression levels was detectable after repetitive stimulation with low IL-2 doses. However, no effects on proliferation and Bcl-2 expression were observed here. Except for a slight increase of proliferation after repetitive stimulation with 10ng/ml IL-2, CD8+ T cells were not affected by IL-2 stimulation concerning CD25 and Bcl-2 expression. In CD3+CD56+ NKT cells, an increase of Bcl-2 expression after repetitive IL-2 stimulation was observed; however, this effect was less prominent in comparison to that observed in the Treg population and rather comparable to the response of the CD4+Foxp3- Tcon population. Thus, concerning the analyzed parameters in vitro IL-2 stimulation only marginally influenced these lymphocyte subsets.
Figure 19: Marginal effects of IL-2 on B cells, CD8+ T cells and NKT cells in vitro. PBMCs from SLE patients (n=10) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) in vitro and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. Bar-diagrams (median + interquartile range) show the fold-change of CD25 expression levels (A), the frequencies of Ki67+ cells (B) and the fold-change of the Bcl-2 MFI (C) among CD3-CD19+ B cells (left), CD3+CD8+ T cells (middle), and CD3+CD56+ NKT cells (right) of IL-2-stimulated samples in comparison to unstimulated samples (Wilcoxon signed-rank test, *=p>0.05, **=p>0.01, ***p=<0.001).
3.5.2 NK cells from SLE patients respond to IL-2 in a dose-dependent manner *in vitro*

CD3-CD56+ NK cells constitutively express the intermediate-affinity IL-2 receptor [114]. Although IL-2 signaling is not essential for NK cell development and maintenance, IL-2 has been shown to induce proliferation and activation of NK cells *in vitro* [114, 212]. No or only marginal effects on proliferation, survival, CD25 (not shown) and CD56 expression were observed in the CD3-CD56+ NK cell population in response to 24h stimulation with IL-2 *in vitro* (Fig. 20A+B). Unlike CD4+ T cells, frequencies and total numbers of NK cells strongly declined in both healthy control and SLE samples during 5-day resting cultures without addition of IL-2 (Fig. 20A). Conversely, in repetitively IL-2-stimulated samples, NK cell frequencies and numbers were rescued by IL-2 in a dose-dependent manner, and this was also reflected by increased Bcl-2 expression levels compared to unstimulated samples (Fig. 20A).

In SLE samples, repetitive stimulation with IL-2 also caused a dose-dependent increase of the percentage of CD56bright cells among NK cells (Fig. 20B). Analysis of Ki67 expression revealed that this was likely to be due to preferred proliferation of CD56bright NK cells in response to stimulation with IL-2 when compared to the CD56dim NK cell population (Fig. 20C). While proliferation was completely lost during 5-day cultures without IL-2, it was dose-dependently maintained by repetitive stimulation with IL-2.

Equally performed analyses in samples from healthy controls revealed a similar dose-dependent expansion of the CD56bright NK cell subset and preferred proliferation of these cells in response to repeated IL-2 stimulations (not shown).

However, the size of the NK cell subsets differed between SLE patients and healthy controls. *Ex vivo* phenotyping revealed that SLE patients had significantly lower levels of total CD3-CD56+ NK cells among peripheral lymphocytes as compared to healthy controls (6.3% vs 18.9%). Conversely, but in accordance with another study [230], the percentage of CD56bright cells among NK cells was significantly higher in SLE patients (15.4% vs 3.5%). In addition, NK cells from SLE patients also showed higher frequencies of proliferating cells *ex vivo* compared to healthy controls (18.5% vs 3.8%, Fig. 20D).

In summary, in addition to Treg, CD56bright NK cells comprise the most responsive lymphocyte subset to IL-2 stimulation. NK cell responses to IL-2 stimulation were, however, dose-dependent and only observed upon repetitive stimulation. This is in contrast to Treg, which responded vigorously to low IL-2 concentrations already after a single stimulation. Thus, a selective targeting of Treg *in vitro* could still be achieved by limiting the IL-2 concentrations and exposure time. Nonetheless, these data also indicate a disturbance of the NK cell composition in SLE patients, and reflected a high dependency of NK cells on γ-chain cytokine signaling.
Figure 20: Dose-dependent response of NK cells to IL-2 in vitro. PBMCs from SLE patients (n=10) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) in vitro and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. Bar-diagrams (median + interquartile range) show frequencies and absolute numbers of CD3-CD56+ NK cells among lymphocytes (A, left), the fold-change of Bcl-2 expression levels among NK cells (A, right), the frequencies of CD56bright cells among NK cells (B) and the frequencies of Ki67+ cells among CD56bright (C, left) or CD56dim (C, right) NK cells in IL-2-stimulated samples in comparison to unstimulated samples. Wilcoxon signed-rank test was used to analyze changes induced by IL-2 compared to untreated samples (asterisks above error bars) or between different IL-2 concentrations (asterisks above horizontal lines, *=p>0.05, **=p>0.01, ***p<0.001). (C) The frequencies of CD3-CD56+ NK cells among lymphocytes (left), and of CD56bright cells (middle) and Ki67+ cells (right) among NK cells were compared between healthy control (HC, n=8) and SLE patient samples in PBMCs ex vivo (Mann-Whitney test).
3.6 Clinical translation of low-dose IL-2 therapy for the treatment of SLE

The in vitro experiments have shown that low CD25 expression in Treg from SLE patients can be selectively recovered with low doses of IL-2 (section 3.4). In addition, they demonstrated that IL-2 in low concentrations had a good selectivity for Treg, with only marginal effects on Tcon and other lymphocytes, with the exception of CD56bright NK cells.

The above presented results, together with pre-clinical studies in lupus mice ([148], Rose et al in preparation) and clinical data published by Saadoun et al and Koreth et al [153, 154], provided the basis for a clinical translation of low-dose IL-2 therapy for the treatment of SLE patients with the aim to restore the pool of suppressive CD25hi Treg.

A low-dose IL-2 treatment strategy was chosen based on the treatment regimen that was used in the study by Saadoun et al. This treatment regimen consisted of four 5-day treatment cycles that were separated by wash-out periods of 9-16 days (Fig. 21). Each 5-day treatment cycle consisted of daily subcutaneous IL-2 injections, starting with 1.5 million IU IL-2 (Proleukin®, aldesleukin) per day in the first cycle. The daily dose was scheduled to be increased to 3.0 million or 4.5 million IU IL-2/day in the subsequent treatment cycles, if the previous dose was tolerated without adverse events and the percentage of Foxp3+CD127lo Treg among CD4+ T cells did not exceed 50%. In case of adverse events or an expansion of Foxp3+CD127lo Treg to more than 50% the IL-2 dose was reduced by half for the following treatment cycle.

Low-dose IL-2 treatment schedule

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Figure 21: Low-dose IL-2 treatment regimen. The IL-2 treatment regimen consists of four 5-day cycles with daily subcutaneous (s.c.) injections of IL-2. Immunologic effects were monitored daily throughout cycle 1 (days 1-6), immediately before cycles 2-4 (days 15, 36, 57) as well as 20h after the last injection of each cycle (days 6, 20, 41, 63), and three weeks after the last IL-2 injection (day 83+/5).

During the course of this thesis, the immunologic effects of low-dose IL-2 therapy, with emphasis on the Treg population, were monitored in five SLE patients undergoing therapy with the above described low-dose IL-2 regimen either 'off-label' ('individueller Heilversuch') or as part of a clinical trial (PRO-IMMUN, EudraCT-Number: 2013-001599-40, [218]). Prior to the start of the low-dose IL-2 treatment the five patients suffered from severe SLE and were either refractory or intolerant to a variety of approved and also experimental therapeutic interventions. The patients presented with substantial skin inflammations, as well as arthritis, myositis, and high hematologic and serologic disease activity. The clinical course before, during and after the IL-2 therapy, including the assessment of adverse events, clinical response parameters, and serological parameters (e.g. anti-dsDNA antibody levels) was monitored by the medical staff (J. Humrich, E. Siegert) of the Rheumatology Unit of the Charité University Hospital. All patients completed the four treatment cycles, which were generally well tolerated. Only mild and transient adverse events, such as fever and sweats, occurred during low-dose IL-2 treatment in
all patients. A response, concerning the improvement of clinical symptoms (e.g. amelioration of skin manifestations) and serological parameters (e.g. reduction of anti-dsDNA antibody levels) was achieved in patients 1, 2 and 4. No obvious clinical response was observed in patients 3 and 5.

3.6.1 Response of Treg to short-term (5-day) low-dose IL-2 treatment during the first treatment cycle

The key aim was to investigate the potential of IL-2 to selectively expand the Treg population and to restore their CD25 expression in SLE patients. Primary analyses were therefore focused on changes within the Treg population during IL-2 therapy. During the first treatment cycle with daily injections of 1.5 million IU IL-2, the response of the Treg population was tightly controlled by flow-cytometric analyses on a daily basis.

The percentage of Foxp3+CD127lo Treg among CD3+CD4+ T cells increased up to 2-fold during the first treatment cycle to 30-44% at day 6 in the five patients, and this was accompanied by a similar rise of absolute Treg counts per µl whole blood (Fig. 22A,B). In parallel, a strong induction of CD25 expression (up to 3-fold) was observed in the Treg population, and the frequencies of CD25hi cells among Foxp3+CD127lo Treg were augmented from below 12% before treatment to 30-65% on day 6 (Fig. 22A,C).

In patients 1, 2 and 5 an initial drop in Treg numbers and frequencies was observed after the first two days of treatment (Fig. 22B). However, Treg recovered during the next treatment days and reached similar levels as in the other two treated patients. These patients also showed a delayed induction of CD25 expression levels in Treg; their CD25 MFI increased only after the 3rd and 4th IL-2 injection, but then continued to rise with every further injection. In contrast, in patients 3 and 4 CD25 levels were strongly increased already after the 2nd injection (day 3), and rose more moderately after the 3rd injection.

Unlike in vitro, where IL-2 stimulation had only marginal effects on the proliferation of T cells (section 3.4.3), low-dose IL-2 treatment in vivo strongly increased the percentage of Ki67+ cells among the Treg population from below 22% at baseline to 38-62% at day 6 (Fig. 22D). Interestingly, the increase of Treg proliferation in the different patients followed a similar pattern reflecting the respective augmentation of Treg frequencies and CD25 expression levels: Treg from patients 3 and 4 presented a gradual increase of proliferation starting already from the first IL-2 injection, while Treg from patients 1, 2 and 5 showed a delayed induction of proliferation starting on day 5.

The delayed response in patients 1, 2 and 5 coincided with comparatively low CD25 expression levels before the start of treatment, but had no effect on the final response at day 6. Thus, although the patients showed different response kinetics, probably attributable to their initial level of IL-2 receptor expression, daily analysis during the first treatment cycle showed that 5-day low-dose IL-2 treatment induced a robust expansion of Foxp3+CD127lo Treg with high CD25 expression levels in all five patients.
3 Results

![Figure 22: Expansion of CD25hi Treg in response to short-term low-dose IL-2 treatment.](image)

PBMCs from five SLE patients were analyzed daily throughout the first 5-day treatment cycle immediately before the first and approximately 20h after each subcutaneous injection of 1.5 million IU IL-2 (Proleukin®). (A) Representative dot-plots showing Foxp3+CD127lo Treg and Foxp3- Tcon (left) and CD25 expression (right) among CD3+CD4+ T cells before (day 1) and after the first treatment cycle (day 6). Frequencies of Foxp3+CD127lo Treg among CD4+ T cells and absolute numbers per µl whole blood (B), CD25 expression levels (MFI) and frequencies of CD25hi cells (C), as well as frequencies of Ki67+ cells (D) among Foxp3+CD127lo Treg are shown for each patient during the first IL-2 treatment cycle.

