The onset of active disease in systemic lupus erythematosus patients is characterised by excessive regulatory CD4+-T-cell differentiation

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Abstract

Objective

An imbalance between CD4+-regulatory T-cells (Tregs) and CD4+-responder T-cells (Tresps) correlates with active disease flares in systemic lupus erythematosus (SLE) patients. Both cell subsets consist of highly proliferating Tregs/Tresps expressing inducible T-cell co-stimulatory molecule (ICOS) and less proliferating ICOS-Tregs/Tresps.

Methods

Six-colour-flow-cytometric analysis was used to examine the effect of ICOS+- and ICOS-Treg/Tresp cell differentiation on the composition of the total CD4+-T-helper cell pool with ICOS+- and ICOS-Tregs/Tresps. The functionality of Tregs was examined using suppression assays.

Results

In 83 healthy volunteers, the ratio of ICOS+-Tregs/ICOS+-Tresps increased significantly with age, while that of ICOS-Tregs/ICOS-Tresps did not change. In 86 SLE patients (SLEDAI <7), disease activity was associated with an age-independently increased ratio of both ICOS+-Tregs/ICOS+-Tresps and ICOS-Tregs/ICOS-Tresps. In these patients, the functional activity of ICOS+-Tregs, but not of ICOS-Tregs, was preserved. In 13 markedly active disease patients (SLEDAI >7), the percentage of both ICOS+-Tregs and ICOS+-Tresps, was strongly increased within total CD4+-T-helper cells. However, the increased ratio of ICOS+-Tregs/ICOS+-Tresps was not maintained in these patients, due to terminal differentiation and accumulation of naïve cells within total ICOS+-Tregs. Despite increased differentiation of both ICOS-Tregs and ICOS-Tresps, the percentage of ICOS-Tregs increased within CD4+-T-helper cells, while that of ICOS-Tresps decreased, resulting in a significantly increased ratio of ICOS-Tregs/ICOS-Tresps independent of age.

Conclusion

Our data reveal a crucial role of Treg immune senescence for the occurrence of disease flares in SLE patients, with ICOS+-Treg cells being most affected.

Key words

systemic lupus erythematosus, regulatory T-cells, responder T-cells, inducible T-cell co-stimulator, T-cell differentiation

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Introduction

Systemic lupus erythematosus (SLE) represents a chronic autoimmune disease with various clinical manifestations ranging from mild disease activity to life-threatening multiple organ involvements. Its pathogenesis still remains elusive. However, it seems that derangement of both the adaptive and innate immune system leads to loss of self-tolerance (1, 2). Immunosuppressive regulatory $CD4^+$-T-cells (Tregs) are significantly involved in the maintenance of self-tolerance, their malfunctioning affects the development of various autoimmune diseases (3), including SLE (4). Numerous murine models suggest that both the quantity and the quality of Tregs are responsible for the appearance of lupus-like disease. Therefore, their number and function have also been extensively investigated in human SLE. However, due to different flow-cytometric characterisation with various surface markers, Treg frequency is subject to strong fluctuations among different studies and functional analysis also shows diverse results (4, 5). Beside suppressive defects of Tregs, a possible resistance of SLE responder T-cells for Treg suppression is documented (6-8).

Tregs do not represent a homologous T-cell population, but consist of different subsets, such as naturally occurring Tregs (nTregs) and peripherally induced Tregs (iTregs). Two distinct nTreg populations with different expression of the inducible T-cell Co-Stimulator (ICOS) molecule are produced in the human thymus. The larger $ICOS^-$-Treg population was found to be conspicuously susceptible to apoptosis, while the smaller highly proliferative $ICOS^+$-Treg population was shown to be much more resistant to activation-induced cell death. Both Treg populations exert their suppressive activity by releasing inhibitory cytokines, with $ICOS^-$-Tregs producing more TGF-β and $ICOS^+$-Tregs producing more IL-10 (9). As a member of the CD28 family, the ICOS molecule is also expressed by $CD4^+$-responder-T-cells (Tresps) and similar as CD28, it is involved in their proliferation and activation. Thereby, its expression is upregulated after T-cell receptor (TCR) engagement and CD28 stimulation (10). Studies of ICOS knockout mice ($ICOS^{-/-}$) have shown that thymic T cell development is largely unaffected by lack of constitutive expression of ICOS (11), but that it is important for the development and effector function of specific T cell subsets (12), particularly of follicular T-helper (Tfh) cells (13, 14). These cells are located within the germinal centers (GCs) of secondary lymphoid tissues in which they provide help for the generation of high-affinity memory-B-cells and long-lived plasma cells (PCs) (15). Circulating Tfh cells (cTfhs) with an activated phenotype showed increased frequency in SLE patients and correlated with disease activity (16, 17). Tfh cells arise in the periphery (lymph nodes and spleen) from conventional naïve $CD4^+$-T-cells after antigenic stimulation and ICOS-Ligand (ICOSL) co-stimulation (18). Furthermore, constitutive expression of ICOS was also observed on developing T-cells in the thymus. Similarly as for the subset of $ICOS^+$-nTregs (19), the ICOS molecule is also important for the development of thymic natural killer T (NKT) cells (20) natural Th17 (nTh17) cells (21) and IL-17 producing γδT-cells (22).

Recent studies by our group revealed the necessity of an appropriate differentiation of both recent thymic emigrants (RTE)-Tregs as well as RTE-Tresps into memory-Tregs/Tresps to ensure their proper functioning. By using CD31 and CD45RA to distinguish between naive ($CD45RA^+$) and more differentiated mature memory T-cells ($CD45RA^-$), we have shown that specific clinical conditions such as end-stage-renal-disease (ESRD), dialysis or long-term transplant patients are associated with age-independent increased differentiation of both $ICOS^+$- and $ICOS^-$-Treg/Tresp cells. As a result, the balance in the ratios of $ICOS^+$-Tregs/$ICOS^+$-Tresps and $ICOS^-$-Tregs/$ICOS^-$-Tresps are severely impaired (23, 24).

Due to the fact that ICOS signalling has a crucial role for germinal center formation and isotype switching as well as for the fate and function of specific effector and regulatory $CD4^+$-T-cell subsets, we aimed to examine the role

of excessive differentiation in response to self-antigens of ICOS$^+$- and ICOS$^-$-Treg/Tresp cells in SLE patients with SLEDAI <7 compared to patients with SLEDAI >7.

Materials and methods

Patient collectives and healthy controls

Blood samples from 83 healthy donors and 99 SLE patients were obtained. Diagnosis was made according to the American College of Rheumatology (ACR) criteria (25, 26). SLE patients were divided according to their SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) into markedly active (SLEDAI ≥7; n=13) and inactive (SLEDAI <7; n=86) disease patients. This cut-off was proposed to differentiate between low and moderate to severe disease activity (27). Blood samples were collected during routine visits at the Department of Nephrology, University of Heidelberg. All patients and healthy controls were fully informed about the aim of the study, and written informed consent was obtained from all participates. The study was approved by the Ethics Committee of the Medical Faculty of Heidelberg and conducted according to the Declaration of Helsinki (study approval no. S523/2012).

