EXTENDED REPORT

Foxp3$^+$ Helios$^+$ regulatory T cells are expanded in active systemic lupus erythematosus

Tobias Alexander,[1,2] Arne Sattler,[3] Lars Templin,[1] Siegfried Kohler,[4] Christian Groß,[5] Andreas Meisel,[3] Birgit Sawitzki,[6] Gerd-Rüdiger Burmester,[1] Renate Arnold,[7] Andreas Radbruch,[2] Andreas Thiel,[8] Falk Hiepe[1,2]

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[1]Medical Department, Division of Rheumatology and Clinical Immunology, Charité–University Medicine, Berlin, Germany
[2]German Rheumatism Research Center (DRFZ) Berlin, Berlin, Germany
[3]Nephrology and Internal Intensive Care Unit, Berlin–Brandenburg Center for Regenerative Therapies (BCRT), Charité–University Medicine, Berlin, Germany
[4]Department of Neurology with Chair in Experimental Neurology, Charité–University Medicine, Berlin, Germany
[5]Clinic for Orthopaedics, Center for Musculoskeletal Surgery, Charité–University Medicine, Berlin, Germany
[6]Institute for Medical Immunology, Charité–University Medicine, Berlin, Germany
[7]Medical Department, Division of Hematology, Oncology and Tumor Immunology, Charité–University Medicine Berlin, Berlin, Germany
[8]Department of Regenerative Immunology and Aging, Charité–University Medicine Berlin, Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany

Correspondence to
Tobias Alexander, Medical Department, Division of Rheumatology and Clinical Immunology, Charité–University Medicine Berlin, Charitéplatz 1, Berlin 10117, Germany;
tobias.alexander@charite.de

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ABSTRACT

Objectives Recent data debate the suitability of Helios, an Ikaros family member, as a marker for thymic-derived regulatory T cells (Treg). Nevertheless, Foxp3$^+$ Helios$^+$ Treg may be of particular relevance in mediating immune tolerance in chronic autoimmunity, such as systemic lupus erythematosus (SLE), as they possess enhanced suppressive function, compared to Foxp3$^+$ Helios$^-$ Treg.

Methods Multicolour flow cytometry was performed to analyse Foxp3 and Helios expression in peripheral blood CD4 T cells from SLE patients, compared to healthy controls (HC) and systemic sclerosis (SSc) and rheumatoid arthritis (RA) patients. Cytokine production, chemokine receptor expression for CXCR3 and CCR4, basal signal transducer and activator of transcription 5 (STAT5)a phosphorylation levels and T-cell receptor (TCR) Vβ repertoire were analysed by flow cytometry, and the methylation status of the Foxp3 locus (Treg-specific demethylated region, TSDR) by real-time PCR.

Results Frequencies of Foxp3$^+$ Helios$^+$ Treg, unlike Foxp3$^+$ Helios$^-$ T cells, were significantly increased in SLE patients and positively correlated with disease activity, whereas they were unaltered in SSc and RA patients. Compared to HC, Foxp3$^+$ Helios$^+$ Treg in SLE predominantly displayed a CD45RA$^-$/CD31$^-$/FoxP3^{low} memory phenotype with increased Ki-67 expression, enhanced basal pSTAT5a levels and a restricted TCR repertoire. Nonetheless, similar to HC, Foxp3$^+$ Helios$^+$ Treg in SLE lacked effector cytokine production, possessed a highly demethylated TSDR and expressed comparable levels of CXCR3 and CCR4.

Conclusions Our data suggest that Helios-expressing Foxp3$^+$ Treg with functional suppressive capacity and migratory potential into inflamed tissues are expanded in active SLE, presumably through γ-chain signalling cytokines and TCR stimulation, to compensate for autoreactive effector responses.

INTRODUCTION

Systemic autoimmune diseases such as systemic lupus erythematosus (SLE) are characterised by a breakdown of peripheral tolerance to self-antigens, followed by activation and expansion of autoreactive effector lymphocytes, which then propagate autoimmune responses in a self-perpetuating process ultimately leading to multiple organ damage.[1] Foxp3$^+$ T regulatory (Treg) cells are key mediators of peripheral self-tolerance that can actively suppress effector T cells, inhibit inflammation and prevent autoimmunity.[2] Although extensively studied over the past few years, conflicting results were reported concerning quantitative and qualitative deficiencies of Treg in SLE.[3–10] One of the reasons for such controversy is certainly the lack of a phenotype that uniquely associates with suppressive function, in particular under conditions of T-cell activation, as the two key Treg markers, namely CD25 and Foxp3, can also be expressed by activated non-Treg.[11 12]

Several microarray studies showed a relative upregulation of the Ikaros family transcription factor Helios in Foxp3$^+$ Treg.[13 14] In addition, it was suggested that Helios expression may distinguish thymic-derived naturally occurring Treg from peripherally induced Treg, as naturally occurring Treg co-expressed Helios but peripherally induced Treg developing in vitro or in vivo did not.[15] However, this notion was recently challenged by studies demonstrating that Helios expression is inducible in CD4 and CD8 T cells and Treg under certain conditions and associated with T-cell activation and proliferation.[16–18] A functional role for Helios in Treg remains less clear. Previous studies demonstrated that Helios binds to the Foxp3 promoter and upregulates its expression.[19] It was also reported that Foxp3$^+$ Helios$^+$ Treg represent a functional subset with associated CD103 and GITR expression and enhanced suppressive potential, as compared to Foxp3$^+$ Helios$^-$ Treg.[20] In addition, Foxp3$^+$ Helios$^+$ Treg were shown to differ from Foxp3$^+$ Helios$^-$ T cells in terms of epigenetic changes at the Foxp3 locus, their capacity to produce effector cytokines and their stability of Foxp3 expression on in-vitro expansion.[21]

Given their unique phenotypic and functional properties, Helios-expressing Treg may represent a subset of Treg with a putative role in mediating immune tolerance in chronic autoimmunity. We therefore aimed to analyse Treg expression of Helios in systemic autoimmunity with chronic T-cell proliferation in patients with SLE, compared to systemic sclerosis (SSc) and rheumatoid arthritis (RA) patients and healthy individuals.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were obtained from 20 SLE patients, age and sex-matched healthy controls (HC), 10 patients with progressive SSc, 10 patients with RA, 10 patients after undergoing thymectomy, seven autoimmune patients (four SLE, one

granulomatosis with polyangiitis, one SSc and one autoimmune-mediated polyneuropathy) after receiving autologous haematopoietic stem cell transplantation (ASCT) as described,[22 23] and from a cohort of elderly patients (aged 50–96 years) undergoing joint replacement for osteoarthritis, following informed consent. Details of the clinical characteristics and treatment regimens of the analysed SLE patients are provided in supplementary table S1 (available online only). All SSc patients received immunosuppressive drugs, their average prednisolone dose was 5.3 mg/day and their average modified Rodnan skin score was 12/51. All RA patients were on disease-modifying antirheumatic drug therapy with an average disease activity score in 28 joints of 4.0. All patients with thymectomy had surgery for myasthenia gravis; the average time after thymectomy was 12 years. Patients after receiving ASCT were in clinical remissions despite discontinuation of immunosuppression; the median time after ASCT was 55 months. Ethics approval was obtained from the Institutional Review Board of the Charité–University Medicine Berlin, Germany (EA1/178/07 and EA1/124/09). All work was carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were freshly isolated from heparinised blood by Ficoll–Hypaque density gradient centrifugation (Pharmacia Biotec). The phenotype of the cells was assessed by flow cytometry using the following antibodies: anti-CD3-PerCP (UCHT1; Biolegend), anti-CD4-Pacific Blue (TT1) or APC Cy7 (RPA-T4; BD Pharmingen), anti-CD31-APC (AC128; Miltenyi Biotec) anti-CD25-PE-Cy7 (2A3; BD Biosciences), anti-CD127-A647 (eBioRDR5; eBioscience), CD45RA-A700 (HI100; Biolegend), anti-CXCR3 PE (G025H7; Biolegend) and anti-CCR4 A647 (TG6; Biolegend). Cells were then fixed and permeabilised using the anti-human Foxp3 staining set (eBioscience) followed by intracellular staining with anti-Foxp3-Alexa488 or APC (PCH101; eBioscience), anti-Helios-PE or Pacific Blue (22F6; Biolegend), anti-Ki-67-FITC (Ki-67; DAKO). Quantification of peripheral blood T-cell subsets was performed with the TrueCount system (BD Biosciences).