3.6.2 Treg are the preferential responders to low-dose IL-2 treatment in vivo

To address the selectivity of the IL-2-induced Treg expansion, also Tcon and other lymphocyte subsets were tightly monitored during the first treatment cycle in all patients. Although some alterations were observed during the first treatment cycle with low-dose IL-2, absolute numbers of Foxp3- Tcon were not increased at day 6 compared to baseline (Fig. 23A). Also no induction of the overall CD25 expression levels (CD25 MFI) among the Foxp3- Tcon population was observed in any of the five patients (Fig. 23B). As in the in vitro experiments, the frequency of CD25hi cells among Tcon was slightly increased in four of the five patients (up to 0.8%, Fig. 22A and 22C). Only in patient 3 the percentage of CD25hi cells among Tcon reached 1.9% after the second IL-2 injection (day 3) and persisted at 1.3% up to day 6 (Fig. 23C).
3 Results

Figure 23: Response of Tcon to short-term IL-2 treatment. PBMCs from five SLE patients were analyzed daily throughout the first 5-day treatment cycle immediately before the first and approximately 20h after each subcutaneous injection of 1.5 million IU IL-2 (Proleukin®). Absolute numbers of CD4+Foxp3-Tcon per µl whole blood (A), CD25 expression levels (MFI) (B) and frequencies of CD25hi cells (C) among Foxp3- Tcon are shown for each patient during the course of the first IL-2 treatment cycle.

Analysis of Ki67 expression revealed that low-dose IL-2 treatment also caused an induction of proliferation in the Tcon population, resulting in a proportion of 11-35% Ki67+ cells at day 6 (Fig. 24A+B). In patients 1, 4 and 5 the induction of proliferation was however stronger in the Treg compartment, thus an improved Treg/Tcon proliferation ratio was observed in these patients at day 6 compared to the baseline (Fig. 24C). In patient 3, low-dose IL-2 induced the proliferation of Treg and Tcon to similar levels, such that the Treg/Tcon proliferation ratio did not change. In contrast, patient 2 showed a stronger proliferative response of Tcon compared to Treg, resulting in a reduction of the Treg/Tcon proliferation ratio during the first 5-day treatment course.

Analysis of other lymphocyte subsets revealed that also CD8+ T cells, NKT cells and NK cells showed augmented proportions of proliferating cells at day 6 compared to the baseline (Fig. 24D, left). In some cases, the fold-increase of proliferation among T cells or NK cells even exceeded that of proliferating Treg. However, only in the Treg population, the strong proliferation also resulted in increased frequencies among total lymphocytes at day 6 (up to 2-fold), while frequencies of other lymphocyte subsets remained stable or declined slightly (Fig. 24D, right). As shown in section 3.6.1 also absolute cell numbers per µl whole blood were uniformly increased in the case of the Treg population, whereas changes in cell numbers of other lymphocyte subsets varied strongly among the treated patients and no uniform trend was observed (not shown).
Figure 24: Preferential expansion of Treg in response to low-dose IL-2 treatment. PBMCs from five SLE patients were analyzed daily throughout the first 5-day treatment cycle immediately before the first and approximately 20h after each subcutaneous injection of 1.5 million IU IL-2 (Proleukin®). (A) Representative dot-plots showing the expression of Ki67 among Foxp3+ and Foxp3- CD4+ T cells before (day 1) and after the first treatment cycle (day 6). Frequencies of Ki67+ cells among Foxp3- Tcon (B) are shown for each patient during the course of the first IL-2 treatment cycle. (C) Frequencies of Ki67+ cells among Foxp3+CD127lo Treg were divided by the frequencies of Ki67+ cells among Foxp3- Tcon to calculate the Treg/Tcon proliferation ratio (D). Bar-diagrams (Mean+SD) represent the percentage of Ki67+ cells (left) and the frequency among lymphocytes (right) of the indicated lymphocyte populations at baseline (gray bars) and after the 5-day treatment cycle (black bars).
In order to further estimate the selectivity of IL-2 signaling for specific cell subsets, phosphorylation of the downstream signaling molecule STAT5 was analyzed in CD4+Foxp3+ Treg in comparison to other lymphocyte subsets in response to IL-2 treatment. For this, freshly isolated PBMCs were stained with an antibody specific for the phosphorylated form of STAT5 (pSTAT5) and the fluorescence intensity, reflecting the amount of pSTAT5 in the cell, was compared between the lymphocyte subsets before and after IL-2 treatment. Before IL-2 treatment the ratio of pSTAT5 in Treg to that in Tcon was below 1 in all patients, indicating a higher amount of basal STAT5 phosphorylation in Tcon compared to Treg. 5-day IL-2 treatment caused a 1.3-1.5-fold increase of the Treg/Tcon pSTAT5 ratio in all patients, indicating that STAT5 phosphorylation was selectively increased in the Treg population compared to Tcon in response to IL-2 (Fig. 25 left). Similar observations were made when comparing STAT5 phosphorylation levels in Treg with pSTAT5 in CD8+ T cells, NK cells and B cells in patients 2 to 5. Also here, lower or similar pSTAT5 levels were present in Treg compared to other lymphocytes at baseline, but after 5-day low-dose IL-2 treatment a relative induction of STAT5 phosphorylation was observed in Treg compared to the other lymphocyte subsets (Fig.25).

In summary, analysis of the responses of Treg and other lymphocyte subsets revealed pleiotropic effects during 5-day low-dose IL-2 treatment, concerning the induction of proliferation in almost all subsets. Nonetheless, favored signaling and superior expansion of the Treg subset demonstrated that Treg were the preferential target of low-dose IL-2 also in vivo and could be efficiently expanded in the five SLE patients.

Figure 25: Selective STAT5 phosphorylation in Treg in response to IL-2. STAT5 phosphorylation was analyzed by flow cytometry in freshly isolated PBMCs from SLE patients before (day 0) and after the 5-day treatment cycle (day 6) with low-dose IL-2. Relative STAT5 phosphorylation was calculated by dividing the pSTAT5 MFI of CD4+Foxp3+ Treg by the pSTAT5 MFI of the indicated lymphocyte subsets (n=5 for Treg/Tcon, n=4 for other lymphocyte subsets).
3 Results

3.6.3 Dose-dependent induction of CD25 expression and proliferation of Treg during cyclic IL-2 treatment

Since the first 5-day treatment cycle with daily injections of 1.5 million IU IL-2 per day was well tolerated and the analysis of Treg, Tcon and other lymphocyte populations suggested a preferential expansion of Treg, the treatment regimen was continued as planned for all five patients. During treatment continuation the effects on peripheral Treg and Tcon were analyzed immediately before the first and approximately 20h after the last injection of each 5-day treatment cycle, as well as 3 weeks after the last day of treatment.

During the 9-day wash-out period after the initial treatment cycle overall Treg frequencies and CD25 expression levels declined, but remained above baseline levels (Fig. 26A,C). The percentage of proliferating cells among both Treg and Tcon was reduced to baseline levels at day 15 (Fig. 26B and 27A).

For the second treatment cycle, the daily IL-2 dose was increased to 3.0 million IU according to the treatment schedule. Treatment with 3.0 million IU IL-2 caused a stronger expansion of the Foxp3+CD127lo Treg population, reaching frequencies of 47-64% among CD4+ T cells on day 20 (Fig. 26A). Also the CD25 expression among Foxp3+CD127lo Treg and the percentage of proliferating Treg were further amplified during the second IL-2 treatment cycle, resulting in up to 80% of Treg expressing high levels of CD25 and more than 60% of Treg being Ki67+ in four of the five treated patients (Fig. 26B+C).

This massive Treg expansion to more than 50% among CD4+ T cells in most patients together with the occurrence of mild adverse events during the treatment cycle with 3.0 million IU IL-2 prompted a reduction of the IL-2 dose to 1.5 million IU per day for the third treatment cycle in all patients. This dose was maintained also for the last cycle, with the exception of patient 5, for whom the dose was further reduced to 0.75 million IU IL-2 due to persistence of a high Treg frequency of 55% following the third treatment cycle. This reduction of the IL-2 dose for the third and fourth treatment cycles resulted in a slightly less pronounced increase of Treg frequencies and induction of CD25 expression levels (MFI) compared to the 2nd cycle; however, higher levels were reached compared to the first treatment cycle with the same single doses of IL-2. In patients 1 and 2 again very high frequencies of CD25hi Treg (approximately 80%) were observed. Thus, it appeared that despite the decline of CD25 expression during the wash-out phases, Treg were primed during the previous treatment cycles and could respond more vigorously to following IL-2 stimulations.

Similar to the first two treatment cycles, proliferation of Treg was also strongly induced in the third and fourth IL-2 treatment cycles. However, the magnitude of this response differed from patient to patient. While all five patients had shown very similar proliferative responses during the first two treatment cycles, weaker Treg responses were observed during the following cycles in patients 3 and 5 compared to the other three patients. This concerned also the induction of CD25 expression levels and the frequencies of CD25hi Treg (Fig. 26B,C).

In summary, although the proliferative response to IL-2 appears to be relatively short-lived, a robust expansion of CD25hi expressing Treg can be induced also by repetitive cycles of low-dose IL-2 treatment, and the magnitude of this Treg response is dose-dependent.
3 Results

Figure 26: Dose-dependent expansion of CD25hi Treg during cyclic IL-2 treatment. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of Foxp3+CD127lo Treg among CD3+CD4+ T cells (A), of Ki67+ cells among Treg (B), and CD25 expression levels as well as frequencies of CD25hi cells among Treg (C) are shown for each patient during the course of the IL-2 treatment period.

3.6.4 Treg-selectivity is maintained upon dose escalation of IL-2

In contrast to Treg, the IL-2 dose escalation in cycle 2 did not further increase the proliferative response within the Foxp3- Tcon population (Fig. 27A). And interestingly, in the third and fourth treatment cycles levels of Tcon proliferation remained below those observed after the first treatment cycle. In addition, the CD25 MFI of Tcon remained unaffected by IL-2 treatment, independent of the applied dose (Fig. 27B, left). Nevertheless, in three patients an increase in the percentage of CD25hi cells among the Tcon subset up to 2.3% was observed after administration of 3.0 IU IL-2 per day (Fig. 27B, right). During the third and fourth treatment cycles the increase of CD25hi cells among Tcon was comparable to the first treatment cycle. Merely patient 3 showed an increase of CD25hi Tcon to up to 3%, which however seemed to be independent of IL-2, as this was observed immediately before the 4th treatment cycle and CD25hi Tcon levels dropped again during IL-2 treatment.
3 Results

Figure 27: Dose-independent Tcon proliferation in response to cyclic IL-2 treatment. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of Ki67+ cells among Foxp3- Tcon (A), and CD25 expression levels as well as frequencies of CD25hi cells among Tcon (C) are shown for each patient during the course of the IL-2 treatment period.

The proliferative response of CD8+ T cells, NK cells and NKT cells in response to IL-2 treatment was not further increased by the IL-2 dose escalation or by the cyclic treatment (Fig. 28A-C, right). In contrast, frequencies of CD8+ T cells among lymphocytes, which are often elevated in SLE patients, were reduced over the whole treatment period to the range of healthy controls and accompanied also by a drop of CD8+ T cell counts (Fig. 28A,D). Conversely, NK cell frequencies and numbers were increased, but only in those patients who had very low numbers of NK cells before the start of treatment (Fig. 28B,D). Frequencies and cell counts of NKT cells did not change during IL-2 treatment with the exception of patient 1, despite a proliferative response (Fig. 28C,D). In contrast, absolute numbers of Treg and especially CD25hi Treg were augmented in all patients when comparing cell counts in peripheral blood at the baseline (day 0) with levels after the last treatment cycle (day 62, Fig. 28D).