Fluorescence-activated cell sorting (FACS) staining

Venous blood samples (9ml) were collected from all participants and isolated peripheral blood mononuclear cells (PBMCs) were analysed using a six-colour flow cytometry. Summarised, 8 x 10^6 PBMCs were surface stained with 10µl peridinin chlorophyll (PerCP)-conjugated anti-CD4 (BD Biosciences, Heidelberg, Germany), 5µl phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-CD127 (eBioscience, Frankfurt, Germany), 5µl allophycocyanin-hilite 7 (APC-H7)-conjugated anti-CD45RA (BD Biosciences), and 20µl Phycoerythrin (PE)-conjugated anti-ICOS (BD Biosciences) mouse monoclonal antibodies. For the detection of FoxP3 we performed intracellular staining (FoxP3 Staining Buffer Set, eBioscience, Frankfurt, Germany), using 5µl fluoresceinisothiocyanat (FITC)-conjugated anti-human FoxP3 (clone PCH101, eBioscience) according to the manufacturer's instructions. Additionally, the fixed cells were incubated with 2ml Alexa Fluor 647-conjugated anti-Ki67 monoclonal antibodies (clone B56, BD Biosciences) for detection of Ki67 positive cells. Negative control samples were incubated with isotype-matched antibodies. Cells were analysed by a FACS Canto cytometer (BD Bisosciences). For doublet and debris discrimination (14%±7%) FSC-H *versus* FSC-A and FSC *versus* SSC gating was used. Statistical analysis was based on at least 100.000 CD4$^+$-T-cells.

Positive selection of CD4$^+$CD127$^{low±}$ CD25$^+$-Treg cells

For purification of CD4$^+$CD127$^{low±}$ CD25$^+$-Treg cells, venous blood samples (50 ml) were collected from 13 healthy controls and 16 SLE remission patients. Cells were isolated using the "Regulatory-T-cell Isolation Kit II" (Miltenyi Biotec, Bergisch Gladbach, Germany) described elsewhere (7).

Sorting and functional testing of the different Treg subsets

To separate the isolated CD4$^+$CD127$^{low±}$ CD25$^+$-Tregs into ICOS$^+$-Tregs, naïve ICOS$^-$CD45RA$^+$-Tregs and ICOS$^-$CD45RA$^-$-memory-Tregs, the cells were stained with 2µl PE-conjugated anti-ICOS (BD Biosciences) and 5µl APC-H7-conjugated anti-CD45RA (BD Biosciences) mouse monoclonal antibodies. Cells were sorted using a FACS Aria II or FACS Aria III cell sorter (BD Biosciences). Potentially living cells (86%±10%) were sorted after doublet and debris discrimination by gating FSC-A *vs*. SSC-A and FSC-A *vs*. FSC-W. The suppressive activity of these isolated Treg subsets was tested using suppression assays described elsewhere (7).

Statistical analysis

To evaluate the influence of age on the composition of the total CD4$^+$-T-helper cell pool with different Treg/Tresp subsets (ICOS$^+$-Tregs, ICOS$^+$-Tresps, ICOS$^-$-Tregs, ICOS$^-$-Tresps) of both healthy controls and SLE patients, linear regression analysis was used. The same approach was used for evaluating the changes with age in the composition of total ICOS$^+$/ICOS$^-$Tregs/Tresps with their subsets (naïve ICOS$^+$/ICOS$^-$CD45RA$^+$-Tregs/Tresps, ICOS$^+$/ICOS$^-$CD45RA$^-$-memory-Tregs/Tresps) and for evaluating age-dependent changes in the percentage of Ki67$^+$-cells within total Tregs/Tresps and their subsets mentioned above. Age-independent differences between healthy volunteers, active SLE patients and inactive SLE patients, concerning the above listed Treg/Tresp subsets, were examined using multiple regression analysis adjusted for the age variable (centered on the mean), wherein an interaction term of the age and the patient group was included. Analysis of continuous data (suppression assays with ICOS$^+$-Tregs, naïve ICOS$^-$CD45RA$^+$-Tregs, ICOS$^-$CD45RA$^-$-memory-Tregs) was made using the Mann-Whitney U-test. A p-value <0.05 was considered significant. For all tests, the software package BiAS for Windows (v. 10.06) was used.

Results

Treg levels are increased within the total CD4$^+$-T-helper cell pool depending on the disease activity of SLE patients

In this study, we investigated the differentiation of highly proliferative ICOS$^+$-, or less proliferative ICOS$^-$-Treg and -Tresp subsets in healthy controls, SLE patients with SLEDAI <7 and SLE patients with SLEDAI >7. For that, we calculated the percentages of ICOS$^+$- and ICOS$^-$- Tregs/Tresps within the total CD4$^+$-T-helper cell pool and further determined to what extend the naïve ICOS$^+$CD45RA$^+$- and ICOS$^-$CD45RA$^+$-Tregs/Tresps differentiated into ICOS$^+$CD45RA$^-$- and ICOS$^-$CD45RA$^-$-memory Tregs/Tresps. In addition, we determined the proliferation capacity of the different Treg and Tresp subsets by measuring their percentages of Ki67$^+$-cells in all study groups. Supplementary Figure S1 shows the gating strategy that was used in all experiments and Table I presents the clinical data of all participants in this study. Since age as confounding factor may interfere with the results, we used a statistical method which eliminates the influence of age, to focus on the influence of disease activity.

Table I. Clinical characteristics of SLE patients and healthy controls.