Measurement of cytokine production

CD4 T cells were enriched using the AutoMACS device (Miltenyi Biotec) with a purity greater than 95%. Purified cells (1×10^6) were resuspended in 500 µl RPMI-1640 (Gibco BRL) supplemented with 10% human AB serum (Lonza) and rested over night at 37°C. Cells were then stimulated with phorbol myristate acetate (PMA) (25 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) for 5 h in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich) for 4 h at 37°C. The cells were then fixed and permeabilised using the anti-human Foxp3 Staining Set (eBioscience), followed by intracellular staining with anti-Foxp3-A488 (PCH101; eBioscience), anti-Helios-PE (22F6; Biolegend), anti-IL-2-PerCP-Cy5.5 (MQ1-17H12; eBioscience) and anti-interferon-γ-PE-Cy7 (4S.B3; eBioscience).

Phospho-flow analysis of STAT5a

PBMC were isolated from peripheral blood as described above with a bed-to-bench time not exceeding 30 min. After surface staining for CD4 cells, cells were fixed with Foxp3-buffer (eBioscience) and permeabilised with 1 ml methanol (Rotipuran 99.9%; Roth, Germany) for 10 min at −20°C as described.[24] Intracellular staining was performed with PE mouse anti-Stat5a (pY694; BD Bioscience), anti-Foxp3-Alexa488 (PCH101; eBioscience) and anti-Helios-Alexa-647 (22F6; Biolegend) after washing with Foxp3 permeabilisation buffer (eBioscience).

Analysis of TCR Vβ repertoire by flow cytometry

T-cell receptor (TCR) Vβ-family expression analysis was performed on freshly isolated peripheral blood CD4 T cells by flow cytometry using 22 TCR Vβ-specific mononuclear antibodies (IOTest Beta Mark; Beckman Coulter Immunotech, Marseille, France), followed by intracellular staining for Foxp3 and Helios as described above. At least 5×10^3 Foxp3$^+$ T cells were acquired for each Vβ family. Normal ranges were established for each Vβ member based on CI of 97.5% determined in 10 healthy individuals. Perturbations of Vβ families were considered to be significant in patients when they were outside of these normal intervals.

DNA methylation analysis of the Treg-specific demethylated region

Freshly isolated PBMC were surface stained for CD3 and CD4, followed by intracellular staining for Foxp3 and Helios and were then FACS sorted with a purity greater than 94%. Genomic DNA was extracted with the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). A minimum of 60 ng bisulfite-treated (EpiTect; Qiagen) genomic DNA was used in a real-time PCR to quantify the Foxp3 Treg-specific demethylated region (TSDR). Real-time PCR was performed in a final reaction volume of 20 µl containing 10 µl FastStart universal probe master (Roche Diagnostics, Mannheim, Germany), 50 ng/µl lamda DNA (New England Biolabs, Frankfurt, Germany), 5 pmol/µl methylation or non-methylation-specific probe, 30 pmol/µl methylation or non-methylation-specific primers and 60 ng bisulfite-treated DNA or a respective amount of plasmid standard. The samples were analysed in triplicate on an ABI 7500 cycler.

Statistical analysis

Data analysis was performed using the GraphPad Prism V.5.0 (GraphPad, California, USA). The Kolmogorov–Smirnov test was used to evaluate the distribution of each parameter. For data with normal distribution and homogeneity of variance, a Student's t test was used. The Wilcoxon signed-rank test was used to compare data with a non-normal distribution. Correlations were assessed by Spearman's rank correlation coefficients. A value of $p<0.05$ was considered significant in all statistical tests.

RESULTS

Increased proportion of Foxp3$^+$ Helios$^+$ Treg in active SLE

Expression analysis of Foxp3 on freshly isolated peripheral blood CD4 T cells revealed significantly increased frequencies of Foxp3$^+$ T cells in SLE patients compared to age and sex-matched healthy individuals (median values 12.7% vs 7.2%, $p=0.001$, figure 1B). When Helios expression was analysed by intracellular staining of CD4 T cells, in healthy individuals on average 69.7% of Foxp3$^+$ T cells were positive. In contrast, Helios expression was significantly higher in Foxp3$^+$ T cells from patients with SLE (median values 83.8% vs 69.7, $p<0.001$) but not with SSc or RA (figure 1B). A combination of Foxp3 and Helios staining in CD4 T cells revealed significantly higher frequencies of Foxp3$^+$ Helios$^+$ Treg in SLE patients compared to HC (median values 11.0% vs 5.0%, $p<0.001$), SSc and RA patients, whereas frequencies of Foxp3$^+$ Helios$^-$ and Foxp3$^-$ Helios$^+$ T cells were unaltered (figure 1C). Nevertheless, absolute numbers of peripheral blood Foxp3$^+$