In summary, the expansion of CD25hi Treg could be safely induced in SLE patients also by repetitive treatment cycles without overly activating Tcon. Furthermore, the magnitude of the Treg response to IL-2 in vivo was dose-dependent, while at the same time dose escalation did not augment the response of Tcon or other lymphocytes subsets, indicating that also the highest administered dose of 3.0 million IU IL-2 per day was still in a range that selectively targets Treg in vivo.
Results

Figure 28: Effects of cyclic IL-2 treatment on different lymphocyte populations. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies among lymphocytes (left) and frequencies of Ki67+ cells (right) among (A) CD3+CD8+ T cells, (B) CD3-CD56+ NK cells, and (C) CD3+CD56+ NKT cells are shown for each patient during the course of the IL-2 treatment period. A black square with error-bars indicates the mean and range of frequencies among lymphocytes observed in healthy controls (n=8). (D) Absolute cell counts of the indicated lymphocyte subsets per µl whole blood before (day 0) and after the last treatment cycle (day 62) of the individual patients.
3 Results

3.6.5 IL-2 treatment restores the homeostatic Treg/Tcon balance

The *ex vivo* characterization of Treg biology had revealed a disturbance of the homeostatic balance between Treg and Tcon, which was associated with an IL-2-deprived Treg phenotype and high disease activity in SLE patients (section 3.2.4). Accordingly, it was of great interest, whether the homeostatic balance between Treg and Tcon could be restored by IL-2 therapy. As described above, low-dose IL-2 treatment substantially induced proliferation of Treg, but also Tcon proliferation was considerably incremented, and during the first 5-day treatment cycle the Treg/Tcon proliferation ratio was differently affected in the different patients. Calculation of the Treg/Tcon proliferation ratio throughout the following treatment cycles, however, showed that IL-2-induced proliferation in the Tcon population was mostly outweighed by induction of proliferation among Treg. This resulted in an improvement of the Treg/Tcon proliferation ratio in all five patients during the second to fourth treatment cycle (Fig. 29). Thus, treatment with several cycles of low-dose IL-2 had a beneficial effect on the homeostatic balance between Treg and Tcon by shifting the Treg/Tcon proliferation ratio in favor of Treg proliferation. And interestingly, this effect was even maintained for several weeks after treatment discontinuation in some patients.

![Figure 29](image-url)

**Figure 29: Low-dose IL-2 improves the Treg/Tcon proliferation ratio.** PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of Ki67+ cells among Foxp3+CD127lo Treg were divided by the frequencies of Ki67+ cells among Foxp3-Tcon to calculate the Treg/Tcon proliferation ratio, which is depicted for each patient during the course of the IL-2 treatment period.

3.6.6 Homeostatic and IL-2-induced proliferation of Treg is associated with low Bcl-2 expression levels

During the *in vitro* stimulation experiments a selective induction of anti-apoptotic Bcl-2 expression in Treg had been observed after IL-2 stimulation (section 3.4.4). Conversely, during low-dose IL-2 treatment cycles *in vivo*, we observed a reduction of Bcl-2 expression levels in Treg, whereas Bcl-2 expression in Tcon was not altered. Accordingly, the ratio between Bcl-2 expression in Treg and Tcon was transiently reduced from approximately 0.6 at baseline down to a minimum of 0.35 during the IL-2 treatment cycles (Fig. 30A). Again, the least prominent effects
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were observed in patient 3. Interestingly, no reduction of the Bcl-2 ratio was observed during the last treatment cycle of patient 5, which consisted of only 0.75 million IU per day, indicating that this effect was also dose-dependent.

The reduction of the Bcl-2 ratio was reciprocal to the above described changes of the proliferation ratio (compare Fig. 29C). Ex vivo analyses of Ki67 and Bcl-2 expression together revealed that proliferation was almost exclusively restricted to the Bcl-2lo subset in both Treg and Tcon before IL-2 treatment (Fig. 30B, day 1). IL-2 treatment caused an increase of Ki67+ cells among Bcl-2lo Treg and only to a small extent also in Bcl-2hi Treg. Conversely, proliferation of Tcon was mainly induced in the Bcl-2hi subset (exemplarily shown for the first treatment cycle in Fig. 30B). Thus, in vivo proliferation of Treg is associated with low Bcl-2 expression levels. However, whether Bcl-2 expression is lost upon proliferation, or whether low Bcl-2 expression in Treg is a prerequisite for preferential IL-2-induced expansion of this subset remains yet to be determined. Interestingly, with the exception of patient 1, no increase of active caspase-3 was observed among either Treg or Tcon after the IL-2 treatment cycles (Fig. A2 of the appendix). Thus, the low Bcl-2 expression levels upon IL-2 treatment were not associated with increased apoptosis of Treg.

Figure 30: Treg proliferation is associated with low Bcl-2 expression levels. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). (A) The Bcl-2 MFI among Foxp3+CD127lo Treg was divided by the Bcl-2 MFI among Foxp3- Tcon (Bcl-2 ratio) and is depicted for each patient during the course of the IL-2 treatment period. (B) Representative dot-plots show the expression of Bcl-2 and Ki67 among Foxp3+CD127lo Treg and Foxp3- Tcon before (day 1) and after the first IL-2 treatment cycle (day 6).
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3.7 Characterization of the in vivo IL-2-expanded Treg population

3.7.1 IL-2 expands thymic-derived genuine Treg in the periphery

To further characterize the expanded population of circulating Treg in IL-2-treated patients, it was addressed whether solely peripheral proliferation, or also increased thymic egress of Treg contributed to the expansion of the peripheral Treg pool. For this, Foxp3+CD127lo Treg were stained for CD31 in addition to CCR7 and CD45RO in order to identify naïve cells that recently emigrated from the thymus and are associated with a CCR7+CD45RO-CD31+ phenotype [231]. Here, differences between patients 1, 2 and 4, and patients 3 and 5 were noticeable already before the start of treatment. Patients 1, 2 and 4 had relatively low frequencies of recent thymic emigrants (RTE, 0.5-4.5%) among their Treg population, in comparison to patients 3 and 5 who exhibited higher frequencies of RTEs of 15 and 18% at baseline, respectively. In general, the proportion of RTEs among Treg decreased during IL-2 treatment cycles, and the magnitude of this effect was more prominent in the patients with high RTE starting values (Fig. 31A). During the wash-out phases, RTE frequencies among Treg recovered, and partly reached even higher levels compared to baseline.

Figure 31: IL-2-induced peripheral expansion of Helios+ Treg. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of CCR7+CD45RO-CD31+ recent thymic emigrants (A) and of Helios+ cells (B) among Foxp3+CD127lo Treg are shown for each patient during the course of the IL-2 treatment period. (C) Representative dot-plots show the expression of Ki67 in Helios+ and Helios- Foxp3+CD127lo Treg before (day 1) and after (day 62) IL-2 therapy.

Thus, the expansion of the overall Treg pool during IL-2 treatment seems to depend on peripheral proliferation, rather than on an increased thymic output of Treg. But also the contraction of the Treg population in between the treatment cycles appears to be restricted to the peripherally
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expanded pool, and might even be counter balanced by increased thymic output during the wash-out phases. Interestingly, peripheral RTE Treg did neither proliferate nor show high CD25 expression before IL-2 treatment. However, during IL-2 treatment cycles, also here frequencies of CD25hi Treg reached up to 40% and up to 60% of peripheral RTE Treg were Ki67+ (not shown).

It was further discriminated whether the peripherally expanded Treg population originated from genuine thymic-derived Treg or whether IL-2 possibly caused an induction of Foxp3 expression in conventional T cells. To this end, analysis of Helios revealed that the proportion of Helios expressing cells among Foxp3+CD127lo Treg was maintained at high levels (75-90%) during IL-2 stimulation in vivo (Fig. 31B). This was true also for the different Treg subsets, subdivided according to their CD25 expression levels (not shown). Furthermore, analysis of Ki67 expression revealed that Helios+ Treg were highly proliferative in response to IL-2, while no induction of proliferation was observed in the Helios- Treg subset (Fig. 31C), indicating that IL-2 expands the population of genuine Treg.

To further substantiate this finding, CD25+ Treg were FACS-isolated from three patients after the fourth IL-2 treatment cycle (day 62) to analyze the methylation status of the TSDR region of the foxp3 promoter. These analyses showed that 70-80% of the IL-2-expanded CD25+ Treg population had a demethylated TSDR, in contrast to the Tcon population, which retained a methylated TSDR (Fig. 32).

In summary, these findings demonstrate that IL-2 therapy results in the peripheral proliferation and expansion of Treg with a phenotype that is associated with thymic-derived bona fide Treg.

Figure 32: IL-2-expanded CD25+ Treg have a demethylated TSDR. CD3+CD4+ Foxp3+CD127loCD25+ Treg and CD3+CD4+ Foxp3- Tcon were FACS-isolated from PBMCs of three SLE patients after completion of four IL-2 treatment cycles (day 62) and subjected to TSDR methylation analysis. The percentage of CpG demethylated copies of the TSDR region is shown.

3.7.2 IL-2-expanded Treg are suppressive

Having shown the selective expansion of bona fide Treg and the induction of high CD25 expression levels, it was of great interest, whether these IL-2-expanded Treg were also functionally intact and suppressive.

Phenotypic analyses showed that, in parallel to the induction of CD25 expression, also the proportion of CD39+ cells increased among Treg during the IL-2 treatment cycles in all patients (Fig. 33A). This effect was independent of the percentage of CD39+ Treg before the start of the
therapy. And as indicated earlier (section 3.3), CD39 expression was strongly associated with high CD25 expression levels (Fig. 33A, right).

Similarly, also the frequencies of Treg expressing the activation marker CD137 were strongly increased during IL-2 treatment cycles, and CD137 expression was exclusively restricted to the CD25+/hi Treg subset (Fig. 33B). A less robust response concerning the expansion of CD137+ Treg was observed in patients 3 and 5 as compared to the other patients, reflecting earlier observations concerning the lower induction of CD25 expression and proliferation in Treg of these patients. Thus, IL-2-expanded CD25hi Treg also expressed high levels of surface molecules that are associated with the suppressive function and activation of Treg.

Figure 33: IL-2-expanded Treg express CD39 and CD137. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of (A) CD39+ and (B) CD137+ cells among Foxp3+CD127lo Treg are shown for each patient during the course of the IL-2 treatment period. Representative dot-plots show the expression of CD25 and (A) CD39 or (B) CD137 in Foxp3+CD127lo Treg before (day 1) and after IL-2 therapy (day 62).

In order to analyze whether the expanded Treg population was indeed able to suppress the proliferation of Tcon, in vitro suppression assays were performed at baseline (day 0) and after the 2nd treatment cycle (day 20) with Treg from patients 3, 4 and 5. For this, FACS-isolated and CFSE-labeled CD4+CD25-CD127+ responder cells (Tcon) were stimulated with bead-bound anti-CD3 and anti-CD28 antibodies and co-cultured with different dilutions of FACS-isolated CD4+CD127loCD25hi Treg (95-99% Foxp3+) from the same patient.

As shown in Figure 34A, CD25hi Treg from patient 4 were able to suppress Tcon proliferation by approximately 80-90% up to a Treg to Tcon ratio of 1:8 before and of 1:4 after IL-2 treatment. In contrast, lower (<60%) suppression was observed by Treg from patient 3 at baseline, which was, however, increased up to 80% after the second treatment cycle (Fig. 34B). No suppression was observed in assays with Treg from patient 5 before IL-2 therapy. However, at day 20, Treg from patient 5 were able to suppress Tcon proliferation by 20-30% at ratios of 1:1 and 1:2.
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Together, these data provide evidence that the CD25hi Treg population has suppressive capacities as expressed by both phenotypic and functional features, which are maintained or even improved during in vivo IL-2-dependendent expansion of this subset. Nonetheless, differences between the patients may also point towards possible intrinsic Treg defects in some patients, which might not be fully reversed by IL-2.

Figure 34: In vitro suppressive capacity of IL-2-expanded CD25hi Treg. CD3+CD4+ CD127+CD25-responder cells (Tcon) and CD127loCD25hi Treg were isolated from PBMCs of patients 3, 4 and 5 on day 0 and day 20 of IL-2 therapy. Tcon were labeled with CFSE and cultured either alone or together with Treg at Treg:Tcon ratios of 1:1 to 1:16. Cells were stimulated for 4 days with anti-CD3- and anti-CD28-coated beads before CFSE dilution was analyzed by flow-cytometry. The degree of Treg-mediated suppression (% suppression) was calculated as outlined in section 2.7, and is depicted for cells from (A) patient 4, (B) patient 3, and (C) patient 5 at day 0 (circles) and day 20 (squares). (A, right) Dot-plots show the CFSE dilution in Tcon after 4-day stimulation in culture either alone (left) or at a Treg:Tcon ratio of 1:4. Gates indicating the sequential cell generations.