	Healthy controls n=83	SLE remission patients n=86	Active SLE patients n=13
Female sex, n (%)	59 (71 %)	70 (81 %)	9 (69 %)
Age (years)	48 ± 18	47 ± 15	45 ± 17
Time since initial diagnosis (months)		171 ± 110	122 ± 105
Renal involvement, n (%)		64 (74 %)	10 (77 %)
SLEDAI		2 ± 2	13 ± 9
ANA titre ≥1:1280, n (%)		54 (63 %)	13 (100 %)
DsDNA antibodies ELISA (IU/ml)		42.9 ± 90.8	349.0 ± 549.8
C3 complement (g/l)		1.1 ± 0.3	0.6 ± 0.3
C4 complement (g/l)		0.22 ± 0.09	0.08 ± 0.06
Erythrocyturia (x μl^{-1})		5 ± 13	57 ± 82
Leukocyturia (x μl^{-1})		36 ± 168	58 ± 80
Serum leucocytes		6.6 ± 2.4	5.4 ± 2.6
Serum creatinine (mg dl^{-1})		0.9 ± 0.5	0.9 ± 0.3
CKD-EPI GFR (ml min^{-1} (1.73 m^2)$^{-1}$)		90.1 ± 29.4	92.5 ± 29.0
Urine-Protein/Urine-creatinine ratio (g mol $creatinine^{-1}$)		50.3 ± 97.0	187.1 ± 224.1
Medication			
No Medication, n (%)		7 (8 %)	0
Antimalarials, n (%)		62 (72 %)	11 (85 %)
Mycophenolic acid (MPA), n (%)		36 (42 %)	4 (31 %)
Azathioprine (AZA), n (%)		17 (20 %)	3 (23 %)
Glucocorticoids, n (%)		51 (59 %)	3 (23 %)
Glucocorticoid dose (mg d^{-1})		2.3 ± 2.3	5.2 ± 15.9

ANA: antinuclear antibodies; CKD-EPI GFR: Chronic Kidney Disease Epidemiology Collaboration estimated Glomerular Filtration Rate; MDRD GFR: Modification of Diet in Renal Disease Study estimated Glomerular Filtration Rate; SLEDAI: SLE Disease Activity Index; n: number.
The data is presented as their mean and standard deviations.

Compared to healthy controls, we found a significant decrease in the percentage of $CD4^+$-T-helper cells in SLE patients with SLEDAI <7. In SLE patients with SLEDAI >7, this decrease of $CD4^+$-T-helper cell frequency was intensified, but not significantly (Fig. 1A). In contrast, SLE patients with SLDAI <7 tended to show an increased proliferation capacity of $CD4^+$-T-helper cells, which was significantly enhanced in patients with SLEDAI >7 (Fig. 1A). Concerning the composition of the total $CD4^+$-T-helper cell pool with Tregs and Tresps, we ascertained a significant increase of Tregs in SLE patients with SLEDAI <7 compared to healthy controls, while the Tresps were complementary diminished (Fig. 1B-C). These changes were even more pronounced in SLE patients with SLEDAI >7, where the percentage of Tregs exceeded that of SLE patients with SLEDAI <7 significantly, while the Tresp pool declined accordingly (Fig. 1B-C). In SLE patients with SLEDAI <7, the proliferation capacity of Treg cells was significantly diminished compared to healthy controls (Fig. 1B) while for Tresp cells a significantly increased proliferation capacity was found (Fig. 1C). Considering disease activity, patients with SLEDAI >7 showed a significant increase in $Ki67^+$-cells mainly for Tresps but also for Tregs, compared to patients with SLEDAI <7 (Fig. 1B-C).

In SLE patients with markedly increased disease activity naïve $CD45RA^+$-Tregs accumulate within the total $ICOS^+$-Treg pool

Compared to healthy controls, we found a strong decrease in the percentage of $ICOS^+$-Tregs and a complementary increase of $ICOS^-$-Tregs in SLE patients with SLEDAI <7. These circumstances were reversed in patients with SLEDAI >7, as $ICOS^+$-Tregs increased strongly, while $ICOS^-$-Tregs declined accordingly. Conspicuously, these changes showed a certain, although not significant age dependency (Fig. 2A-D).

During a healthy course of life, naïve $CD45RA^+$-Treg/Tresp cells decrease (Fig. 2B, E, H and K), while $CD45RA^-$-memory-Tregs/Tresp increase significantly (Fig. 2C, F, I, and L) within total $ICOS^+$ and $ICOS^-$-Tregs/Tresps, suggesting an increased differentiation of $ICOS^+$- and $ICOS^-$-Tregs/Tresps with age. Such an age-dependent differentiation of all Treg/Tresp subsets was also detected in SLE patients with SLEDAI <7, with the exception of $ICOS^+$-Tregs, whose naïve and memory subsets did not decrease or increase significantly with age (Fig. 2B-C). Furthermore, naïve $CD45RA^+$-Tregs of SLE patients >7 showed a significant age-independent accumulation with a complementary decline of $CD45RA^-$-memory-Tregs within the total $ICOS^+$-Treg pool compared to naïve $CD45RA^+$-Tregs of SLE patients with SLEDAI <7. This suggests an exhaustion of $ICOS^+$-Treg differentiation in patients with SLEDAI >7 (Fig. 2B-C). In contrast, a significantly increased age-independent differentiation of naïve $ICOS^-CD45RA^+$-Tregs into $ICOS^-CD45RA^-$-memory-Tregs was detected in SLE patients with SLEDAI <7, which was further strengthened in patients with SLEDAI >7. This seems to cause the $ICOS^-$-Tregs to lose their age-dependent differentiation capacity in these patients (Fig. 2E-F). These effects shifted the composition of the total Treg pool in favour of $ICOS^-$-Tregs in SLE patients with SLEDAI <7, while these cells decreased with age in patients with SLEDAI >7 (Fig. 2A and D). Such findings propose that there is a strong immune response of Tregs in SLE patients, where $ICOS^+$-Tregs of patients with SLEDAI <7 lose their differentiation capacity with age, when $ICOS^-$-Tregs are still expanded. During markedly active disease, $ICOS^+$-Tregs have already lost their differentiation capacity at a young age, when $ICOS^-$-Tregs still differentiate strongly, but are not able to increase their differentiation capacity with age.

$ICOS^-$-Tresps are diminished within the total Tresp pool in SLE patients with markedly increased disease activity

Similarly as described for the Treg pool, a shifting in the composition of the Tresp pool in favour of $ICOS^+$-Tresps was found (Fig. 2G and J). Thereby, an increased differentiation of naïve $ICOS^+CD45RA^+$-Tresps into $ICOS^+CD45RA^-$-memory Tresps could neither be observed between healthy controls and SLE patients with SLE-