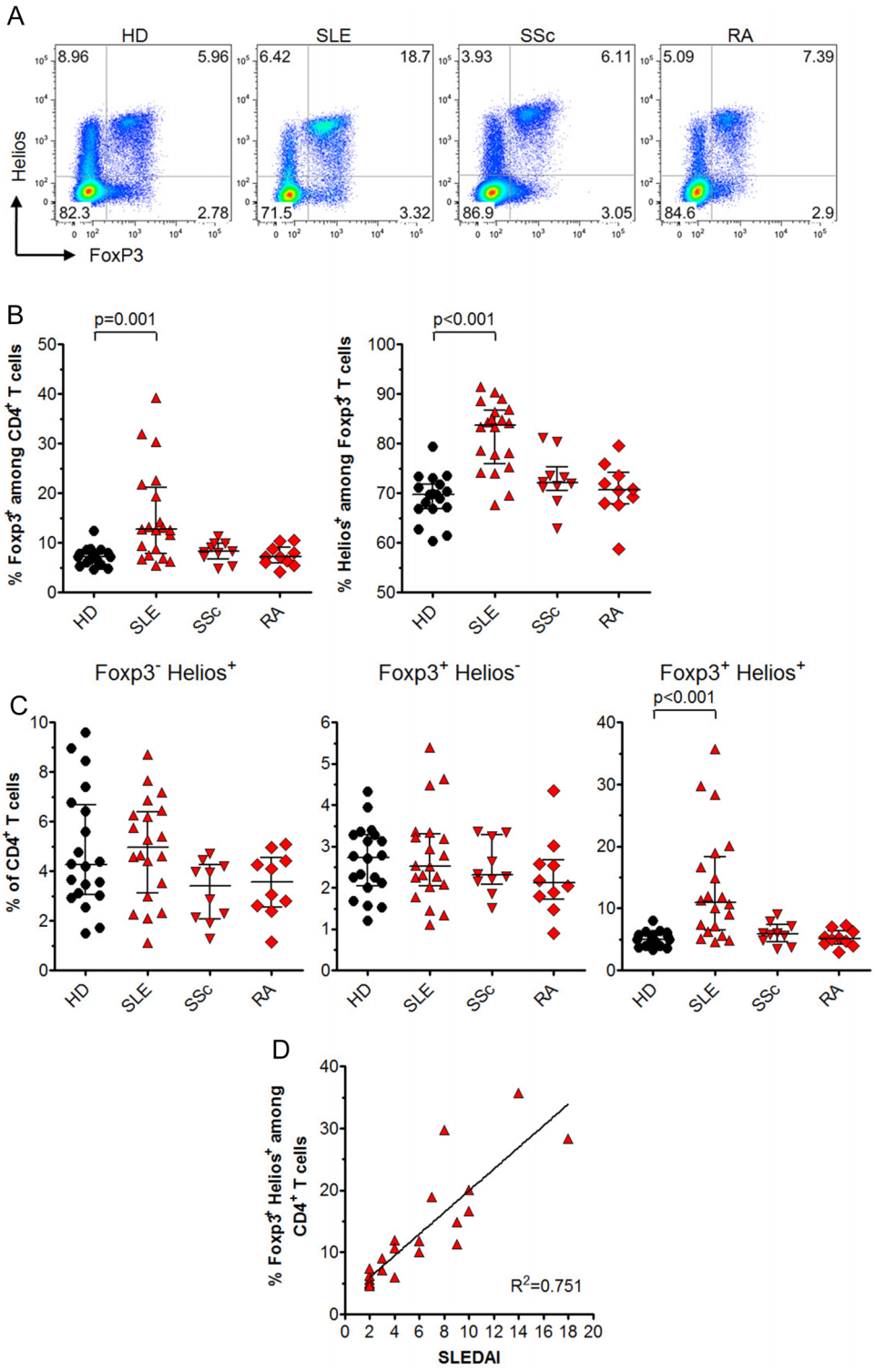

Figure 1 Expression analysis of Foxp3 and Helios in CD4 T cells. (A) Expression analysis of Foxp3 and Helios among CD4 T cells in representative peripheral blood samples from a healthy donor (HD) and patients with systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and rheumatoid arthritis (RA). (B) Frequencies of $Foxp3^+$ CD4 T cells and frequencies of Helios-expressing $Foxp3^+$ T regulatory cells (Treg) in HD (n=20), SLE patients (n=20), SSc (n=10) and RA patients (n=10) (median/interquartile range values). (C) Frequencies of $Foxp3^-$ $Helios^+$, $Foxp3^+$ $Helios^-$ and $Foxp3^+$ $Helios^+$ cells among CD4 T cells in HD (n=20), and patients with SLE (n=20), SSc (n=10) and RA (n=10) (median/interquartile range values). (D) Correlation between frequencies of Helios-expressing $Foxp3^+$ Treg among CD4 T cells in SLE patients with their disease activity based on SLE disease activity index (SLEDAI).

$Helios^+$ Treg were decreased in SLE patients compared to HC (27.3/μl vs 56.7/μl, $p<0.001$, data not shown). Notably, the frequency of these Treg in SLE positively correlated with disease activity at the time point of blood donation as determined by the SLE disease activity index (SLEDAI; $R^2=0.751$, figure 1D). Otherwise, no correlations were found with patient's age, disease duration, treatment regimens or organ manifestations.

Foxp3$^+$ Helios$^+$ in SLE predominantly display a Foxp3^{low}/CD45RA$^-$/CD31$^-$ memory phenotype

To investigate the phenotype of Helios-expressing Foxp3$^+$ Treg in SLE, we first performed expression analysis of the interleukin (IL)-2 receptor alpha-chain (CD25) that is constitutively expressed on Treg,[25] and the IL-7 receptor subunit alpha (CD127), which is known to correlate inversely with Foxp3 expression and suppressive Treg function.[26] Similar to what has been described for HC,[20] Foxp3$^+$ Helios$^+$ Treg from SLE samples displayed significantly higher expression levels for CD25 and lower expression levels for CD127 compared to Foxp3$^+$ Helios$^-$ T cells (figure 2A). Nonetheless, CD25 expression levels on Foxp3$^+$ Helios$^+$ Treg from SLE patients were lower compared to those from HC (p=0.026), as suggested earlier in studies on Treg phenotypes in SLE.[6]

We next analysed CD4 T cells for their expression of CD45RA and Foxp3, as this combination analysis enables the delineation of human Foxp3$^+$ cells into three subsets: CD45RA$^+$ Foxp3^{low} resting Treg, CD45RA$^-$ Foxp3^{high} activated Treg, both of which are suppressive in vitro, and cytokine-secreting CD45RA$^-$