3.7.3 Low-dose IL-2 treatment expands Treg expressing tissue-homing chemokine receptors

Cutaneous lupus has been associated with reduced numbers of Treg in skin lesions, compared to other chronic inflammatory diseases of the skin [232]. In addition, an altered migratory capacity via the skin-homing chemokine receptor CCR4 has been described for Treg in SLE [191]. Motivated by the finding that some of the IL-2-treated patients showed a significant and fast amelioration of skin-related symptoms in response to IL-2 treatment, it was aimed to analyze whether this phenomenon could be associated to changes in the potential of Treg to migrate to the skin. Thus, the expression of chemokine receptors responsible for homing to the skin and inflamed tissue, namely CCR4 and CCR6, was analyzed in Treg of SLE patients and healthy controls \textit{ex vivo}, and after IL-2 therapy of patients 3, 4 and 5.

Although the number of available samples was limited (n=5), these analyses revealed that the percentage of CCR4+ cells among Treg was similar in healthy controls and SLE patients, while CCR6+ Treg appeared to be lower in SLE patients (Fig. 35). However, during the IL-2 treatment cycles, the frequencies of both, CCR6+ and CCR4+ cells were increased among Treg. Thus, although the analyses are yet limited to phenotypic observation, it appears that IL-2 might improve the migratory potential of Treg.

![Figure 35: Increase of chemokine receptor expressing Treg by IL-2 therapy.](image)

(Frequencies of (A) CCR4+ and (B) CCR6+ cells among Treg from healthy donors (HC, n=5) in comparison to Treg from SLE patients (n=5, left), and among Treg from patients 3, 4 and 5 during the course of the IL-2 treatment cycles (right).)
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3.8 Characterization of Tcon and NK cells during low-dose IL-2 therapy

3.8.1 Loss of CD28- Tcon and reduction of Tfh cell frequencies

In section 3.6.4 it was demonstrated that also the Foxp3- Tcon population showed a considerable proliferative response to IL-2 treatment, although this did not lead to an overall expansion of the Tcon population. No obvious alterations were observed in the percentages of naïve, Tcm, Tem or Term cells among Tcon during the IL-2 treatment cycles in the five patients (not shown). Also, no considerable activation, as determined by CD25 (Fig. 27) and CD137 (not shown) expression was observed among Tcon.

Since IL-2 signaling is implicated in the control of CD4+ Th subset differentiation, proportions of Th1-differentiated (T-bet+CXCR3+CCR6-CCR4-), Th2-differentiated (CXCR3-CCR6-CCR4+) and Th17-differentiated (CXCR3-CCR6+CCR4+) cells among circulating effector memory Tcon were analyzed during IL-2 therapy. Although some alterations were observed during the treatment period, changes in the proportions of these Th cell subsets differed largely between the patients and also between the treatment cycles, and could therefore not be conclusively attributed to IL-2-dependent effects (not shown).

However, analysis of CD28 expression in patients 3 to 5, revealed that CD28- cells, which were exclusively found within the Th1 effector cell compartment, decreased continually throughout the IL-2 treatment period from 4-5% to approximately 1.5% among effector memory Tcon and remained low also after treatment discontinuation (Fig. 36A).

Finally, in patient 5, Tcon were also stained for the chemokine receptor CXCR5 in combination with CCR7 and CD45RO in order to determine the presence of circulating Tfh cells. Within the central memory Tcon compartment of this patient, CXCR5+ Tfh cells were continually reduced from 25% before the start of treatment down to 16% by the end of the last treatment cycle (Fig. 36B).

In summary, the exact role of IL-2 on Th cell subset differentiation cannot be answered yet, due to the limited amount of data. But the available results indicate that IL-2 treatment may cause a reduction of circulating Tfh cells and leads to the loss of CD28- EM T cells.

Figure 36: IL-2-induced reduction of Tcon subsets. PBMCs from patients 3, 4 and 5 were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration. Frequencies of (A) CD28- cells among CCR7-CD45RO+ effector memory (EM) Tcon and (B) CXCR5+ cells CCR7+CD45RO+ central memory (CM) Tcon are shown during the course of the IL-2 treatment period.
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3.8.2 Low-dose IL-2 treatment expands immature NK cells

As demonstrated in the in vitro stimulation experiments (section 3.5.2), CD3-CD56+ NK cells represented one of the main target populations of in vitro IL-2 signaling besides Treg. Therefore, the effects of low-dose IL-2 treatment on the NK cell population were analyzed in detail in the five treated SLE patients.

Proliferation of CD3-CD56+ NK cells was strongly enhanced by IL-2 treatment, reaching similar levels of Ki67+ cells as the Treg population (shown earlier in Fig. 24 and 28). Similar to the effects of in vitro IL-2 stimulations, preferential proliferation was observed in the CD56bright NK cell subset (up to 93% Ki67+) in response to IL-2 treatment as compared to CD56dim NK cells (up to 78% Ki67+, Fig. 37A). This high proliferative response of the CD56bright NK cell subset was uniformly detected in all five treated patients and was reflected by increased proportions of CD56bright cells among NK cells, as well as by augmented absolute numbers of CD56bright NK cells per µl blood in most patients after the IL-2 treatment cycles (Fig. 37B).

Further phenotypic investigations showed that the CD56bright NK cells lacked the expression of CD57, CD16 and KIRs, showed low expression levels of perforin and granzyme B, but expressed high levels of CD62L, CD122, IL18Rα and NKG2A. This is the typical phenotype of the CD56bright NK cell subset, representing less differentiated cells, which are associated with a high capacity to produce cytokines but low cytotoxicity [16, 233]. After IL-2 treatment, this phenotype was maintained among the expanded CD56bright NK cell population with even increased levels of CD62L expression (shown for CD62L, NKG2A, CD57 and perforin in Fig. 37C).

The CD56dim NK cell subset generally showed a more differentiated NK cell phenotype with large proportions of cells expressing CD16, CD57, KIRs and high levels of the cytolytic molecules perforin and granzyme B, and less cells expressing CD62L, IL18Rα or high levels of CD122 (Fig. 37C, and not shown). Conversely, also within the CD56dim NK cell compartment, an IL-2-dependent expansion was observed among the less differentiated cell subset: The proportions of cells positive for CD62L and NKG2A increased among the CD56dim NK cells, while the percentage of CD57+ and KIR+ cells among the CD56dim NK cells was reduced after IL-2 therapy (Fig. 37C). No distinct alterations were observed concerning the expression levels of perforin and granzyme B in either NK cell subset. Thus, these data indicate that IL-2 causes a preferential expansion of the less differentiated cytokine-producing NK cell subsets in SLE patients in vivo.
Figure 37: IL-2 expands immature NK cells. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after each 5-day cycle of low-dose IL-2 treatment, as well as three weeks after the last IL-2 administration. (A) Frequencies of Ki67+ cells among CD56bright (left) and CD56dim NK cells (right), and (B) percentages and absolute numbers of CD56bright cells are shown for each patient during the course of the IL-2 treatment period. (C) Representative dot-plots showing the expression of CD56 and CD62L, NKG2A, CD57 or perforin in NK cells before (day 0) and after IL-2 treatment (day 62).
4 Discussion

4.1 IL-2 deficiency in SLE

Deficient IL-2 production by exogenously stimulated T cells from SLE patients has been described more than 30 years ago [203, 204]. Here, it was aimed to investigate whether a reduced IL-2 availability could be detected in the sera of SLE patients. With the available immunoassays, which had a reported theoretical sensitivity for the detection of IL-2 of 3pg/ml, it was not possible to reliably detect IL-2 in the majority of serum samples, both from SLE patients and from healthy controls. Yet, the detection of IL-2 in serum samples from healthy controls, or patients with various diseases has been reported in the literature. However, also in those reports the measured IL-2 levels were just in the range of the lower detection limits [234–236]. Thus, in contrast to mice, where IL-2 can be readily detected in plasma [148], peripheral IL-2 levels in humans appear to be particularly low, which underscores the importance for its localized mechanism of action [138].

Therefore, the analysis of production of IL-2 in resting cultures of complete PBMCs, was used as an alternative approach to resemble the physiological setting in vitro. Here, lower production of IL-2 was found in SLE samples, which was independent of the frequency of CD4+ T cells. In addition, CD4+ T cells from SLE patients, which are the main producers of IL-2 in healthy individuals [107, 129], showed reduced IL-2 mRNA expression after resting cultures. Intrinsic defects of CD4+ T cell have also been described by others to be responsible for a defective IL-2 production in response to TCR stimulation in SLE. IL-2 transcription is activated by binding of pCREB to the promoter, and repressed by binding of phosphorylated CREMα [237]. In line with this, G. Tsokos and colleagues showed that stimulated T cells from SLE patients exhibited lower levels of pCREB and higher levels of pCREM, leading to reduced IL-2 expression in SLE patients [238, 239]. CD28-dependent signaling is necessary for full activation of CREB [240]. Accordingly, the high degree of chronic activation among T cells from SLE patients, as detected here by loss of CD28 expression, high Blimp-1 expression levels, and acquisition of a Th1 phenotype, might contribute to the low IL-2 expression in SLE T cells. It cannot be excluded that increased frequencies of Foxp3+ Treg among CD4+ T cells might suppress IL-2 production, lead to higher IL-2 consumption, or bias the number of IL-2-producing cells in SLE. However, the low CD25 expression levels and activity of the Treg population in SLE patients, as well as their lack of CD25 up-regulation during resting cultures do not support the first two possibilities. In addition, no correlation was observed between ex vivo Treg frequencies and 24h IL-2 mRNA expression in CD4+ T cells from SLE patients (not shown).

These findings, although not providing a direct proof for IL-2 deficiency in SLE in vivo, were highly indicative for a perturbed IL-2 production, as a result of chronic activation of CD4+ T cells in SLE.
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4.2 Treg phenotype and homeostasis reflect an in vivo IL-2 deficiency in SLE

Since IL-2 acts directly in a para- or autocrine fashion, deficient IL-2 production is likely to have effects at the local sites of immune reactions, even if changes in systemic IL-2 protein levels are not detectable. Thus, it was hypothesized that if IL-2 deficiency did play a role in SLE, this could be reflected by phenotypic changes in IL-2-dependent cells, namely in CD4+Foxp3+ Treg. Indeed, a primary hallmark of IL-2 deprivation, the loss of surface CD25 expression, was clearly visible in Treg from SLE patients, exhibiting low percentages of CD25hi and increased proportions of CD25neg cells. Although CD25 expression could not be directly linked to the in vivo availability of IL-2, low CD25 expression levels were attributed to reduced spontaneous IL-2 production by SLE lymphocytes in vitro.

Another hallmark of IL-2 deficiency, as revealed by studies with IL-2/-/- mice or IL-2-neutralization [111, 112] and our own work with lupus-prone mice that acquire an IL-2 deficiency [148], is a homeostatic imbalance between Treg and Tcon, where Treg are incapable to keep Tcon proliferation in check. This phenomenon was also evident in the SLE patient cohort, where Treg proliferation was not changed, but Tcon proliferation increased in association with disease activity, resulting in a significantly reduced Treg/Tcon proliferation ratio. Both hallmarks of IL-2 deficiency, the reduced CD25 expression among Treg and the disturbed homeostatic balance between Treg and Tcon, correlated with each other. This was also confirmed by the finding, that a high proliferative activity is associated with high CD25 expression levels in Treg. Further in this line, enhanced levels of apoptosis among Treg, which - in contrast to Tcon - could not be attributed to a high proliferative rate, corroborated the hypothesis that a substantial Treg survival factor is lacking in SLE.

Thus, together with the impaired in vitro IL-2 production by CD4+ T cells, the phenotypic and homeostatic perturbations of the Treg population provide strong evidence for a relevant IL-2 deficiency in SLE also in vivo.

4.3 Importance of IL-2 deficiency for SLE pathogenesis

The IL-2-dependent abnormalities in Treg biology were associated with a high disease activity in SLE patients, suggesting a role for IL-2 deprivation of Treg in the pathogenesis of SLE. In fact, a phenotype that is associated with activation (CD137+, Ki67+) and suppressive function (CD39+) of Treg was linked to high CD25 expression levels in Treg from both healthy individuals and SLE patients. Consequently, the lack of CD25hi Treg in SLE patients can be associated with a loss of highly metabolically active and suppressive Treg. Thus, IL-2 deficiency in SLE is reflected by a deficit in functionally active Treg, which in turn might contribute to the loss of peripheral tolerance and perpetuation of autoimmunity in SLE (Fig. 38). This is in line with our animal studies, where experimental induction of IL-2 deficiency by injection of IL-2-neutralizing antibodies resulted in acceleration of disease in lupus-prone mice in parallel to the loss of CD25+ Treg and activation of conventional T cells [148]. Accordingly, it was hypothesized that a correction of the IL-2 deficit might restore a healthy Treg phenotype and consequently re-establish Treg function in SLE.