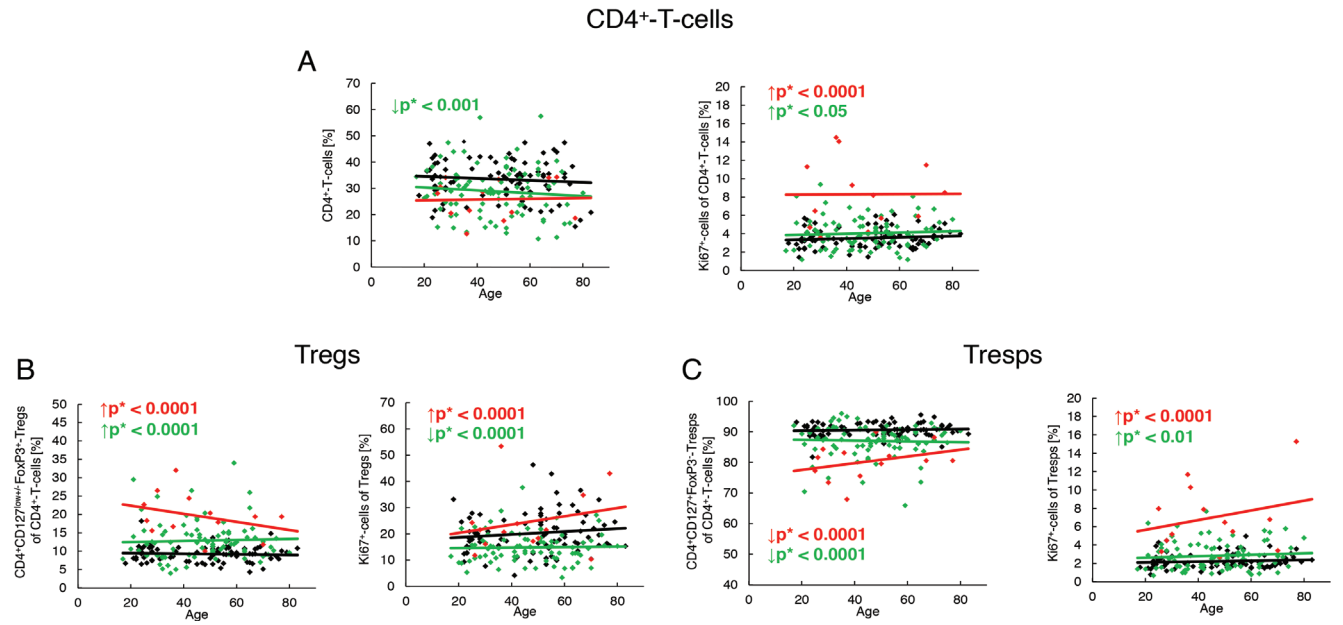

Fig. 1. Percentages and Ki67 expression of CD4+-T-cells, total CD4+CD127low+/-FoxP3+-Tregs and total CD4+CD127+FoxP3--Tresps during course of life in healthy volunteers (n=83), SLE patients with low disease activity (SLEDAI <7, n=86) and SLE patients with moderate to high disease activity (SLEDAI >7, n=13).
The percentages of CD4+-T-cells within PBMCs (**A**), Tregs (**B**) and Tresps (**C**) within the CD4+-T-helper pool, as well as their Ki67 expression are shown for healthy volunteers (◆), SLE patients with SLEDAI <7(◆) and patients with SLEDAI >7 (◆). The figures present the regression lines concerning changes in percentages of the individual T-cell subsets together with their Ki67 expression with increasing age. Significant age-independent differences between healthy volunteers and SLE remission patients are marked by green arrows (↑↓) and green *p**-values, differences between SLE remission patients and SLE active patients are marked by red arrows (↑↓) and red *p**-values.

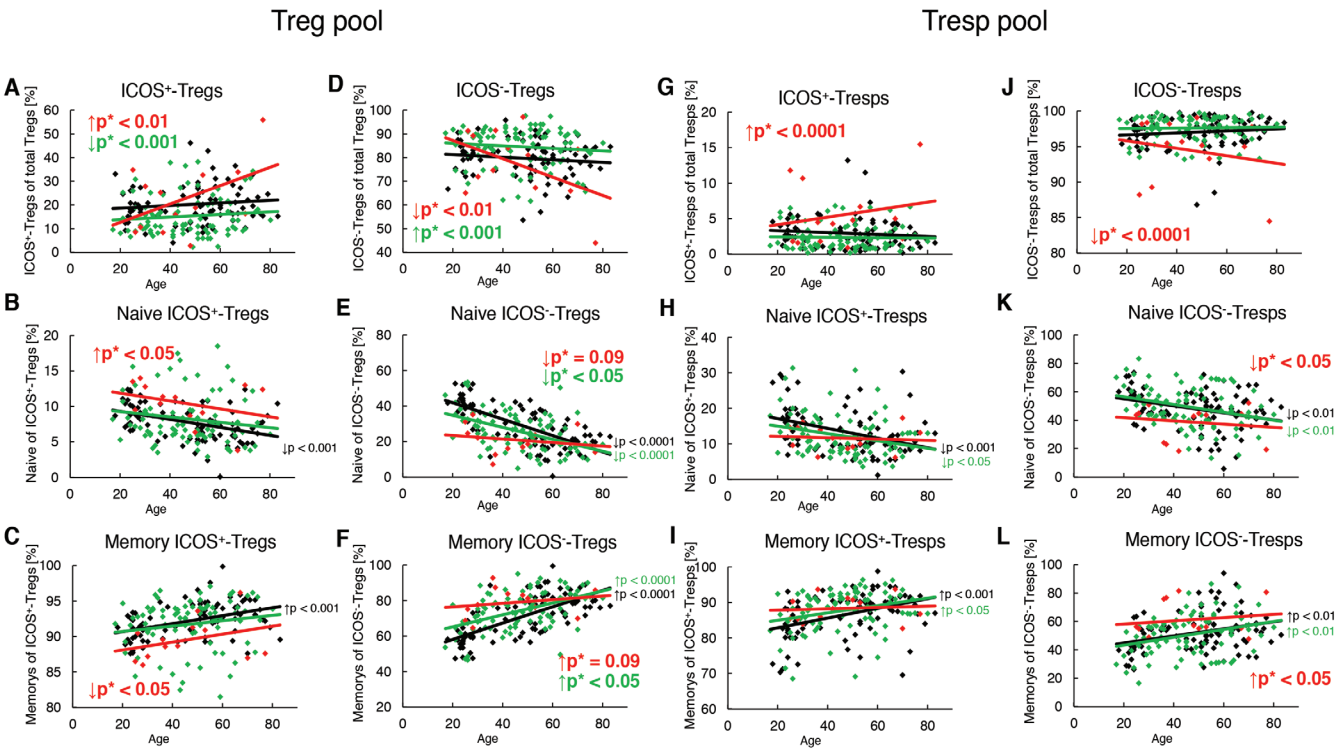

Fig. 2. Changes in composition and differentiation of the total Treg and Tresp pool with ICOS+- and ICOS--T-cells in healthy volunteers (n=83) SLE patients with low disease activity (SLEDAI <7, n=86) and SLE patients with moderate to high disease activity (SLEDAI >7, n=13) throughout life.
The percentages of ICOS+- and ICOS--Tregs (**A** and **D**), as well as ICOS+- and ICOS--Tresps (**G** and **J**) within total Tregs or Tresps together with their respective naïve (**B**, **E**, **H** and **K**) and memory subsets (**C**, **F**, **I**, and **L**) were estimated in healthy volunteers (◆), SLE patients with SLEDAI <7 (◆) and patients with SLEDAI >7 (◆). The figures present the regression lines concerning the changes in the percentages of the different Treg/Tresp subsets with increasing age. Significant changes are indicated by black (healthy volunteers), green (patients with SLEDAI <7), or red (patients with SLEDAI >7) *p*-values and arrows. Age-independent differences between healthy volunteers and SLE patients with SLEDAI <7 are indicated by green *p**-values and green arrows, differences between SLE patients with SLEDAI <7 and patients with SLEDAI >7 are indicated by red *p**-values red arrows.