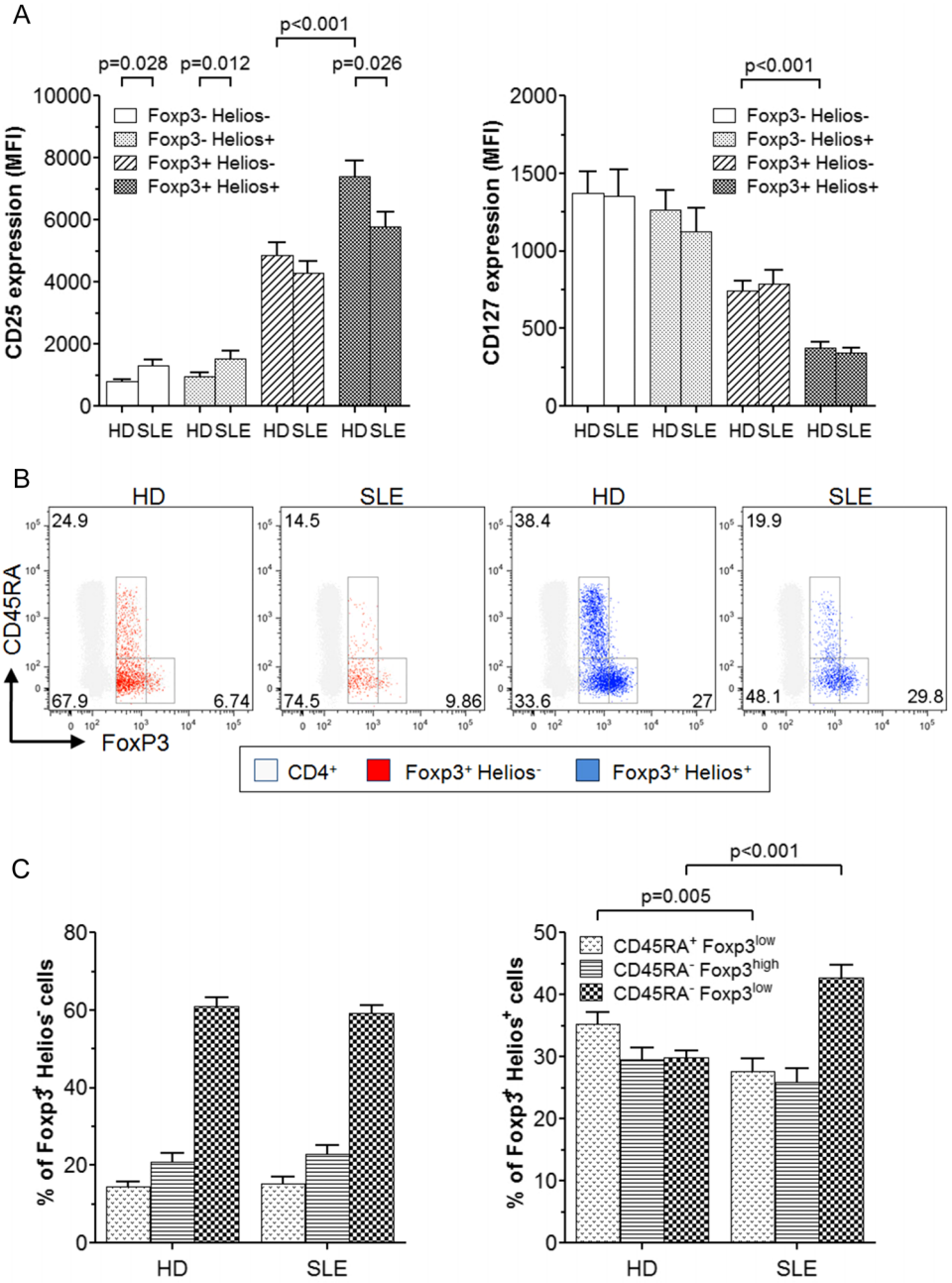

Figure 2 Phenotypic characterisation of Helios-expressing Foxp3$^+$ T regulatory cells (Treg) in systemic lupus erythematosus (SLE). (A) Expression levels for CD25 and CD127 based on median fluorescence intensity (MFI) values (mean values±SEM) in Foxp3$^-$ Helios$^-$, Foxp3$^-$ Helios$^+$, Foxp3$^+$ Helios$^-$ and Foxp3$^+$ Helios$^+$ T-cell subsets from healthy donors (HD) (n=20) and SLE patients (n=20). (B) Foxp3$^+$ Helios$^-$ (red, left dot plots) and Foxp3$^+$ Helios$^+$ (blue, right dot plots) Treg subsets were gated and analysed for their expression of CD45RA and Foxp3 and overlaid with expression in total CD4 T cells (grey) in representative samples from a HD and a SLE patient. (C) Frequencies of CD45RA$^+$Foxp3^{low}, CD45RA$^-$Foxp3^{high} and CD45RA$^-$Foxp3^{low} Treg (mean values±SEM) among either Foxp3$^+$ Helios$^-$ (left column) or Foxp3$^+$ Helios$^+$ Treg (right column) in HD (n=20) and SLE patients (n=20).

Foxp3^{low} cells, which are less suppressive.[27] While Foxp3$^+$ Helios$^+$ T cells from healthy individuals were nearly equally distributed among the aforementioned subsets, such cells in SLE were significantly more confined to the CD45RA$^-$ Foxp3^{low} subset ($p<0.001$) at the expense of CD45RA$^+$ Foxp3^{low} resting Treg cells ($p=0.005$) (figure 2C). Conversely, the majority of Foxp3$^+$ Helios$^-$ T cells displayed a CD45RA$^-$ FoxP3^{low} memory phenotype in both HC and SLE patients.

To evaluate whether Foxp3$^+$ Helios$^+$ Treg in SLE have the potential to migrate into inflamed tissues, we investigated their chemokine receptor expression for CXCR3 and CCR4 that are instrumental for the recruitment into inflamed tissues[28] or the skin,[29] respectively. Here, we found similar expression levels for CXCR3 and CCR4 in Helios$^+$ Foxp3$^+$ Treg from 10 SLE patients with lupus nephritis when compared to HC, suggesting that such Treg have migratory potential into inflamed tissue, including the skin (see supplementary figure S1, available online only). Only co-expression levels for CXCR3 and CCR4 were slightly decreased in both Helios$^-$ and Helios$^+$ Foxp3$^+$ Treg from SLE samples, possibly reflecting Treg tissue reallocation.

Foxp3$^+$ Helios$^+$ Treg in SLE lack effector cytokine production and possess a demethylated TSDR

To analyse whether Foxp3$^+$ Helios$^+$ T cells in SLE patients represent activated conventional T cells or resemble a T-cell subset with functional regulatory potential, we first assessed their capacity to produce effector cytokines. When enriched CD4 T cells were stimulated with PMA and ionomycin for 5 h and cytokine production by Foxp3$^+$ T cells was analysed by intracellular staining, in healthy individuals low, but considerable numbers of IL-2 and IFN-γ-secreting cells were detected; and in accordance with previous findings,[15] these cells were strictly confined to the Foxp3$^+$ Helios$^-$ T cell population (figure 3). When samples from SLE patients were investigated, Foxp3$^+$ Helios$^+$ T cells also lacked effector cytokine production for IL-2 and IFN-γ, whereas Foxp3$^+$ Helios$^-$ T cells contained significant proportions of such cells with even more IL-2-secreting cells compared to HC (median values 29.5% vs 23.5%, $p=0.002$). These findings suggest that Helios-expressing Foxp3$^+$ T cells in SLE, in contrast to Foxp3$^+$ Helios$^-$ T cells, were not enriched for effector T cells.

In-vitro suppression assays of Helios-expressing Treg are not feasible as their staining requires cell fixation/permeablisation and no surrogate markers are available in humans allowing for cell sorting. We therefore used an alternative approach by investigating the methylation status of the TSDR of the Foxp3 gene.[30] Demethylation of the TSDR correlates with stable Foxp3 expression and defines a permanent suppressor cell lineage. We isolated three different CD4 subsets (Foxp3$^-$ Helios$^-$, Foxp3$^+$ Helios$^-$ and Foxp3$^+$ Helios$^+$) by FACS sorting from five HC and five active SLE patients, and analysed the methylation status of the TSDR in each subpopulation (figure 3C). In HC, Foxp3$^-$ T cells expressed a fully methylated TSDR, whereas Foxp3$^+$ Helios$^+$ Treg were fully demethylated and Foxp3$^+$ Helios$^-$ T cells were approximately 30% demethylated, which is in line with previous findings.[21] When CD4 T-cell subsets from SLE patients were analysed, an identical TSDR