As discussed in the introduction and later in this chapter, IL-2 is a pleiotropic cytokine with im-
important functions for the homeostasis and functional differentiation also of other lymphocytes besides Treg [106]. Thus, the loss of functional CD25hi Treg is most likely not the only mechanism of how IL-2 deficiency contributes to SLE pathogenesis. While our data suggest that low Treg CD25 expression could be used as a surrogate marker for a lack of IL-2, more systemic investigations in the form of transcriptome analyses of whole blood or of IL-2-responsive lymphocyte subsets could be a promising approach to estimate the global effects of IL-2 deficiency in SLE, similar to studies performed in IL2 deficient mice [111] and recently in IL-2-treated healthy volunteers [241]. Such investigations would allow to determine a global ‘IL-2-deprivation signature’ and possibly to detect new biomarkers to more accurately define the level of IL-2 deprivation in individual patients.

**Figure 38**: Simplified schematic overview of the proposed association between chronic activation of CD4+ T cells, IL-2 deficiency, defects in Treg biology and SLE pathogenesis. Deficient IL-2 production by CD4+ T cells in SLE can probably be attributed to chronic activation of CD4+ T cells, due to persistent autoantigen presentation and ongoing inflammation, which results in the loss of CD28 expression and up-regulation of the IL-2-repressor Blimp-1. IL-2 deficiency in turn leads to an impaired homeostatic balance between Foxp3+ Treg and Foxp3- Tcon with uncontrolled proliferation of the latter. IL-2 deprivation further results in loss of CD25 expression in Foxp3+ Treg, which is accompanied by reduced activation and suppressive function. Impaired Treg activity in turn contributes to the loss of self-tolerance and the progression of self-directed immunity, such as activation of autoreactive T and B cells and consequent autoantibody production. Whether IL-2 deficiency also impacts (regulatory) NK cell functions remains to be resolved.

### 4.4 Discrepancies between increased Treg frequencies and the IL-2 deprivation of Treg in SLE

Alterations in frequencies and numbers of Treg in SLE have also been described earlier [61]. However, inconsistent definition of the Treg population has led to conflicting results and interpretations. In earlier studies, where Treg were defined only on the basis of CD25 expression, mostly reduced numbers of CD4+CD25+ or CD4+CD25hi Treg were reported [189, 190]. In contrast, here, and also in other studies, increased frequencies of total Foxp3+ (CD127lo) Treg...
among CD4+ T cells have been demonstrated [79, 193, 195, 242]. Taking into consideration the low CD25 expression in Treg from SLE patients, as was demonstrated here and by others, the early studies most likely underestimated the number of total Treg since they excluded Foxp3+ CD25 negative or CD25 low Treg. Thus, these data underline the importance to define Treg by surrogate markers such as the master transcription factor Foxp3 when analyzing their quantities. CD25 expression itself is a phenotypic trait that is subject to change depending on the functional and metabolic state of the cell and is influenced by IL-2 availability.

Nonetheless, the high proportion of total Foxp3+CD127lo Treg among CD4+ T cells found in SLE appears to be contradictory to the reduced Treg/Tcon proliferation ratio and the concept that IL-2 deprivation impairs Treg homeostasis and functional activity in SLE. Several explanatory attempts for this discrepancy can be made:

One approach to explain this discrepancy is promoted by M. Miyara and H. Yang and colleagues. Their work suggests that the majority of the alleged CD25neg or CD25low Treg are in fact effector T cells with induced Foxp3 expression and the ability to produce effector cytokines [71, 224]. However, it was shown in the present study that all Treg subsets in SLE patients express Helios at very high frequencies, which in combination with Foxp3 has been associated with TSDR demethylation and absence of cytokine production, i.e. with bona fide Treg, in SLE [79, 80]. Although less TSDR demethylation was observed among CD25neg Treg, sorted from one SLE patient during this study, compared to the CD25+ population from the same patient, 56% were still demethylated, indicating that at least half of these CD25neg cells were genuine Treg. Nonetheless a larger number of samples will of course be necessary to substantiate this finding. In addition, at least a subset of the Foxp3+CD25neg population is able to up-regulate CD25 expression upon in vitro IL-2 stimulation to similar levels as Foxp3+CD25+ Treg. Our findings are also in line with investigations from M. Bonelli and colleagues who found that the majority of the enlarged subset of Foxp3+CD25neg cells in SLE expressed Helios [243]. Furthermore, they demonstrated that CD4+CD127-CD25- T cells from SLE patients, which contained up to 53% Foxp3+ cells, were able to suppress T cell proliferation but not IFN-γ production in vitro [223]. Thus, there is mounting evidence that the population of Foxp3+ CD127lo CD25neg cells in SLE patients comprises a large proportion of bona-fide Treg, which are probably in a metabolically and functionally impaired state. Accordingly, the expansion of the Foxp3+ T cell population in SLE cannot be exclusively attributed to an enhanced proportion of effector T cells with induced Foxp3 expression.

Another possible explanation for the expanded population of Foxp3+ Treg in SLE could be an increased thymic output of Treg. In fact, analyses in a limited number of SLE patients revealed a correlation between frequencies of RTE among Treg and the percentage of Treg among CD4+ T cells (n=7, r=0.714, not shown). Conversely, RTE Treg but not RTE Tcon frequencies correlated inversely with the age of the SLE patients (own observations) and generally decreased levels of recent thymic emigrant T cells have been associated with a high disease activity in SLE [244, 245]. Thus, a relative increase in the thymic output of Treg, but not Tcon might indeed be a mechanism of the immune system to counteract the homeostatic imbalance between
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Treg and Tcon in SLE. This finding is also supported by data from Humrich et al, who showed a progressive increase of Foxp3+ Treg frequencies and absolute numbers in the thymus of (NZBxNZW)F1 lupus mice [148].

In addition, other cytokines besides IL-2 might contribute to the survival of Treg, without rescuing their IL-2-dependent functional activity. In line with this, relatively high levels of Bcl-2 expression were observed in the CD25neg Treg subset when compared to their CD25+ counterparts. At the same time, CD25-Treg also expressed comparatively high amounts of CD127 (IL-7R) as opposed to Treg with higher CD25 and Foxp3 expression levels (not shown). Thus, other members of the common γ-chain family cytokines might play a role in the maintenance of a large Treg pool despite the lack of IL-2. In line with this, increased serum levels of IL-15 and excess soluble IL-7 receptor, which potentiates IL-7 activity, have been associated with SLE [246–249]. This hypothesis would also be in consistence with the finding of T. Alexander and colleagues that basal phosphorylation of STAT5 is increased in both Tcon and Treg of active SLE patients compared to healthy controls [79].

Most of the SLE patients included in this study have long-standing disease and have been treated with a variety of immuno-modulatory drugs (table A1 in the appendix). However, concerning the frequencies of Foxp3+CD127lo Treg among CD4+ T cells, no differences were observed between untreated SLE patients with newly diagnosed disease and patients receiving glucocorticoids and/or other immunosuppressive agents with the exception of patients treated with belimumab (n=4), who showed increased percentages of Treg (Fig. A3 of the appendix). Thus, the generally high frequencies of Treg seem not to be attributable to SLE therapy.

Finally, all analyses were restricted to circulating lymphocytes of the peripheral blood. Thus, no conclusion can be drawn about the proportions and numbers of Treg and Tcon in secondary lymphoid organs or at the sites of inflammation. The finding that Treg from SLE patients expressed little CCR6 could however indicate an inefficient re-localization of Treg into the inflamed organs. This would be also in line with findings from Lee at al, who demonstrated decreased frequencies of circulating CCR4+ Treg in SLE and consequently lower migration of Treg towards CCR4 ligands CCL22 and CCL17 in vitro [191]. Similarly, reduced numbers of Foxp3+ Treg have been observed in skin lesions of patients with cutaneous lupus, when compared to other chronic inflammatory skin diseases [232].

In summary, the origin of the high frequencies of total Foxp3+CD127lo Treg in the peripheral blood of SLE patients cannot be finally answered, yet. However, based on the available data it can be hypothesized that due to the lack of IL-2 signaling, a large proportion of Treg is not sufficiently activated and therefore lacks the ability to migrate to the site of inflammation and is instead retained in a resting state. At the same time, the survival of this Treg population is maintained by signals from cytokines such as IL-7, and their pool might even be increased due to elevated levels of thymic output as a means to counteract the relative deficiency of functional Treg and the hyperactivity of conventional T cells.
4 Discussion

4.5 Selective correction of the Treg phenotype as a prerequisite for the translation of an IL-2-based immunotherapy for SLE

In vitro stimulation with IL-2 showed that low doses of 1 ng/ml and a short stimulation time of 24h were sufficient to restore CD25 expression in Treg from SLE patients. Interestingly though, Treg from SLE patients did not reach the same percentages of CD25hi cells as did Treg from healthy controls stimulated with the same amount of IL-2. Thus, an intrinsic limitation might exist in SLE Treg. Nonetheless, CD25 expression up to physiological levels was not compromised, providing the basis for an IL-2-based immunotherapy for SLE patients.

Having confirmed the reversibility of this Treg defect by IL-2 stimulation, the major concern for the translation of an IL-2-based therapy for the treatment of SLE patients was the risk of unwanted activation or expansion of effector T cells or other lymphocytes by IL-2.

Importantly, stimulation with low IL-2 doses specifically induced CD25 and Bcl-2 expression in Treg and had less prominent effects on Tcon. Also B cells, CD8+ T cells and NKT cells showed virtually no response to in vitro IL-2 stimulation. However, the lack of proliferative activity of T cells in vitro also indicated that not all effects that are expected in vivo could be monitored in vitro. In this case we profited from the two clinical studies with low-dose IL-2, which were published at that time, and from where also the administered IL-2 doses had been adopted [153, 154]. Although the proliferative responses of lymphocyte subsets to IL-2 were not presented in those studies, they showed a specific expansion of numbers and frequencies of Treg but not of Tcon or CD8+ T cells. In addition, pre-clinical data from studies with (NZBxNZW)F1 mice from our group, where even higher IL-2 doses had been applied in a similar treatment regimen, showed a selective induction of Treg proliferation and concomitant Treg expansion in response to IL-2 without activation of Tcon or CD8+ T cells in vivo (Rose et al manuscript in preparation).

Nevertheless, Treg were not the only responders to IL-2 stimulation in vitro. Five-day repetitive IL-2 stimulation dose-dependently increased proliferation and frequencies of NK cells in comparison to unstimulated controls. Due to their constitutive expression of the intermediate-affinity IL-2R subunits and the reported expression of CD25 on a subset of NK cells [114] this was not unexpected. However, IL-2 stimulation merely supported the maintenance of input numbers and frequencies of NK cells, but did not induce an expansion of NK cell numbers in culture. Interestingly, the proliferation and increased frequencies of CD56bright NK cells in response to IL-2 stimulation indicated a preferential expansion of immature NK cells, which is in line with early studies investigating the effect of IL-2 stimulation on NK cell proliferation [250]. It can, however, not be excluded, that IL-2 stimulation also induced cytolytic effector functions of NK cells. SLE patients have generally low frequencies and numbers of peripheral NK cells and their cytotoxic capacity has been shown to be impaired [187], which might be due to the high proportions of CD56bright cells. In this context, a link between IL-2 deficiency and defective NK cell function has been proposed already in 1985 by G. Tsokos, who showed an improvement of SLE NK cell cytotoxicity by IL-2 stimulation in vitro [186]. Thus, it was hypothesized that activation or expansion of the NK cell subsets by IL-2 stimulation did not necessarily present a risk factor in SLE, but might actually have beneficial effects by restoring a functional NK cell pool, which might contribute to immune regulation.
4 Discussion

The *in vitro* effects of IL-2 presented in this study were limited to doses from 1 to 10ng/ml. The stimulation experiments with supernatants from healthy controls (section 3.4.2), however, indicated that probably even lower IL-2 concentrations would suffice to significantly increase CD25 expression in Treg. Thus, to gain more information about the dose-dependency of IL-2-induced responses, experiments with doses below 1ng/ml would be valuable to find the most Treg-selective IL-2 concentrations. Nonetheless, within the range of IL-2 concentrations used here, the effects on NK cells and Tcon were clearly dose-dependent. This gave reason to expect a highly selective response of Treg and less pronounced effects in the NK cell compartment when using these low doses also *in vivo*.