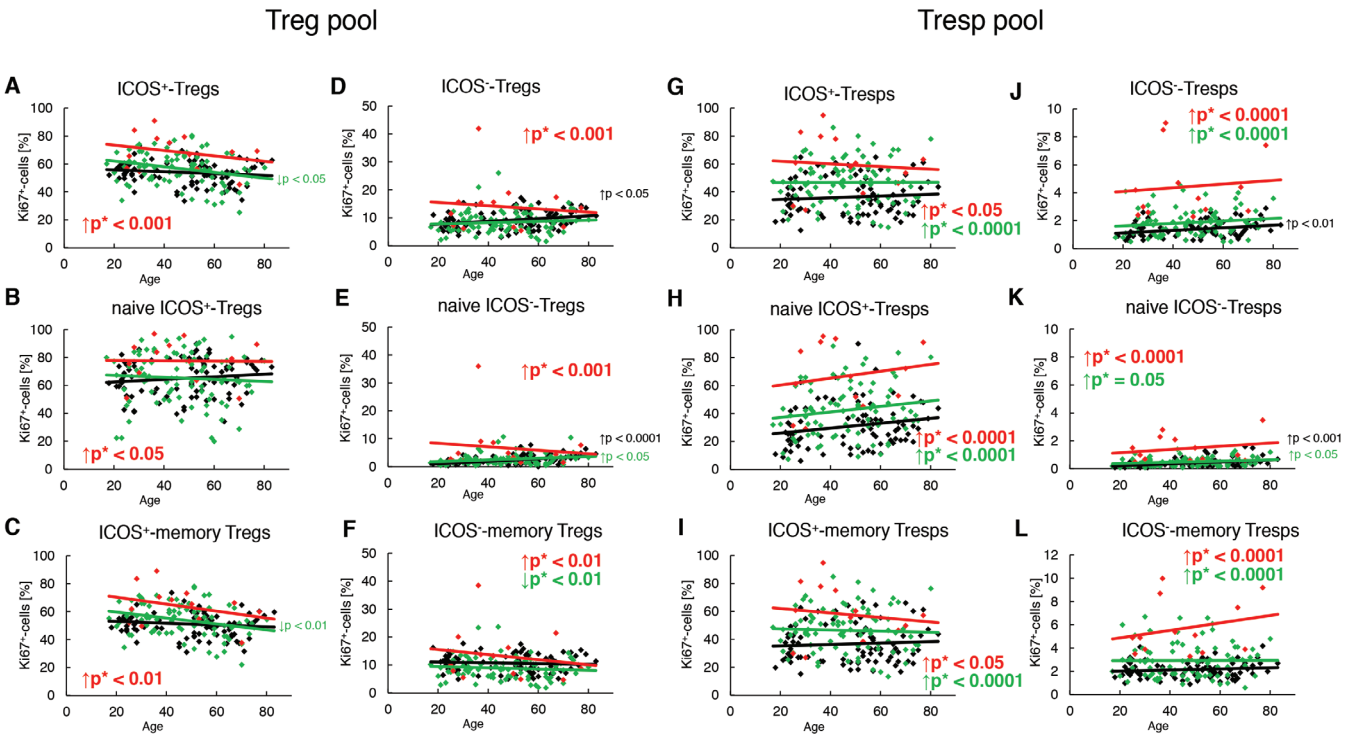

Fig. 3. Changes in the Ki67 expression of ICOS$^+$- and ICOS$^-$-Tregs/Tresps and their subsets in healthy volunteers (n=83) SLE patients with low disease activity (SLEDAI <7, n=86) and SLE patients with moderate to high disease activity (SLEDAI >7, n=13) throughout life.
The percentages of Ki67$^+$-cells in ICOS$^+$- and ICOS$^-$-Tregs (**A** and **D**), in ICOS$^+$- and ICOS$^-$-Tresps (**G** and **J**) as well as in their respective naïve (**B**, **E**, **H** and **K**) and memory subsets (**C**, **F**, **I**, and **L**) is shown for healthy volunteers (◆), SLE patients with SLEDAI <7 (◆) and patients with SLEDAI >7 (◆). The figures present the regression lines for age-related changes in the percentages of Ki67$^+$-cells for the individual Treg/Tresp subsets. Significant changes are indicated by black (healthy volunteers), green (SLE patients with SLEDAI <7), or red (patients with SLEDAI >7) *p*-values and arrows. Significant age-independent differences between healthy volunteers and SLE patients with SLEDAI <7 are indicated by green *p**-values and green arrows, differences between SLE patients with SLEDAI <7 and patients with SLEDAI >7 are indicated by red *p**-values and arrows.

DAI <7, nor between patients with SLEDAI <7 and patients with SLEDAI >7 (Fig. 2H and I). For ICOS$^-$-Tresps, a significantly increased differentiation of naïve ICOS$^-$CD45RA$^+$-Tresps into ICOS$^-$CD45RA$^-$-memory-Tresps was observed in patients with SLEDAI >7 compared to patients with SLEDAI <7 (Fig. 2K and L). Such findings suggest that the differentiation of ICOS$^-$-Tresps is particularly restricted to episodes of markedly active disease. Nevertheless, this enhanced differentiation leads to a decrease of these cells within the entire Tresp pool (Fig. 2G and J).

Increased disease activity is associated with extensive proliferation of the entire CD4$^+$-T-helper cell system

Compared to healthy controls Ki67-staining revealed a comparable proliferation capacity of both ICOS$^+$- and ICOS$^-$-Tregs and their subsets in SLE patients with SLEDAI <7 (Fig. 3A-F), with the exception of ICOS$^-$-memory Tregs whose proliferation capacity was significantly reduced (Fig. 3F). In contrast, the proliferation capacity of both ICOS$^+$- and ICOS$^-$-Tresps including their subsets, was substantially increased in these patients (Fig. 3G-L). These findings suggest that Treg cells are sufficiently suppressed by the immunosuppressive therapy, while Tresp cells are still activated in SLE patients with SLEDAI <7. Patients with SLEDAI >7 showed a significant increase concerning the proliferation capacity of all Treg and Tresp subsets compared to SLE patients with SLEDAI <7, demonstrating an extensive activation and proliferation of the entire CD4$^+$-T-helper cell system (Fig. 3A-L).