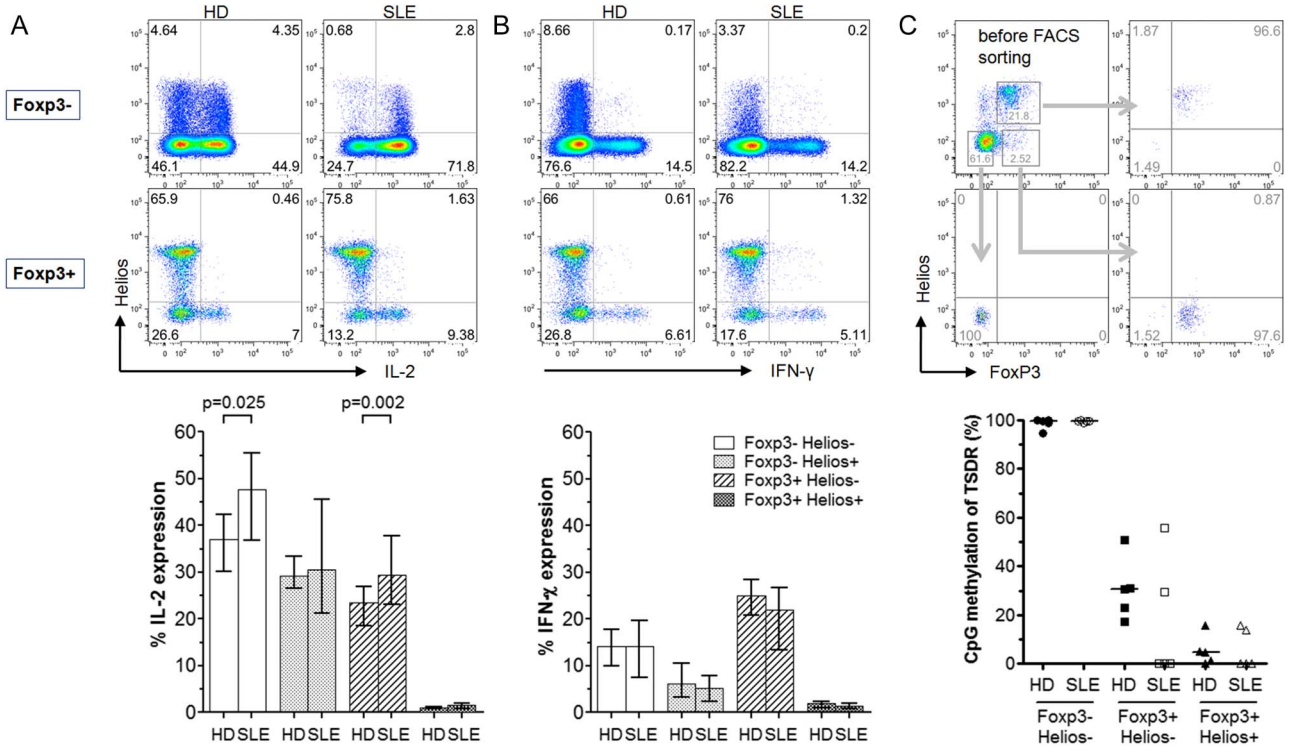

Figure 3 Foxp3$^+$ Helios$^+$ T regulatory cells (Treg) do not secrete effector cytokines and possess a demethylated Treg-specific demethylated region (TSDR). Purified CD4 T cells from healthy donors (HD) and systemic lupus erythematosus (SLE) patients were intracellularly analysed for cytokine production for IL-2 (A) and IFN-γ (B) after stimulation with phorbol myristate acetate/ionomycin for 5 h in the presence of brefeldin A for 4 h. The upper row shows representative samples from a HD and a SLE patient. The lower panel shows their respective expression levels among Foxp3/Helios-expressing CD4 T cell subsets (median/interquartile range values). (C) Freshly isolated peripheral blood CD4 lymphocytes were stained for intracellular expression of Foxp3 and Helios and fluorescence-activated cell sorter (FACS) sorted with purity greater than 94% into three different fractions: Foxp3$^-$ Helios$^-$, Foxp3$^+$ Helios$^-$ and Foxp3$^+$ Helios$^+$. Upper plots show the staining before and after sorting in one representative SLE sample. TSDR methylation was performed in each fraction from five HD and five active SLE patients. Median values are shown.

methylation pattern was observed with a highly demethylated TSDR in Helios-expressing Foxp3$^+$ Treg (figure 3C), suggesting that such Treg possess functional suppressive properties.

Helios expression in Foxp3$^+$ T cells is not related to thymic output conditions

To evaluate whether Helios expression correlates with thymic activity as suggested earlier,[15] we investigated its expression in Foxp3$^+$ CD4 T cells under different thymic output conditions in correlation to surface expression of CD31, a marker of recently emigrated, thymic-derived Treg.[31 32] First, Helios expression was analysed in Foxp3$^+$ T cells from cord blood samples and a cohort of healthy individuals over the age range from 18 to 96 years. We found that Helios was stably expressed at an average of approximately 70% during ageing (R^2=0.307), while co-expression levels of CD45RA and CD31 within this subset significantly declined from a median of 66.4% in cord blood to 3.8% in a 96-year-old individual (R^2=0.865, figure 4A).

Next, Helios expression was investigated under conditions with impaired thymic output in samples from thymectomised patients, and under conditions with increased thymic output from autoimmune patients after receiving ASCT.[22 23] Surprisingly, despite their differences in thymic activity, Helios expression in Foxp3$^+$ T cells was similar in both groups, with comparable levels to age-matched HC (figure 4B). However, Helios-expressing Treg differed in their co-expression of CD45RA and CD31, with significantly lower levels observed in patients after thymectomy (median values 7.1% vs 20.7%, p=0.009) and higher levels in patients after ASCT (median values 26.4% vs 20.7%, p=0.016) compared to age-matched HC, suggesting that Helios expression in Foxp3$^+$ Treg is not related to thymic output conditions.

Foxp3$^+$ Helios$^+$ Treg in active SLE co-express Ki-67 and display increased basal pSTAT5a levels

Based on previous observations that Helios expression in CD4 T cells was associated with cellular activation and proliferation,[17] we tested samples from SLE patients and healthy individuals for co-expression of Helios and a marker for cellular proliferation, Ki-67. In HC, the fraction of proliferating cells was higher in Helios-expressing T cells compared to Helios$^-$ T cells in both Foxp3$^-$ and Foxp3$^+$ T cells (figure 5A). As suggested earlier,[27] T cellular proliferation was significantly higher in SLE patients compared to HC and this was evident throughout each subset of Foxp3$^-$ and Foxp3$^+$ T cells, yet with the highest proliferation rates observed within the Foxp3$^+$ Helios$^+$ Treg subset. Of note, cellular proliferation was clearly linked to SLE disease activity, which may account for the expansion of Helios-expressing Treg in these patients.