4.6 Mechanisms of low-dose IL-2 treatment-dependent effects in SLE patients

Based on our pre-clinical data and the two described clinical trials with low-dose IL-2 it was assumed that the benefit-to-risk ratio of a low-dose IL-2-based immunotherapy would be reasonably high for the treatment of SLE patients. Thus, five patients with severe refractory SLE were treated with low-dose IL-2 in the context of an 'off-label' therapy ('Individueller Heilversuch', patients 1 and 2) or as part of the clinical phase I/IIa study PRO-IMMUN (patients 3-5) with the primary goal to selectively expand functional CD25hi Treg in order to strengthen the endogenous tolerance mechanism and thereby ameliorate disease. The intended effect of low-dose IL-2 treatment on the Treg population was more than satisfactorily achieved with a remarkable expansion of bona fide Foxp3+CD127lo CD25hi Treg in all five patients during the IL-2 treatment cycles and without overt activation of conventional T cells. Comparison of cell numbers, frequencies, proliferation and STAT5 phosphorylation among different lymphocyte subsets showed that Treg were the preferential responders to low-dose IL-2 therapy also *in vivo*. In parallel, also a satisfactory clinical response to low-dose IL-2 therapy was achieved in patients 1, 2 and 4, where disease activity was reduced within the first two treatment cycles.

Treatment results of only five patients does not allow to draw correlative conclusions between the immunologic effects and the clinical outcome of low-dose IL-2 therapy. Nevertheless, based on the collected data it can be speculated on the mechanisms of action of how IL-2 induces a clinical response in SLE patients *in vivo*. These mechanisms are discussed in the following and are summarized in Figure 39, p. 86.

4.6.1 Suppressive capacity of Treg

In the first parts of this thesis it was shown that the low CD25 expression in IL-2-deprived Treg from SLE patients was associated with a phenotype of diminished Treg activation and suppressive capacity as well as with increased disease activity. Accordingly, induction of high CD25 expression in Treg by IL-2 therapy was expected to increase also their suppressive function. In line with this, a good clinical response was observed in those patients, who also showed the highest induction of CD25 expression among Treg in response to IL-2 treatment (patients 1, 2 and 4).

Furthermore, the induction of CD25hi Treg was associated with increased percentages of...
4. Discussion

CD39+ Treg. This finding is in line with a study by Barron et al, which investigated the IL-2-dependent maintenance of Treg function in mice and indicated a higher suppressive capacity of Treg through the ability to convert pro-inflammatory ATP to adenosine \[251\]. In addition, CD137 expression was increased in CD25hi Treg upon IL-2 treatment. CD137 expression has been associated with recent TCR-dependent activation of Treg \[227\], but has also been shown to be induced by administration of IL-2/anti-IL-2 antibody complexes in mice \[252\] and own observations). Whether in this case the CD137+ Treg population represents indeed a subset of recently antigen-stimulated Treg, or whether IL-2 alone caused the induction of CD137 expression needs further clarification. Next generation sequencing of the TCR in continuation of this project will allow to determine whether CD137+ Treg represent clonally expanded cells, or whether up-regulation of CD137 is independent of specific TCR stimulation. In either case, up-regulation of the co-stimulatory receptor is a sign of Treg activation, and in mice CD137 expression in Treg has been linked with higher suppressive capacity \[253\].

In vitro suppression assays also confirmed that IL-2-induced CD25hi Treg were able to suppress Tcon proliferation, indicating that the IL-2-induced population of CD25hi CD39+ CD137+ Treg confers a high suppressive capacity to the Treg pool. However, it remains elusive whether IL-2 improves the suppressive capacity on a single cell basis or whether the mere expansion of already suppressive Treg is responsible for the suppressive function of the IL-2-expanded Treg population. The ability of CD25hi Treg from patient 4 to suppress Tcon proliferation already before IL-2 treatment, is speaking in favor of the latter alternative. In contrast, CD25hi Treg from patients 3 and 5 showed an impaired suppressive capacity before IL-2 treatment, which could be restored at least partially by IL-2 treatment and could therefore be indicative for an intrinsic defect of Treg which is in part repaired by IL-2.

Finally, the dose-independent increase of the Treg/Tcon proliferation ratio during the IL-2 treatment cycles, and the maintenance of elevated levels even three weeks after the last IL-2 administration in some patients, is suggestive for an improved ability of the Treg population to suppress Tcon activity also \textit{in vivo}.

4.6.2 Migratory capacity of Treg

The increased proportions of CCR4+ and CCR6+ cells suggest an improved potential of Treg to migrate into inflamed tissues such as the skin in response to IL-2 treatment, supporting the hypothesis that the clinical improvement is at least in part due to the expansion of CD25hi Treg. A recent pilot study with five patients suffering from alopecia areata reported a notable increase of Treg counts in lesional skin after low-dose IL-2 treatment \[157\], underpinning the assumption that the amelioration of skin lesions in the treated patients might be attributed to induction of Treg migration and function. Lately, investigations from the group of G. Tsokos also showed that IL-2 treatment diminished skin inflammation in lupus-prone MRL/lpr mice \[254\]. The authors attributed the beneficial effects to the reduction of CD4-CD8- DN T cells, but a contribution of the increased pool of Treg could not be excluded. In the present study we did not find any conclusive effects of IL-2 therapy on the frequencies or numbers of CD4-CD8- DN T cells (not shown), thus further supporting the role of Treg for the amelioration of skin lesions in human SLE.
4.6.3 Activation-induced cell death

AICD is a controlled apoptotic mechanism to limit the expansion of effector T cells that were repeatedly stimulated by persistent antigen, such as self- or tumor-antigens and stop an immune reaction after the pathogen has been cleared \[255\]. IL-2 can sensitize CD4+ T cells to AICD, which requires Fas receptor-dependant signaling \[256\]. One proposed mechanism of IL-2-dependent immune-regulation is therefore an increase of AICD in effector T cells \[226\]. In the IL-2-treated patients we did not observe increased apoptosis of Tcon, as determined by active Caspase-3 or Bcl-2 expression. However, absolute numbers of Tcon and of CD8+ T cells were reduced and also the loss of CD28- Tcon detected after IL-2 treatment may have been a result of AICD. In fact, CD28 expression is associated with protection from AICD through the prevention of Fas ligand expression and up-regulation of the anti-apoptotic protein Bcl-x upon co-stimulation \[257\], suggesting that CD28- T cells might be more susceptible to IL-2-dependent AICD than their CD28+ counterparts. Thus, together with Treg-dependent suppression of proliferation, AICD might contribute to the IL-2-induced attenuation of immune-reactions in SLE.

4.6.4 Natural killer cell cytotoxicity

NK cell cytotoxicity against activated T cells may represent a physiological mechanism for the termination of adaptive immune responses. Therefore, another possible mechanism of IL-2-dependent immune-regulation is the induction of NK cell cytotoxic function. The IL-2 defect in SLE has been linked to defects in NK cell cytotoxicity and it was proposed from in vitro studies that IL-2 stimulation could restore NK cell cytotoxic function \[186\]. As expected, NK cells were the second-strongest responders to IL-2 therapy after Treg in terms of proliferation and expansion, and interestingly, the strongest expansion was observed in those patients who had very low frequencies of NK cells before the start of the IL-2 treatment. Similar to the in vitro observations, the CD56bright NK cell subset preferentially proliferated in response to IL-2 also in vivo. And also among the CD56dim NK cell subset, it was the more immature cells, which showed preferential proliferation in response to IL-2, while they did not show increased expression of the cytolytic molecules perforin and granzyme B. Thus, in contrast to the proposed induction of cytotoxic activity, IL-2 appeared to cause an expansion of the cytokine-producing NK cell population. CD56bright NK cells have been associated with regulatory functions, due to their potential to produce IL-10 \[258\]. However, they can also produce high amounts of pro-inflammatory cytokines and have been associated with a detrimental role in rheumatoid arthritis where they induce the differentiation of monocytes to Th1-promoting dendritic cells \[259\]. Thus, further analysis of their cytokine-profile and cytotoxicity will be necessary to elucidate the function of the expanded NK cell population.

Interestingly, treatment of Multiple Sclerosis patients with Daclizumab, a CD25 blocking antibody, caused a similar expansion of CD56bright NK cells. This phenomenon was associated with an increased availability of T cell-derived IL-2 due to blockade of CD25, and was linked to the inhibitory effect of daclizumab on Multiple Sclerosis disease activity \[260, 261\]. Although, also there no changes in perforin expression were observed, both CD56dim and CD56bright NK cells were shown to be cytotoxic towards activated autologous T cells in vitro after Da-
clizumab therapy, and correlations were found between the expansion of CD56bright NK cells and the contraction of CD8+ and CD4+ T cell numbers. Thus, the role of *in vivo* cytotoxicity of NK cells might be underestimated by mere phenotypic analyses.

Early studies searching for methods to inhibit cytokine activity *in vivo* revealed that complex formation of free cytokines with monoclonal anti-cytokine antibodies did increase the half-life of cytokines in serum from minutes to days [262]. Further exploitation of such cytokine-antibody complexes as a method to increase the half-life and activity of cytokines revealed that different monoclonal anti-IL-2 antibodies had different effects on the cellular specificity of the cytokine response. While for example murine IL-2 complexed with the anti-IL-2 clone S4B6 preferentially stimulates proliferation of CD8+ T cells and NK cells, complexes with clone JES6-1 do not stimulate CD8+ T cells and NK cells but retain the ability to stimulate Treg in mice [263]. This distinct cellular specificity, which is most probably due to differential binding capacities to the high- or intermediate-affinity IL-2R complexes [264], renders these IL-2/anti-IL-2 complexes a useful tool for the investigation of IL-2-dependent effects. Studies with the different IL-2/anti-IL-2 complexes in lupus mice could thus help to better define the impact of IL-2-dependent expansion of Treg cells versus cytotoxic cells in the treatment of SLE. In fact, treatment of MRL/lpr lupus mice with IL-2/anti-IL-2 clone S4B6 has been shown to reduce the number of CD4-CD8- DN T cells, which could be attributed to the expansion of cytotoxic lymphocytes [254]. Similarly, enhanced killing of effector T or B cells by the expanded NK cell population might represent an additional mechanism of IL-2-dependent immune-regulation. But functional analyses of NK cell cytotoxicity and their cytokine profile will be essential to decipher their role in SLE pathogenesis and during IL-2 therapy.

### 4.6.5 Inhibition of autoantibody production

An unexpected finding was the reduction of circulating anti-dsDNA antibodies upon IL-2 therapy in the three responding SLE patients. In contrast, total Ig levels remained stable and the loss of autoantibodies could not be associated with changes in circulating B cell or plasma cell counts or frequencies (not shown). This suggests that IL-2 specifically targets (auto-) antigen specific antibody production without affecting the plasma cell population as a whole.