The significantly increased ratio of ICOS$^+$-Tregs/ICOS$^+$-Tresps in SLE patients cannot be maintained with increased disease activity

We further investigated whether alterations in the differentiation and proliferation of the different Treg/Tresp subsets in SLE patients could affect the composition of the total CD4$^+$-T-helper cell pool with ICOS$^+$-Tregs/Tresps or ICOS$^-$-Tregs/Tresps and whether such changes affect the ratio of ICOS$^+$-Tregs to ICOS$^+$-Tresps or that of ICOS$^-$-Tregs to ICOS$^-$-Tresps. In patients with SLEDAI <7, we did not detect changes in the percentage of ICOS$^+$-Tregs within total CD4$^+$-T-helper cells compared to healthy controls (Fig. 4A), but we found significantly reduced percentages of ICOS$^+$-Tresps (Fig. 4B), resulting in an significantly increased ratio of ICOS$^+$-Tregs/ICOS$^+$-Tresps (Fig. 4C). However, the age-dependent increase of this ratio in healthy controls could not be maintained in patients with SLEDAI <7 (Fig. 4C). In contrast, ICOS$^-$-Tregs were strongly increased (Fig. 4D), while ICOS$^-$-Tresps were considerably decreased (Fig. 4E) within total CD4$^+$-T-helper cells, resulting in a significantly increased ratio of ICOS$^-$-Tregs/ICOS$^-$-Tresps (Fig. 4F) in SLE patients with SLEDAI <7.
With markedly increased disease activi-

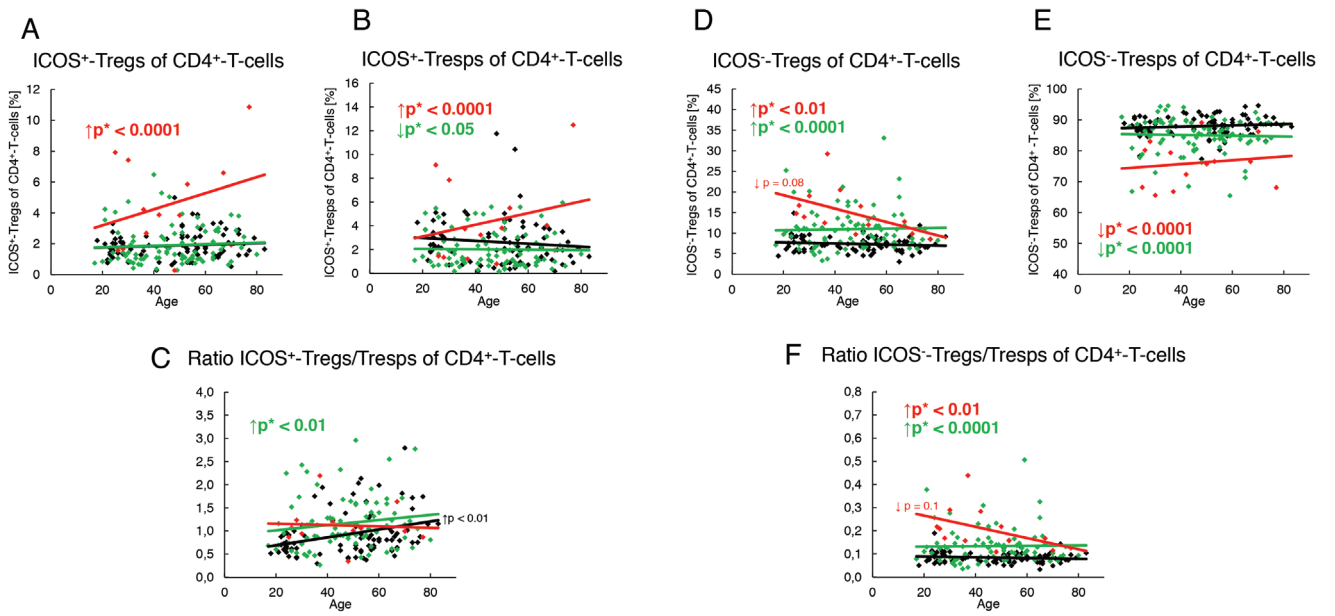

Fig. 4. Composition of the CD4+-T-cell-pool with ICOS+-Tregs/Tresps and ICOS-Tregs/Tresps, and ratios of ICOS+-Tregs/ICOS+-Tresps or ICOS-Tregs/ICOS-Tresps in healthy volunteers (n=83) SLE patients with low disease activity (SLEDAI <7, n=86) and SLE patients with moderate to high disease activity (SLEDAI >7, n=13) throughout life.
The percentage of ICOS+-Tregs (**A**), ICOS+-Tresps (**B**), ICOS-Tregs (**D**) and ICOS-Tresps (**E**) within total CD4+-T-helper cells, as well as the ratio of ICOS+-Tregs/ICOS+-Tresps (**C**) and ICOS-Tregs/ICOS-Tresps (**F**) throughout life is shown for healthy volunteers (◆), SLE patients with SLEDAI <7 (◆) and patients with SLEDAI >7 (◆). Significant age related changes in the percentages of the different subsets within total CD4+-T-helper cells were not found (**A**, **B**, **D** and **E**). A significant age-related increase in the ratio of ICOS+-Tregs/ICOS+-Tresps was detected in healthy volunteers (indicated as black *p*-value and arrow), but not in SLE patients with SLEDAI <7 or patients with SLEDAI >7 (**C**). Significant age-independent differences between healthy volunteers and SLE patients with SLEDAI <7 or patients with SLEDAI <7 and patients with SLEDAI >7 are indicated by green *p**-values and arrows or red *p**-values and arrows. A significant age-independently increased ratio of ICOS+-Tregs/ICOS+-Tresps was found in SLE patients with SLEDAI <7 compared to healthy volunteers, but it could not be maintained in patients with SLEDAI >7 (**C**). The ratio of ICOS-Tregs/ICOS-Tresps was age-independently increased in both SLE patients with SLEDAI <7 and patients with SLEDAI >7 (**F**).

ty, we detected a strong increase of both ICOS+-Tregs (Fig. 4A) and ICOS+-Tresps (Fig. 4B), abolishing the effect of the increased ratio of ICOS+-Tregs/ICOS+-Tresps seen in patients with SLEDAI <7 (Fig. 4C). In contrast, the effect of strongly increased percentages ICOS-Tregs (Fig. 4D), but decreased percentages of ICOS-Tresps (Fig. 4E) was significantly enhanced in patients with SLEDAI >7 compared to patients with SLEDAI <7. Thereby, the significantly increased ratio of ICOS-Tregs/ICOS-Tresps was exceeded, especially in younger individuals. However, an almost significant age dependent decrease of this ratio was indicated in these patients, supporting our data concerning an intensified differentiation, but age-dependent exhaustion of ICOS-Treg differentiation during active disease (Fig. 4F).