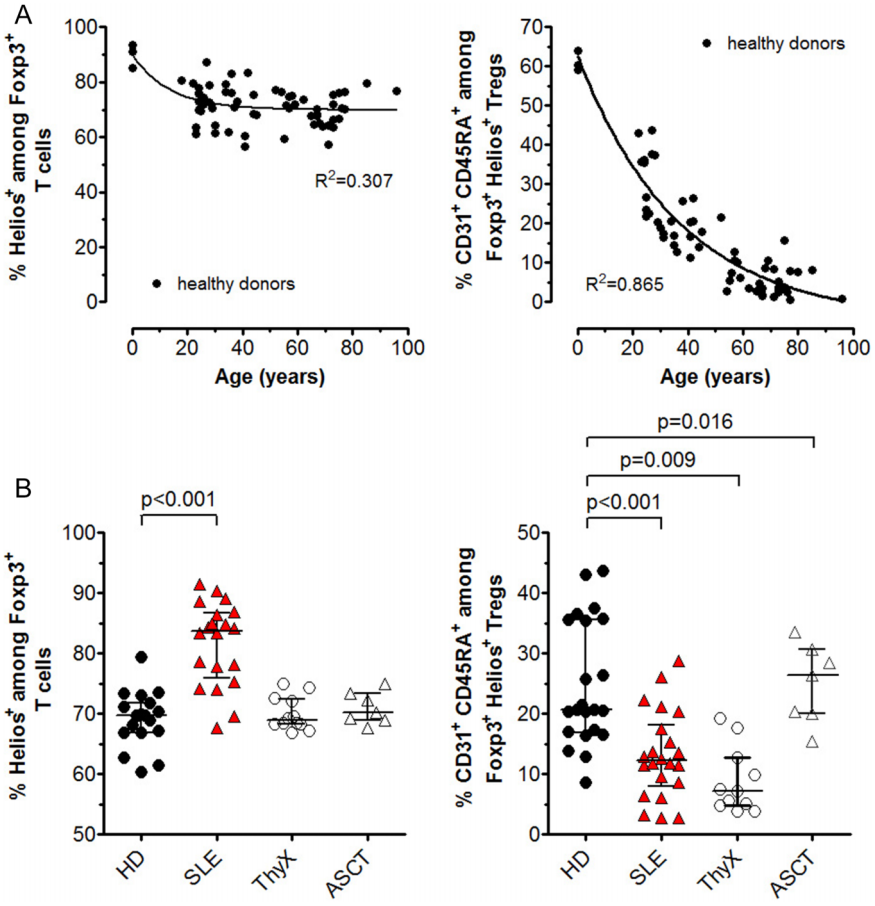

Figure 4 Helios expression in Foxp3$^+$ T regulatory cells (Treg) is unrelated to thymic activity. (A) Helios expression levels among Foxp3$^+$ Treg (left graph) and co-expression levels for CD45RA and CD31 on Foxp3$^+$ Helios$^+$ Treg (right graph) were analysed in peripheral blood samples from cord blood and healthy individuals over age. (B) Helios expression levels among Foxp3$^+$ T cells (left graph, median/IQR values) and co-expression levels for CD45RA and CD31 on Foxp3$^+$ Helios$^+$ Treg (right graph, median/IQR values) from healthy donors (n=20) compared to those from age-matched patients with systemic lupus erythematosus (SLE) (n=20), and patients after undergoing thymectomy (ThyX) (n=10) or autologous haematopoietic stem cell transplantation (ASCT) for autoimmune disease (n=7).

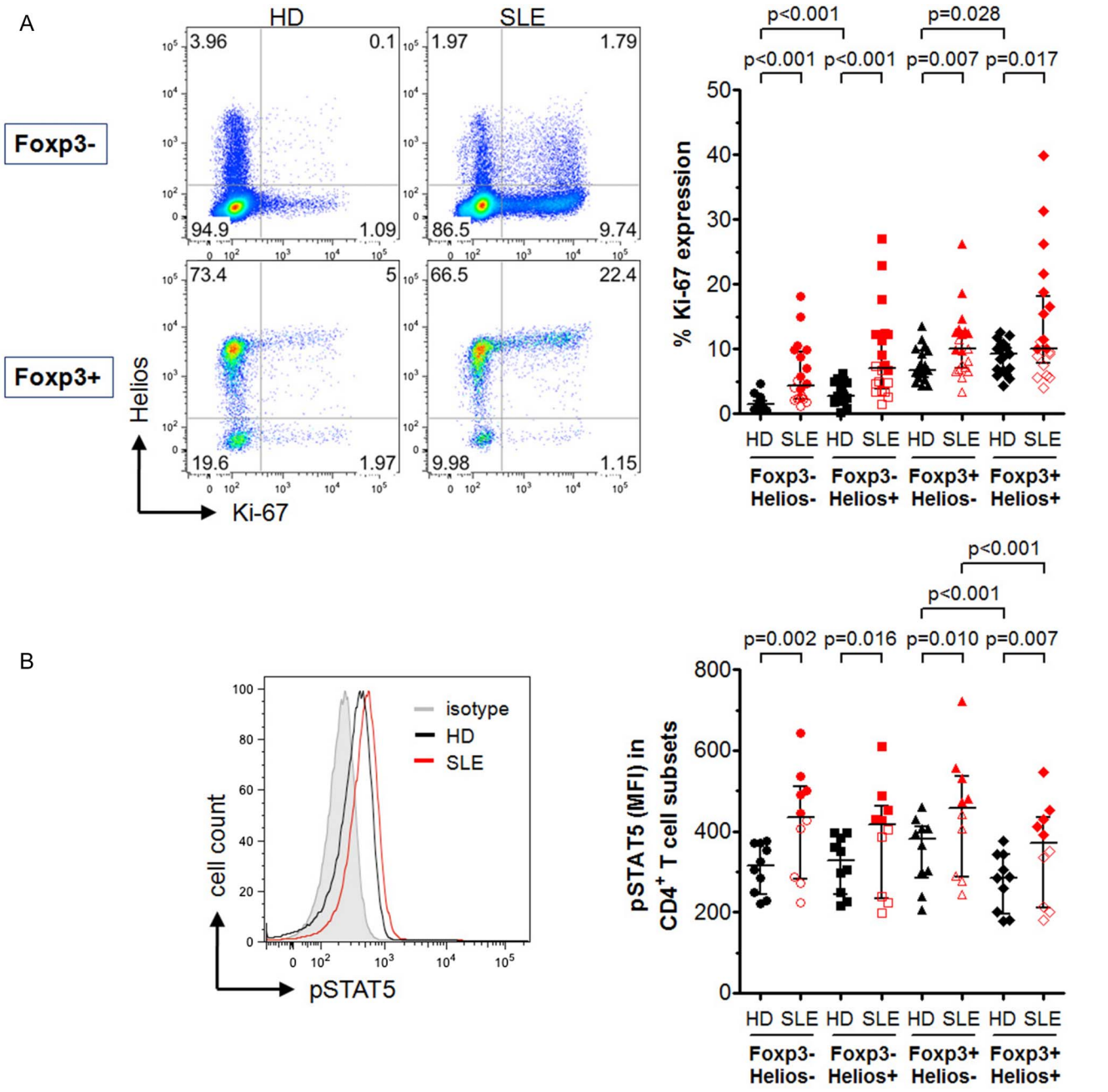

Figure 5 Compared to healthy controls, Helios-expressing Foxp3$^+$ T regulatory cells (Treg) from active systemic lupus erythematosus (SLE) patients display higher basal expression levels for Ki-67 and pSTAT5a. (A) Co-expression analysis for Helios and Ki-67 in Foxp3$^-$ and Foxp3$^+$ CD4 T cells subsets (median/IQR values) in peripheral blood samples from healthy donors (HD) (n=20) and SLE patients (n=20). SLE patients with inactive disease (SLE disease activity index; SLEDAI < 6) are depicted with open symbols, and those with active disease (SLEDAI ≥ 6) with closed symbols. (B) CD4 T cells were analysed for the co-expression of Helios, Foxp3 and pSTAT5a in peripheral blood samples from healthy individuals (n=10) and SLE patients (n=10). SLE patients with inactive disease (SLEDAI < 6) are depicted with open symbols, and those with active disease (SLEDAI ≥ 6) with closed symbols, median/interquartile range values are shown. The dot plot shows representative samples of pSTAT5 expression in CD4 T cells from a HD and a patient with active SLE compared to isotype control.

Common cytokine receptor γ-chain family cytokines play crucial roles in the development, proliferation, survival and differentiation of multiple cell lineages including both conventional and regulatory T cells.[33] To investigate their role in promoting the enhanced T cellular proliferation in SLE, we analysed basal phosphorylation levels of signal transducer and activator of transcription 5 (STAT5)a, which acts downstream of the IL-2R common γ-chain, in CD4 T-cell subsets from healthy individuals and SLE patients using phospho-specific antibodies and flow cytometry. Indeed, compared to HC, pSTAT5a expression levels in SLE patients were not only significantly increased in conventional CD4 T cells as indicated earlier,[34] but also in both Helios$^+$ and Helios$^-$ Foxp3$^+$ T-cell subsets, and this was clearly related to their disease activity (figure 5). These data suggest that T-cell proliferation in active SLE is at least partly driven by common γ-chain cytokines.