Whether IL-2 directly acts on B cells or plasma cells, or whether the observed effects are an indirect result, remains elusive. Conversely, IL-2 has been associated with plasma cell differentiation, enhanced Ig secretion and proliferation of activated human B cells [265, 266], as well as with induction of IgM production by naïve B cells when combined with dendritic cell stimulation *in vitro* [267]. In lupus mouse models IL-2 treatment had no effect on anti-dsDNA antibody levels ([254] and our own observations). Thus, it appears more likely that IL-2 indirectly influences autoantibody production, rather than directly acting on plasma cell activity or differentiation. This would be in line with the proposed role of the expanded population of suppressive Treg, which might inhibit the provision of CD4+ T cell help for B cell differentiation or directly inhibit plasma cell generation in the germinal center. Along this line, Treg have been implicated in the control of germinal center responses in mice, as inhibition of Treg function resulted in larger germinal center responses and increased frequencies of class-switched B cells [268]. In addition, we have shown earlier that adoptive Treg transfer, as an add on therapy to cyclophosphamide-
induced disease remission in lupus mice, enhanced the reduction of plasma cell frequencies \[199\]. In early studies investigating the role of IL-2 deficiency in SLE, IL-2 normalized the enhanced Ig production in mixed-lymphocyte reactions of SLE lymphocytes, which was attributed to a restoration of suppressor T cell generation \textit{in vitro} \[269\]. Further, CD4+ Tfh cells are specialized in promoting B cell proliferation, differentiation and class-switching in B cell follicles. Importantly, differentiation of CD4+ T cells into Tfh cells is negatively regulated by IL-2 signaling \[270\]. Therefore, these cells might be a potential target during IL-2 therapy, causing a reduction of T cell dependent B cell differentiation and the consequent decline of autoantibody-producing cells. According to their function, Tfh cells are predominantly found in secondary lymphoid organs and are identified by constitutive expression of the B cell follicle homing receptor CXCR5 \[37\]. Recently, a population of circulating memory CD4+ T cells that express CXCR5 and efficiently induce B cells to produce Ig \textit{in vitro} has been detected in human peripheral blood, and is considered to represent circulating Tfh cells \[271\]. In SLE patients, this population has been shown to be expanded and to correlate with anti-dsDNA antibody levels and frequencies of plasma blasts \[272\], suggesting that these circulating Tfh cells might be indicative for germinal center reactions. First results from patient 5 showed that frequencies of circulating Tfh cells were reduced during IL-2 therapy, indicating that IL-2 may indeed have a negative regulatory effect on Tfh cell differentiation or expansion and might thus influence antibody production.

In parallel to the ongoing study presented here, a clinical study to assess the safety and efficacy of low-dose IL-2 therapy in SLE is currently performed at the Beijing University People’s Hospital in China \[273\]. Interestingly, they have recently reported a reduction of anti-dsDNA antibody levels in response to IL-2 treatment in SLE patients \[274\], confirming our results. In addition, the clinical study performed by Saadoun et al also reported that anti-nuclear antibodies present in one of the treated patients with HCV-induced vasculitis were no longer detectable after low-dose IL-2 treatment \[154\]. Thus, in summary, there is cumulative evidence that low-dose IL-2 therapy indeed affects autoantibody production, although the exact mechanisms are not fully understood.

### 4.7 Dose-dependency of IL-2 effects

Comparison of the first and second IL-2 treatment cycles indicated a dose-dependent increase of the Treg response in terms of higher CD25 expression and stronger induction of proliferation, as well as CD137 and CCR4 expression in response to 3.0 million IU Proleukin compared to 1.5 million IU. A slightly lower response was observed again in the subsequent treatment cycles where the dose had been reduced again to 1.5 million IU. However, at least in patients 1, 2 and 4 the induction of CD25 expression and the expansion of the Treg pool was still more robust in the third and fourth cycles compared to the first treatment cycle although the same IL-2 doses were applied. These observations indicate that, despite the transience of many of the observed effects of IL-2, Treg appear to be primed from previous treatment cycles allowing for more robust responses to following IL-2 stimulation.

In contrast to the dose-dependency of the Treg response, no such differences were observed in the response of Tcon or CD8+ and NK cells, indicating that the use of 3.0 million IU Proleukin
Figure 39: Simplified schematic overview of proposed mechanisms of IL-2 treatment-dependent effects in SLE patients. Low-dose IL-2 treatment results in the expansion of the bona fide Foxp3+ Treg population and the induction of high CD25 expression levels. A high suppressive capacity of the CD52hi Treg population is likely to be responsible for the diminution of auto-inflammatory processes through the suppression of self-reactive CD4+ T cells and B cells, also at the site of inflammation. IL-2 may also inhibit the differentiation of CD4+ T cells into Tfh cells, thereby inhibiting further B and plasma cell differentiation. Whether IL-2-dependent expansion of CD56bright NK cells contributes to disease amelioration due to increased cytotoxicity or anti-inflammatory cytokine production, or whether it results in the opposite needs to be elucidated, yet. In addition, IL-2 may increase the susceptibility of activated T cells to activation-induced cell death (AICD), thereby limiting the expansion of auto-reactive cells. was still in the range of high Treg specificity. However, the dose increase did not correlate with the clinical response since many effects were already apparent before the start of the second treatment cycle. In contrast, dose escalation caused an increase of mild adverse events in form of fever and sweats in all patients.

The observed dose-dependency of minor adverse events was comparable to those reported in other clinical studies with low-dose IL-2: During the treatment of patients with HCV-induced vasculitis, receiving the same low-dose IL-2 regimen as the SLE patients in our study, flu-like symptoms were reported only after dose escalation to 3.0 million IU Proleukin per day [154]. Similarly, in a placebo-controlled trial with type-1 diabetes patients to evaluate dose-dependent safety and biological efficacy of low-dose IL-2, dose-dependent flu-like symptoms were reported upon 5-day treatment with daily doses of 3.0 million IU IL-2 [156]. Also the study of Koreth et al, in which patients with GvHD were treated with daily low-dose IL-2 injections for a duration of eight weeks, reported a maximum tolerated dose of 1.0 million IU per square meter body-surface area and the occurrence of fever and malaise at a dose of 3.0 million IU per square meter. In these studies, also the expansion of the Treg pool was associated with the
applied IL-2 dose, and in HCV patients a significant correlation between clinical response and Treg numbers was shown [153, 154, 156]. In line with the observed dose-dependency, the Treg responses of patient 3, who had received the lowest amount of Proleukin when calculated in relation to body weight, were lower during all cycles compared to the other patients. Nonetheless, the dose escalation was also not well tolerated by this patient. Together, these observations imply that the adverse events are IL-2 dose-dependent, but independent of the cellular responses detected by our analyses.

As indicated in section 4.5, one limitation of the present study is the lack of data on the potential effects of even lower IL-2 doses. Some information in this regard can, however, be retrieved from patient 5, who received a dose of only 0.75 million IU IL-2 per day during the fourth treatment cycle. Here, although less robust, an induction of CD25 expression and proliferation among Treg was still detectable, however, also proliferation of CD56bright NK cells was strongly induced. In line with this, the effects of ‘ultra-low dose’ IL-2 administrations (0.1 and 0.2 million IU/m$^2$/day) have been investigated in a recent study with healthy volunteers. Here, 5-day treatment induced the expansion and suppressive function of Treg and also increased the pool of IFN-$\gamma$-producing CD56bright NK cells [241]. Thus, the administration of lower IL-2 doses appears to suffice for the expansion of Treg in vivo, but does not increase its selectivity when comparing Treg and CD56bright NK cell responses. Also conclusions about clinical effects cannot be drawn, yet.

4.8 Differential effects of IL-2 on Treg homeostasis

A major discrepancy between in vitro stimulation with IL-2 and in vivo IL-2 therapy were the differential effects on proliferation and Bcl-2 expression in Treg. While basically no proliferation but a selective induction of Bcl-2 expression in CD25+ Treg had been observed by IL-2 stimulation in vitro, the opposite appeared to be true in vivo. The lack of T cell proliferation in vitro can most probably be attributed to the absence of TCR-dependent stimulatory signals, although whole PBMCs had been used.

Further analyses showed that both homeostatic and IL-2-induced proliferation in vivo were associated with low Bcl-2 expression levels in Treg. These data did not disclose whether proliferation causes the loss of Bcl-2 expression or whether low Bcl-2 expression is a prerequisite for Treg proliferation. However, findings by Bonnefoy-Berard and co-workers, which describe Bcl-2 as a negative regulator of proliferation [275], are indicative for the latter alternative. Thus, the induction of Bcl-2 expression by IL-2 in vitro, might have further contributed to the lack of a proliferative response of IL-2-stimulated T cells in vitro. The pre-existence of low Bcl-2 expression in Treg might potentially serve as a biomarker for the expected proliferative response of Treg in response to low-dose IL-2 in vivo. Interestingly, others have previously associated high CD25 expression with low Bcl-2 expression levels [276, 277] and this was also observed here. In fact this association might also be linked to the higher percentage of proliferating cells in the CD25hi Treg subset, as demonstrated earlier in this work.

At the same time the impact of low Bcl-2 expression on the viability of Treg is not clear. Low homeostatic Bcl-2 expression levels of Treg ex vivo have been associated with high CD95 expression and increased susceptibility to apoptosis in vitro [278]. However, we could not link the
low Bcl-2 expression after IL-2 treatment cycles to an increase of apoptosis (active Caspase-3+). Thus, investigation of the expression of other molecules involved in the regulation of apoptosis will be necessary in order to understand the impact of IL-2-induced proliferation and reduction of anti-apoptotic Bcl-2 on the homeostasis of Treg in SLE. In fact, signaling through CD137, which is expressed in the IL-2-expanded population of CD25hi Treg, is known to improve survival through the elevation of anti-apoptotic Bcl-xl and down-regulation of anti-apoptotic Bim [278], might thus represent a mechanism of how low Bcl-2 levels are counter-balanced in highly proliferative Treg.

Conversely, a study investigating the biology of Treg in pancreatic islets of non-obese diabetic (NOD) mice, in which low CD25 and low Bcl-2 expression in Treg were linked to a local IL-2 deficiency, administration of IL-2 restored both CD25 and Bcl-2 expression [147]. Furthermore, our data are in contrast to investigations in GvHD patients receiving daily low-dose IL-2 injections for eight weeks. Here Bcl-2 expression was reported to be specifically elevated in Treg and linked to an increased resistance to Fas-induced apoptosis [279]. However, Treg from those patients also showed generally lower frequencies of Ki67+ cells both before (median 4%) and in response to IL-2 treatment (median at peak 15.5%, [279]), compared to SLE patients in our study. Furthermore, Bcl-2 levels started to increase only after the proliferative response of Treg had peaked and returned to baseline levels. Interestingly, in the mentioned study, also frequencies of RTE Treg increased from the second week of treatment after the proliferative response had ceased.

Together these differential homeostatic responses indicate a complex role of IL-2 in the control of lymphocyte homeostasis, which reaches beyond mere induction of proliferation, and is dependent on the initial composition and homeostatic situation of the responder cell population. Also the observed IL-2-dependent reduction of CD8+ T cell proportions to a normal range, and the expansion of NK cells in patients with low baseline values are in close agreement with this intricate function of IL-2 for lymphocyte homeostasis.

4.9 IL-2 therapy in the context of other clinical studies

Since the first clinical trials with HCV and GvHD patients had proven safety and efficacy of low-dose IL-2 in diseases associated with aberrant immune activation in 2011 [153, 154], IL-2 based immunotherapies have gained increasing attention as a tool to re-establish tolerance in autoimmune diseases. In the meantime, besides the already mentioned trials, two studies investigating the effect of low-dose IL-2 in the treatment of type-1 diabetes [155, 156], and a pilot study with patients suffering from alopecia areata [157] have been published. As mentioned above, first results from a clinical study investigating safety and efficacy of low-dose IL-2 therapy in patients with SLE in China have also been presented [273, 274]. In addition, a broader scale clinical study assessing the safety and biological efficacy of low-dose IL-2 as a Treg inducer in a set of 11 autoimmune and auto-inflammatory diseases, including SLE, is currently ongoing in France [280].

Qualitative and/or quantitative defects in Treg biology have been associated with all these diseases in question [61, 281], and besides for SLE, evidence for genetic associations with defects in the IL-2–Treg axis has been shown for type-1 diabetes [282]. It is tempting to speculate that
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Diseases associated with IL-2 deficiency would profit more from an IL-2-based immunotherapy than others. But the limited data that is available up to now does not allow for any assumptions in this direction. Similar IL-2 doses were applied in all of the mentioned trials. And except for the GVHD study [153] and the Chinese SLE study [273], a similar treatment schedule, which was also applied in our study, consisting of one or more 5-day treatment cycles was used. As indicated earlier, also all studies reported a dose-dependent occurrence of minor adverse events. A dose-dependent expansion of the Treg population was reported in all cases, and was associated with the clinical response in the case of HCV. Overlapping results with the present study were also observed concerning the expansion of (CD56bright) NK cells, migration of Treg, and loss of anti-dsDNA antibodies [153, 154, 157].