SLE patients show deficiencies concerning their suppressive activity of ICOS- but not of ICOS+-Tregs

To examine whether there were differences in the suppressive activity of separated ICOS+-Tregs, naïve ICOS-CD45RA+-Tregs and ICOS-CD45RA-memory-Tregs, the total CD4+CD127low+/-CD25+-Treg pool of 13 healthy volunteers (mean age 41 years ± 17 years) and 16 SLE patients with SLEDAI <7 (mean age 49 years ± 13 years) was isolated by MACS and sorted into the above mentioned Treg-subsets. Subsequently, the isolated Treg subsets were analysed separately for their suppressive capacity. We found that neither the maximum suppressive activity (Fig. 5A), nor the ratio of ICOS+-Tregs/Tresps up to which the IOCS+-Tregs could be diluted to achieve a minimum suppressive activity of 15% (Fig. 5D), was significantly reduced compared to healthy controls. However, both parameters were significantly decreased for both naïve ICOS-CD45RA+- and ICOS-CD45RA-memory Tregs (Fig. 5B, C, E and F). Such findings suggest that the impaired functionality of ICOS-Tregs in patients with SLEDAI <7 may be caused by the reduced proliferation capacity, detected most notably in ICOS--memory Tregs (Fig. 3F).

Discussion

Although new knowledge on the pathogenesis of human SLE is constantly being gained (28), the particular role of immunosuppressive CD4+-Tregs or stimulating responder CD4+-Tresps remains elusive. Comprehensive systemic review and meta-analysis reported loss of CD4+-Tregs to be the most important cause of the disturbed CD4+-T-cell signalling in active disease patients (29). In contrast, our data reveal a significant decrease of total CD4+-T-helper cells in SLE patients, but surprisingly indicate a shift in the composition of the total CD4+-T-helper cell pool in favour of Tregs. This shift was significantly more pronounced in patients with SLEDAI >7 compared to patients with SLEDAI <7. Since previous studies which characterised Tregs as CD4+CD25+FoxP3+-T-cells confirm these data, discrepancies in Treg cell numbers cannot only be explained by

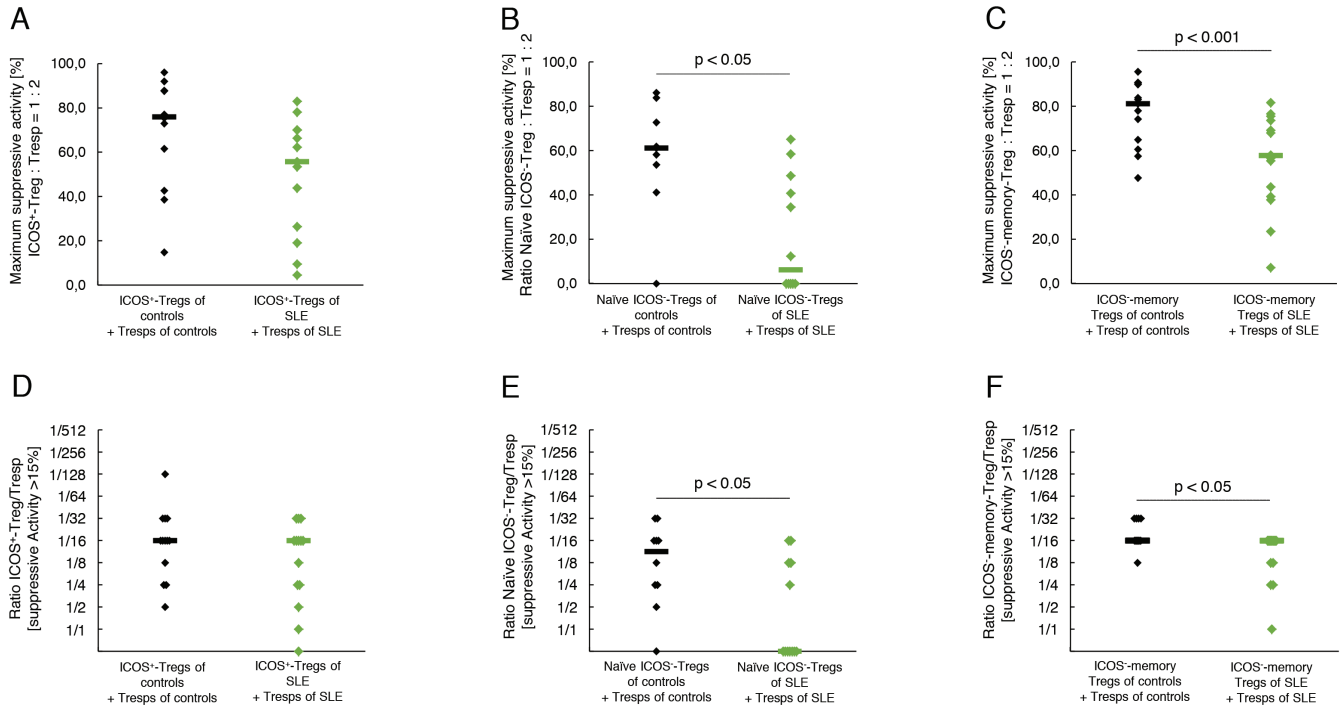

Fig. 5. Suppressive activity of ICOS$^+$-Tregs, naïve ICOS$^-$-Tregs and memory ICOS$^-$-Tregs in healthy volunteers (n=13) and SLE patients with SLEDAI <7 (n=16). The suppressive activity of the different Treg subsets was estimated by suppression assays for healthy volunteers (◆) and SLE remission patients (◆). The figure shows the maximum suppressive activity of ICOS$^+$-Tregs (**A**), naïve ICOS$^-$-Tregs (**B**) and ICOS$^-$-memory Tregs (**C**) at a Treg/Tresp ratio of 1:2. Furthermore, it shows the ratio of Tregs to Tresps up to which purified ICOS$^+$-Tregs (**D**), naïve ICOS$^-$-Tregs (**E**) and ICOS$^-$-memory Tregs (**F**) could be diluted to achieve a minimum suppressive activity of at least 15%. Significant differences in the suppressive activity between healthy volunteers and SLE patients with SLEDAI <7 were detected for both subsets of ICOS$^-$-Tregs but not for ICOS$^+$-Tregs.

the additional use of CD127 for quantitative determination of Tregs (30). Rather it appears that deficiencies in Treg cell numbers and function were documented due to the fact that CD25 either alone or in combination with other markers was used to identify human Treg cells. Previously, increased proportions of CD4$^+$FoxP3$^+$-T-cells, and in particular of CD4$^+$CD25$^-$FoxP3$^+$-T-cells, correlating with disease activity, were found in SLE patients (31-33) and subsequently also in patients with other autoimmune diseases such as rheumatoid arthritis (34) and multiple sclerosis (35). These CD4$^+$CD25$^-$FoxP3$^+$-T-cells were shown to share numerous properties of conventional CD4$^+$CD25^{high+}Foxp3$^+$-Tregs, such as demethylation of the Treg specific epigenetic control region in FoxP3 (TSDR), constitutive expression of the transcription factor HELIOS and lack of IL-2 production. Compared to conventional CD4$^+$CD25^{high+}Foxp3$^+$-Tregs, more of these cells were shown to be in cell cycle (Ki67$^+$) and expressed the late-stage inhibitory receptor PD-1, but revealed reduced expression of the early-stage inhibitory receptor CTLA-4. As the number of these cells correlated with the proportion of CD25^{high+}Foxp3$^+$Ki67$^+$-T-cells in cell cycle, it is assumed that these cells represent thymus derived Treg cells which were recently expanded in response to an autoimmune reaction (36).