Expanded Foxp3$^+$ Helios$^+$ Treg in active SLE have a skewed TCR repertoire

Naturally occurring Treg exhibit a broad TCR repertoire recognising various self and non-self antigens.[35 36] Such antigens not only stimulate self-reactive conventional T cells but may also activate natural Treg, thereby maintaining dominant self-tolerance.[37] Under autoimmune conditions with an accumulation of self-antigens, such as active SLE, one should expect an activation and clonal expansion of naturally occurring Treg. To investigate this issue, we analysed the TCR repertoire of Foxp3$^+$ Helios$^+$ Treg by using a panel of TCR Vβ family-specific antibodies and flow cytometry in three active SLE patients with increased proportions of Helios-expressing Treg and three SLE patients in remission compared to 10 healthy individuals. Indeed, based on normal ranges established for each TCR Vβ member in 10 HC, Foxp3$^+$ Helios$^+$ Treg from active SLE patients showed a highly skewed TCR repertoire, whereas SLE patients in remission exhibited a completely normal TCR repertoire (figure 6).

DISCUSSION

While the suitability of the Ikaros transcription family member Helios as a marker for naturally occurring Treg is currently debated, it has become evident that Foxp3$^+$ Helios$^+$ Treg, in contrast to Foxp3$^+$ Helios$^-$ Treg, have enhanced suppressive potential,[20] and differ in terms of epigenetic changes at the Foxp3 locus and their capacity to produce effector cytokines.[21] Helios expression may therefore define a subset of regulatory T cells with a putative role in mediating self-tolerance and could thus be of particular relevance in autoimmunity. The present study is the first to investigate Helios expression in Treg from patients with chronic autoimmunity such as SLE, SSc and RA. We found that frequencies of Foxp3$^+$ Helios$^+$ Treg, unlike their Foxp3$^+$ Helios$^-$ or Foxp3$^-$ Helios$^+$ counterparts, were significantly increased in SLE and positively correlated with disease activity, whereas they were unaltered in SSc and RA patients when compared to age and sex-matched HC. These observations raise the question about the origin and functionality of Helios-expressing Treg in SLE as well as the factors that mediate such Treg expansion. Our data indicate that Foxp3$^+$ Helios$^+$ Treg in SLE are peripherally expanded, exhibit increased basal pSTAT5a levels and a restricted TCR repertoire but lack, in contrast to Foxp3$^+$ Helios$^-$ T cells, effector cytokine production and possess a highly demethylated TSDR of the Foxp3 gene, suggesting a proliferation of Treg with suppressive potential in active disease to compensate for autoimmune responses.

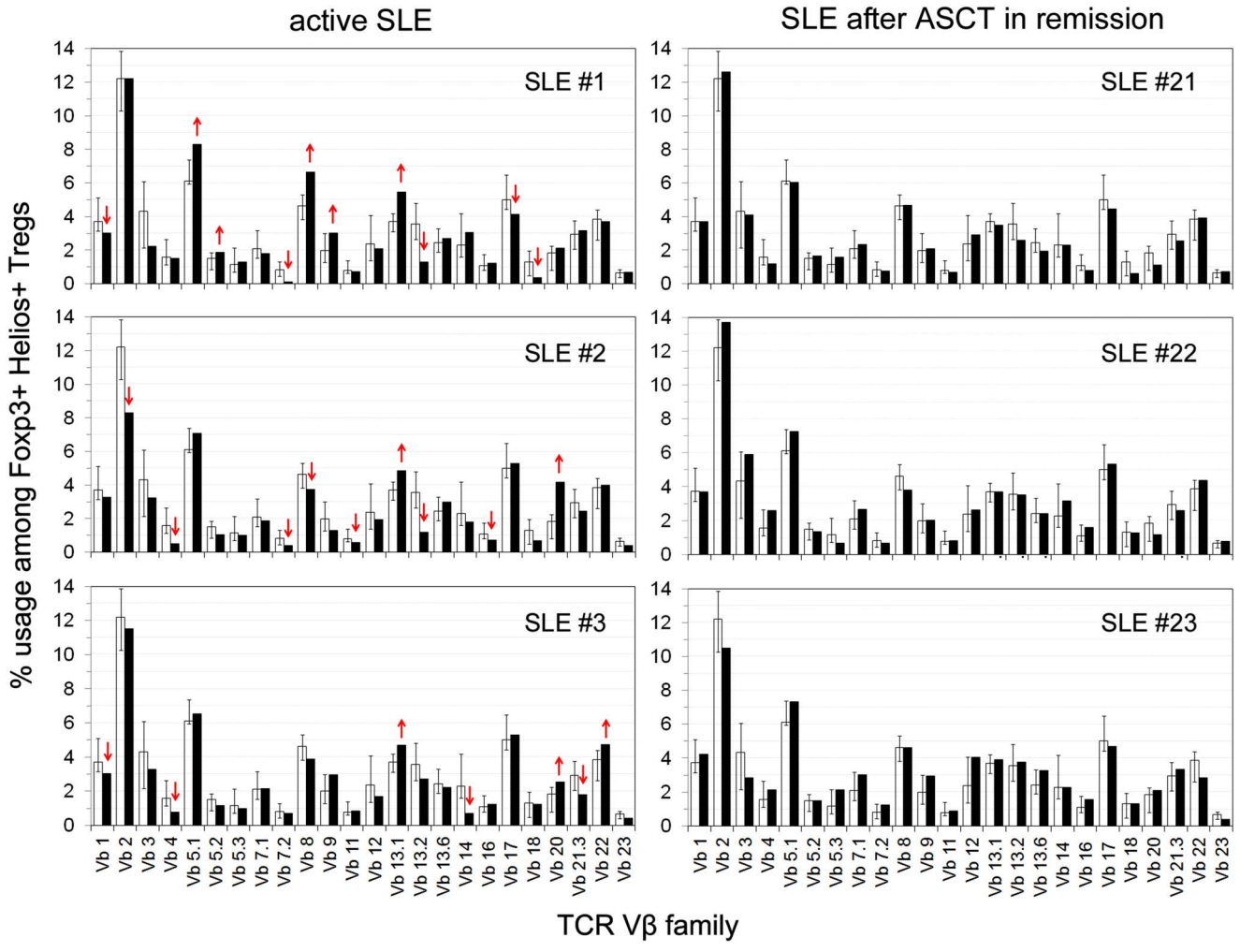

Figure 6 Helios-expressing T regulatory cells (Treg) from systemic lupus erythematosus (SLE) patients show a restricted T-cell receptor (TCR) repertoire. The TCR repertoire of Foxp3$^+$ Helios$^+$ CD4 Treg was analysed by using a panel of TCR Vβ family-specific antibodies and flow cytometry in three patients with active SLE (left panel) and three SLE patients in remission after autologous haematopoietic stem cell transplantation (ASCT) (right panel). Normal ranges were established for each TCR Vβ member based on CI of 97.5% determined in 10 healthy individuals (open bars); perturbations of TCR Vβ families were considered to be significant in patients when they were outside of these normal intervals (depicted with red arrows).