A comparison of detailed results concerning the expansion of Treg is rather difficult due to the lack of comprehensive information concerning for example the assessment of CD25 expression levels and proliferation in most of the other studies. In addition, investigations of Treg expansion in other diseases are based on very different starting situations, as indicated also in section 4.8. While high levels of overall Foxp3+CD127lo Treg are found in SLE and low-dose IL-2 was applied with the aim to expand CD25hi Treg here, the above mentioned studies aimed at an overall expansion of Treg, which mostly had a low prevalence in the respective diseases. Nonetheless, the aim to selectively improve the balance between highly functional Treg and auto-reactive effector T cells by low-dose IL-2 therapy was achieved in all situations. Collectively, the present study, together with the other reports, promotes low-dose IL-2 as a safe and efficient therapeutic tool to induce immuno-regulatory mechanisms in SLE and other autoimmune-related diseases.

4.10 Possibilities for the future development of low-dose IL-2 therapy for SLE

While both clinical and immunologic results of the applied low-dose IL-2 regimen are very promising, the follow-up data also showed that most of the cellular effects were transient and short-lived and the duration of clinical remission was limited. This opens the question whether long-term IL-2 treatment strategies or combinations with other therapeutics could improve the sustainability of the therapeutic effects of IL-2 in SLE.

The safety of low-dose IL-2 treatment in humans in terms of absence of immune activation has been convincingly demonstrated in the here presented and in the above mentioned studies. However, another possible risk of prolonged low-dose IL-2 treatment could be an impairment of protective immune responses due to chronically high levels immune-regulation. Recent investigations in NOD mice injected with an IL-2-producing adenoviral vector showed that continuous low-dose IL-2 release for 25 weeks did not impair vaccine or anti-viral responses and had no effects on pregnancy or tumor-growth [283]. This indicates that prolonged exposure to IL-2 does not impair normal immune responses despite a continuous stimulation and expansion of Treg in mice.

Considering the strong activation of Treg during the 5-day IL-2 treatment cycles and the rapid contraction of the CD25hi Treg pool thereafter, a maintenance regimen with low-dose IL-2 injections every two to three days could thus be a possible alternative solution in order to sustain high levels of active Treg for a longer period of time. In fact, an off-label low-dose IL-2 therapy was continued in two of the responding SLE patients after completion of the four scheduled
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treatment cycles. Here, patients received a 5-day induction cycle and thereafter treatment was continued with two injections of 1.5 million IU IL-2 per week. Unfortunately, blood collection from these patients for phenotypical analyses was only possible every two to three weeks. Nonetheless, these limited analyses revealed a sustained elevation of overall Treg levels, but induction of CD25 expression appeared to be very short-termed and could only be detected if blood was drawn up to one day after the respective last IL-2 injection. Thus, tighter monitoring intervals will be necessary to evaluate whether this low-dose IL-2 treatment regimen is sufficient to maintain high levels of functional CD25hi Treg. Also, whether continuous IL-2 treatment is safe and effective in human patients with autoimmune diseases requires larger scale clinical investigations.

An alternative approach to avoid the repeated injections of IL-2, which were accompanied by inflammatory responses at the injection site, and to further reduce the administered doses, is the development of IL-2 variants with an increased half-life and activity. Elongation of the in vivo half-life of IL-2 could allow for alternative treatment strategies and might potentially reduce the dose and the frequency of injections. IL-2/anti-IL-2 complexes or IL-2-fc-fusion proteins are promising developments and have been successfully applied in animal models [151, 284]. In this context, a recent study from Bell and colleagues with cynomolgus monkeys is of particular interest: Treatment with a single dose of a high-avidity bivalent IL-2 fusion protein was shown to induce similar Treg specific effects, concerning STAT5 phosphorylation, proliferation and CD25 expression, compared to repetitive administration of IL-2 (Proleukin) at 10-fold higher doses [285]. On the other hand, the short-lived nature of the effects of unmodified IL-2, provides a higher degree of safety and flexibility, allowing for the individualized adaptation of dose and timing.

Another approach for the improvement of IL-2-based immunotherapies could be the modification of IL-2 in a way that it more specifically activates Treg, while avoiding the activation of NK cells. In view of this, IL-2 variants, which specifically bind to the high-affinity but not the intermediate-affinity IL-2R variants, have been developed [286], but data on Treg-specific expansion and therapeutic efficiency in autoimmune-related diseases are not available, yet. In this context, also the Treg-specific IL-2/anti-IL-2 complexes come into play [106]. But as discussed in sections 4.5 and 4.6.4 it cannot be excluded that the expansion of NK cells could actually have beneficial effects in the treatment of SLE. Thus the application of more Treg-specific forms of IL-2 is not necessarily beneficial compared to unmodified IL-2 for the treatment of SLE.

An additional strategy to improve the therapeutic efficacy of low-dose IL-2 therapy is based on the specific combination of IL-2 with other therapeutic concepts. In the presented study, glucocorticoid treatment was continued during low-dose IL-2 therapy in all five patients, and its dose could be reduced during the treatment period. In fact, it has been shown in mice that Treg are less susceptible to dexamethasone-induced cell death and that dexamethasone treatment amplifies the IL-2-induced expansion of Treg in vivo [287, 288]. Thus, glucocorticoid treatment might specifically boost the effects of low-dose IL-2 therapy on Treg expansion.

In addition, low-dose IL-2 therapy may also be valuable as an add-on therapy in combination with other therapeutic agents in SLE. We have recently shown that treatment of lupus mice with cyclophosphamide inhibits Treg proliferation and causes a decline of Treg numbers. However,
adoptive Treg transfer to restore the Treg pool size after such a cyclophosphamide regimen significantly prolonged the interval of remission induced by cyclophosphamide [199]. Similarly, expansion of the endogenous Treg pool by low-dose IL-2 treatment might enhance the immunosuppressive effect of cyclophosphamide also in human SLE patients.

4.11 Conclusions and future perspectives

In this work it was shown that defects in Treg biology, including a reduced CD25 expression, a disturbed homeostasis and insufficient functional activity, are associated with SLE disease activity. These disturbances are linked to a defective IL-2 production and excessive proliferation of Tcon in SLE patients. IL-2 supplementation is able to selectively restore CD25 expression in Treg in vitro. Finally, an IL-2-based therapy with the aim to restore a functional Treg pool in vivo was successfully translated for the treatment of patients with SLE.

Several findings propose that the observed disease amelioration in response to low-dose IL-2 can be attributed to an enhanced suppressive capacity of the IL-2-expanded population of CD25hi Treg. In addition, the expansion of NK cells, the loss of Tfh and CD8+ cells, and the reduction of autoantibody levels indicate that IL-2 plays an important role for the homeostatic balance among all lymphocyte subsets. Further in-depth investigations and larger clinical trials will be necessary to precisely reveal the mechanisms of action of low-dose IL-2 in vivo and the contribution of different lymphocyte subsets to the associated clinical effects.

In this context, more detailed characterization of the Treg function before and after IL-2 therapy will be of importance. This includes the analysis of the expression of regulatory cytokines and cytotoxic molecules, suppression of effector cytokines, antigen-specificity, as well as the migratory capacity of Treg.

In line with this, analyzing the effects of IL-2 on NK cell function beyond phenotypic characterizations will be of great interest in order to understand the role of NK cells and especially CD56bright NK cells for SLE pathogenesis and during IL-2 therapy. The same holds true for the population of CD8+ T cells whose role in SLE pathogenesis is largely unclear, but which are known to be influenced by IL-2 signaling both during priming and memory formation [106]. Assessment of NK cell and CD8+ T cells cytotoxicity in vitro will be a first approach to answer this question. Furthermore, as described above, IL-2/anti-IL-2 antibody complexes of different cellular specificity provide a valuable tool to study the impact of NK cells and CD8+ T cells in the mouse model.

In addition, very little is known about how low-dose IL-2 affects the humoral immune system and leads to the specific reduction of auto-antibody levels. The characterization of circulating B cell and plasma cell subsets has not revealed any conclusive data so far, but analyses in a larger cohort of IL-2-treated patients might help to shed some more light on the B cell-dependent mechanisms. In addition, the identification of circulating Tfh cells did provide first information about possible influences of IL-2 on the germinal center reaction.

IL-2-dependent effects on non-lymphocyte subsets, have not been investigated here. However, dendritic cells, for example, have been shown to express CD25 and to produce higher amounts of immuno-(regulatory) cytokines in response to IL-2 [289]. In view of this, peripheral leukocytes including dendritic cells, monocytes, and granulocytes granulocytes, are monitored in the
IL-2-treated patients in cooperation with the group of Dr. Grützkau (DRFZ, Berlin). Finally, the detection of biomarkers or signatures to identify patients, who (i) display an IL-2 deficiency and (ii) will respond to low-dose IL-2 therapy, is one of the main goals for the future. With regard to this, whole blood RNA samples are collected before, during and after IL-2 treatment from all patients. With detailed gene-expression profiling it is aimed to gain more information about the systemic effects of IL-2-deficiency on the one hand, and the effects of IL-2 therapy on the other hand. In regard of potential biomarkers for the response to IL-2 therapy, the most obvious difference between the two non-responding patients and the three responders to low-dose IL-2 were the different proportions of RTE Treg before the start of treatment. Whether this was by chance or whether an association between the clinical response and RTE Treg frequencies indeed exists can only be answered by treatment of a larger cohort of SLE patients. In addition, the presented suggest a role of Bcl-2 expression levels for the proliferative response of Treg. Thus, low Bcl-2 expression levels might discriminate patients with a more robust Treg response.

In summary, while several open questions about the exact mechanisms remain, this work demonstrates that low-dose IL-2 therapy represents a feasible and safe novel therapeutic approach for the treatment of SLE, which directly targets the pathogenic mechanisms of the disease.
Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Appendix
Supplemental data

Figure A1: CD25 expression in healthy control CD4+ T cells upon *in vitro* IL-2 stimulation. PBMCs from healthy controls (HC) (n=8) were repetitively stimulated every 24h with 1.0, 2.5, 4.0 or 10ng/ml IL-2 (Proleukin®) *in vitro* and analyzed by flow cytometry 24h after the first (24h) or the last (5d) stimulation. Bar-diagrams show the fold-change of CD25 expression levels (MFI, left), and the frequencies of CD25hi cells (right) among Treg (A) or Tcon (B) of IL-2-stimulated samples compared to unstimulated samples. Bars indicate the median with inter-quartile range. Wilcoxon signed-rank test was used to analyze changes induced by IL-2 compared to untreated samples (asterisks above error bars) or between different IL-2 concentrations (asterisks above horizontal lines, *p>0.05, **p>0.01, ***p<0.001).
Appendix

Figure A2: Active Caspase-3 expression during low-dose IL-2 therapy. PBMCs from five SLE patients were analyzed immediately before and approximately 20h after the last injection of each 5-day treatment cycle with low-dose IL-2, as well as three weeks after the last IL-2 administration (day 83). Frequencies of active caspase-3+ cells among Foxp3+CD127lo Treg (left) and Foxp3- Tcon (right) is depicted for each patient during the course of the IL-2 treatment period.

Figure A3: Expansion of Foxp3+CD127lo Treg frequencies is treatment-independent. SLE patients were grouped according to their current therapy and frequencies of Foxp3+CD127lo Treg among CD3+CD4+ T cells were compared between the different groups. GC, glucocorticoids; CTX, cyclophosphamide; Aza, azathioprine; HCQ, hydroxychloroquine; MMF, mycophenolate-mofetil.
## Table A1: SLE patient data

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SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; CTX, Cyclophosphamide; HCQ, Hydroxychloroquine; MTX, Methotrexate; MMF, Mycophenolate-Mofetil
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SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; CTX, Cyclophosphamide; HCQ, Hydroxychloroquine; MTX, Methotrexate; MMF, Mycophenolate-Mofetil
Appendix

Publications

Published articles


Oral presentations

• Annual European Congress of Rheumatology (EULAR) 2014, Paris, France

• 41. Kongress der Deutschen Gesellschaft für Rheumatologie (DGRh) 2013, Mannheim, Germany

• 13. Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS) 2013, Boston, USA

• 10. International Conference on Immunosuppression 2013, Barcelona, Spain

• 40. Kongress der Deutschen Gesellschaft für Rheumatologie (DGRh) 2012, Bochum, Germany
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Eidesstattliche Erklärung


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(Ort, Datum)                        (Caroline Gräfin von Spee-Mayer)