Our data document an increased proportion of cells in cell cycle (Ki67$^+$) in both naïve and memory ICOS$^+$- or ICOS$^-$-Tregs/Tresps in SLE patients with SLEDAI >7 compared to patients with SLEDAI <7. Accordingly, Ki67$^+$-urinary CD4$^+$-T-cells were already described to correlate with the occurrence of active lupus nephritis (37). However, when considering the differentiation of these cells, our data showed that naïve ICOS$^+$-Tregs were enriched, while ICOS$^+$-memory-Tregs were reduced within the total ICOS$^+$-Treg pool in SLE patients with SLEDAI >7. Such findings propose that similar as assumed for CD4$^+$CD25$^-$FoxP3$^+$-T-cells, the enrichment of naïve ICOS$^+$-Tregs in patients with SLEDAI >7 may be the result of a permanent inflammatory reaction, which is attempted to be suppressed by the increased differentiation of naïve ICOS$^+$- and ICOS$^-$-Tregs into ICOS$^+$- and ICOS$^-$-memory Tregs. However, it seems that this ongoing differentiation can largely be maintained for ICOS$^-$-Tregs but not for ICOS$^+$-Tregs. Recently published data, demonstrating an increased number of naïve CD45RA$^+$FoxP3$^+$-Tregs with impaired suppressive function in active SLE patients also support such conclusions (38).

As recently published, the immunosuppressive therapy has a considerable effect on the composition of the total CD4$^+$-T-helper cell pool regarding Tregs and Tresps (7). Obviously, in SLE patients with SLEDAI <7, the percentage of ICOS$^+$-Tresps is stronger reduced within the total CD4$^+$-T-helper cell pool than that of ICOS$^+$-Tregs. Thus, the ICOS$^+$-Treg/ICOS$^+$-Tresp ratio remains significantly increased in these patients. Therefore, our findings may propose that the immunosuppressive therapy has a stronger effect on the differentiation of ICOS$^+$-Tresps than on

ICOS$^+$-Tregs. In patients with markedly elevated disease activity (SLEDAI >7), the percentage of both ICOS$^+$-Tregs and ICOS$^+$-Tresps rises within the total CD4$^+$ T helper cells, but ICOS$^+$-Tregs apparently do not rise strongly enough to significantly increase the ICOS$^+$-Treg/ICOS$^+$-Tresp ratio in these patients. Similarly, the exaggerated differentiation of ICOS$^-$-Tregs is inhibited by the administered therapy in patients with SLEDAI <7. However, in patients with increased disease activity, this suppressive effect is removed. Due to the additional tremendous decrease of ICOS$^-$-Tresps, the ratio of ICOS$^-$-Tregs/ICOS$^-$-Tresps rises strongly. Thereby, it seems that such an exaggerated Treg cell differentiation is only a response mechanism to a misdirected increased differentiation of ICOS$^-$-Tresps, whereby malfunctioning defective ICOS$^-$-Tresps with low expansion capacity and low sensitivity for Treg suppression may arise.

Functional testing of Tregs from patients with SLEDAI <7, that have not yet been carried out in this way, revealed an impaired suppressive activity of ICOS$^-$-Tregs, but not of ICOS$^+$-Tregs. Such findings may propose that either the functionality of ICOS$^-$-Tregs is strongly disturbed in SLE patients, so that even an increasing proportion of ICOS$^-$-Tregs is not able to inhibit a decreasing proportion of ICOS$^-$-Tresps in active disease, or that the immunosuppressive therapy may be responsible for the loss of functionality of these cells. This conclusion could be considered since the percentage of Ki67$^+$-T cells in the ICOS$^-$-memory Tregs of patients with SLEDAI <7 was significantly reduced compared to healthy controls, but increased again in patients with SLEDAI >7. Therefore it seems more likely that a stable increased ICOS$^+$-Treg/ICOS$^+$-Tresp ratio is necessary for the avoidance of disease flares in remission patients. Experiments in mice support these assumptions, as in a lupus-prone mouse model (B6.Sle1 mice) it was shown that the elimination of ICOS expressing CD4$^+$-T-cells resulted in a significant loss in anti-nucleosome autoantibodies, suggesting that loss of tolerance is controlled by ICOS (39).

Meanwhile, it is known that ICOS is predominantly expressed on both Tfh and regulatory follicular helper cells (Tfr) which regulate the quantity and quality of humoral immunity. Activation of this molecule in CD4$^+$-T-helper cells was shown to induce Bcl-6-dependent functional differentiation of both cell subsets (40). Since Tfr cells act antagonistic to Tfh cells, their functional balance may be critical for the humoral immune homeostasis. In humans, both Tfh and Tfr cells were identified as circulating blood cells (41) and alterations of their numbers were found to be related to the pathogenesis of SLE (16, 17, 42-44), wherein in particular the increased ratio of circulating Tfh/Tfr cells was found to be correlated with disease activity in SLE patients (44). In contrast, an increased prevalence of Tfr cells and an increased ratio of Tfr/Tfh cells were also found to correlate with autoantibodies and SLEDAI scores in SLE patients (45). In our study the ICOS$^+$-Tregs/Tresps were not further characterised by Tfr or Tfh specific markers, such as of Bcl-6, CXCR5, or PD-1. However, both published data evaluating Tfr/Tfh ratios, as well as our data evaluating ICOS$^+$-Treg/ICOS$^+$-Tresp ratios in active SLE patients provide evidence that highly proliferating ICOS$^+$-Tregs/Tresps reveal an increased prevalence within the entire CD4$^+$-T-helper cell pool. Thereby, the dual role of ICOS signalling on both Treg and Tresp expansion (46) may contribute to sensitive changes in their ratio in active disease patients. Our data rather suggest that normal age-dependent differentiation is disproportionately increased in SLE patients, especially for ICOS$^+$-Tregs. Therefore, it is likely that the progressive immune senescence of these cells prevents the required increase in the ICOS$^+$-Treg/ICOS$^+$-Tresp ratio in active disease patients.

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