Thornton *et al*[15] suggested that Helios expression is related to thymic Treg development. However, we found no relationship between thymic activity and Helios expression in Treg when samples were analysed from cord blood, elderly persons and patients after undergoing ASCT or thymectomy, suggesting that Helios expression is either maintained during Treg homeostasis or peripherally re-induced on T-cell activation. When surface expression of CD45RA and a marker of recently emigrated, thymic-derived Treg, CD31,[31 32] was analysed on Foxp3$^+$ Helios$^+$ Treg, we found significantly lower co-expression levels for both markers in SLE samples compared to HC, suggesting that such Treg were not recently generated in the thymus but rather peripherally expanded. Indeed, as indicated by Ki-67 expression analysis, Foxp3$^+$ T cells from SLE patients were highly proliferative compared to those from HC, and consistent with previous findings,[17] most dividing Ki-67$^+$ T cells were Helios$^+$ in both Foxp3$^-$ and Foxp3$^+$ T cell subsets.

It was recently reported that Helios expression is not associated with Treg lineage commitment but is simply linked to CD4 T-cell activation and proliferation.[17] In contrast, our data on their phenotypic, functional and epigenetic properties provide support for the notion that Foxp3$^+$ Helios$^+$ T cells in SLE, although peripherally expanded, resemble a Treg subset with functional, suppressive capacity. Consistent with findings in healthy individuals, Helios$^+$ Treg from SLE samples displayed higher expression levels for CD25 and lower levels for CD127 as compared to Foxp3$^+$ Helios$^-$ T cells.[17 20] We also noticed that, although primarily confined to the CD45RA$^-$ Foxp3^{low} memory Treg subset, a phenotype that is reportedly enriched for cytokine-secreting T cells,[27] Helios-expressing Treg in SLE lacked effector cytokine secretion for IL-2 and IFN-γ. Finally, we observed that Foxp3$^+$ Helios$^+$ Treg in SLE, similar to HC,[21] have completely demethylated TSDR regions of the Foxp3 locus, an epigenetic imprinting that is critical for stable Foxp3 expression and a permanent suppressor cell lineage.[30] Collectively, these data indicate that Foxp3$^+$ Helios$^+$ Treg, whether naturally occurring or peripherally induced, display distinct phenotypic and functional Treg properties,[20 21] and such properties seem to be preserved even under autoimmune conditions with chronic T-cell activation such as SLE.

To evaluate whether Foxp3$^+$ Helios$^+$ Treg in SLE have the capacity to exert their suppressive function at the site of inflammation, we investigated their migratory potential by analysing the chemokine receptor expression for CXCR3 and CCR4 that are critical for T-cell recruitment into inflamed tissue and the skin, respectively.[28 29] Here, we could demonstrate that the expression levels for both chemokine receptors was similar in Foxp3$^+$ Helios$^+$ Treg from SLE samples when compared to HC with approximately 40% CXCR3 and approximately 50% CCR4 expression. Although only the identification in tissue samples would indicate their role in controlling autoimmune responses in end organs, such as lupus nephritis[38] or the skin, our data at least suggest that Foxp3$^+$ Helios$^+$ Treg possess migratory capacity for trafficking into inflamed tissues. This may be of particular relevance as CXCR3$^+$ Treg have, for example, recently been shown to be involved in mediating immune tolerance in renal allotransplant recipients.[39]

Based on the assumption that the enhanced T cellular proliferation in SLE was driven by common γ-chain family cytokines, we sought to investigate basal phosphorylation levels of STAT5 by flow cytometry. Indeed, compared to HC, pSTAT5a expression levels in SLE patients were not only significantly increased in conventional CD4 T cells as indicated earlier,[34] but also in Foxp3$^+$ Treg subsets, suggesting recent in-vivo Treg stimulation with common γ-chain cytokines such as IL-2, IL-7, IL-15 and IL-21. Among these common γ-chain cytokines, IL-2 may be the most obvious candidate for driving Treg proliferation in SLE as its administration either in vitro[17] or in vivo as a treatment option for renal cell carcinoma[40] has been reported to enhance Helios expression in Treg. Nonetheless, SLE is rather regarded as a disease with IL-2 deprivation, leading to a homeostatic imbalance of regulatory and conventional effector T cells.[41] As Foxp3$^+$ Helios$^+$ Treg showed the highest proliferation rates among CD4 T cells in SLE, we expected the highest pSTAT5a expression levels within this subset. However, we were surprised to find that Foxp3$^+$ T cells with the highest basal pSTAT5a expression levels were Helios$^-$. This may reflect differences in tissue reallocation, expression levels of the respective cytokine receptor or kinetics of γ-chain cytokine signalling in the aforementioned T-cell subsets. It has recently been reported that oligodeoxynucleotides stabilise Helios-expressing Foxp3$^+$ human Treg cells during in-vitro expansion.[21] As vast amounts of self-antigens, including DNA nucleotides, are accumulating in active SLE,[42] it is tempting to speculate that such oligodeoxynucleotides are involved in mediating the expansion of Helios-expressing Treg in SLE. This may also explain the fact that Helios$^+$ Treg are expanded in SLE but not in other chronic autoimmune diseases with increased serum levels of pro-inflammatory cytokines such as SSc or RA.

TCR specificity is thought to play a crucial role in Treg development and function.[35 36] We were recently able to demonstrate that the TCR repertoire of CD4 T cells in SLE is highly restricted,[22] but the TCR diversity of naturally occurring Treg in SLE is largely unknown. As self-antigens that are accumulating in active SLE[42] not only stimulate self-reactive conventional T cells but may also activate naturally occurring Treg cells, we wondered whether the TCR repertoire of SLE Treg is retracted in a similar way. Indeed, flow cytometric analysis of the TCR Vβ family usage revealed a highly skewed TCR repertoire in Foxp3$^+$ Helios$^+$ Treg from active SLE patients when compared with age-matched healthy individuals. In contrast, SLE patients in remission with normal frequencies of Helios-expressing Foxp3$^+$ Treg displayed a completely normal TCR Vβ repertoire usage of such Treg. These findings indicate that Helios-expressing Treg in active SLE are clonally expanded, suggesting a role for (auto) antigens in driving Treg activation and proliferation.

In contrast to our findings, some groups reported similar or decreased levels of circulating Treg in SLE when compared to HC.[5 8 10] This discrepancy may be related to the use of CD25 for Treg analysis in those studies, a marker that is decreased in Foxp3$^+$ T cells in SLE,[6 43] resulting in an underestimation of Treg frequencies. In addition, SLE Treg predominantly display a Foxp3^{low} memory phenotype,[27] which may lead to difficulties in their cytometric analysis and that provides a rationale for a combination staining of Foxp3, for example, with Helios, as performed in our study.

In conclusion, our data show that Helios-expressing Foxp3$^+$ Treg are peripherally expanded in active SLE and these Treg, whether naturally occurring or peripherally induced, seem to possess functional suppressive capacity and migratory potential into inflamed tissue. Our data also indicate that Foxp3$^+$ Helios$^+$ Treg in SLE are not enriched for effector T cells but rather are actively involved in controlling chronic autoimmune responses. Nevertheless, although expanded in vivo, lupus Treg may not fully compensate for the ensuing autoreactive effector responses. Based on these findings, Foxp3$^+$ Helios$^+$ Treg may serve as a source for Treg-based interventions in future therapeutic approaches, and may be utilisable as a biomarker for disease activity in SLE.

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Foxp3$^+$ Helios$^+$ regulatory T cells are expanded in active systemic lupus erythematosus

Tobias Alexander, Arne Sattler, Lars Templin, Siegfried Kohler, Christian Groß, Andreas Meisel, Birgit Sawitzki, Gerd-Rüdiger Burmester, Renate Arnold, Andreas Radbruch, Andreas Thiel and Falk Hiepe

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