Two separate effects contribute to regulatory T cell defect in systemic lupus erythematosus patients and their unaffected relatives

N. Costa,* O. Marques,†
S. I. Godinho,* C. Carvalho,†
B. Leal,† A. M. Figueiredo,†
C. Vasconcelos,‡ A. Marinho,‡
M. F. Moraes-Fontes,*§
A. Gomes da Costa,¶ C. Ponte,¶
R. Campanilho-Marques,¶**
T. Cóias,¶ A. R. Martins,††
J. F. Viana,†† M. Lima,‡ B. Martins†
and C. Fesel [ID]*

*Instituto Gulbenkian de Ciência, Oeiras, Portugal, †UMIB, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Portugal, ‡Hospital de Santo António, Centro Hospitalar do Porto, Unidade Imunologia Clínica, Porto, Portugal, §Hospital de Curry Cabral, Centro Hospitalar de Lisboa Central, Unidade de Doenças Auto-imunes, Lisbon, Portugal, ¶Hospital de Santa Maria, Lisbon, Portugal, **Instituto Português de Reumatologia, Lisbon, Portugal, and ††Centro Hospitalar de Lisboa Ocidental, Lisbon, Portugal

Summary

Forkhead box P3 (FoxP3)$^+$ regulatory T cells (T$_{regs}$) are functionally deficient in systemic lupus erythematosus (SLE), characterized by reduced surface CD25 [the interleukin (IL)-2 receptor alpha chain]. Low-dose IL-2 therapy is a promising current approach to correct this defect. To elucidate the origins of the SLE T$_{reg}$ phenotype, we studied its role through developmentally defined regulatory T cell (T$_{reg}$) subsets in 45 SLE patients, 103 SLE-unaffected first-degree relatives and 61 unrelated healthy control subjects, and genetic association with the CD25-encoding *IL2RA* locus. We identified two separate, uncorrelated effects contributing to T$_{reg}$ CD25. (1) SLE patients and unaffected relatives remarkably shared CD25 reduction *versus* controls, particularly in the developmentally earliest CD4$^+$ FoxP3$^+$ CD45RO$^-$CD31$^+$ recent thymic emigrant T$_{regs}$. This first component effect influenced the proportions of circulating CD4$^+$FoxP3^{high} CD45RO$^+$ activated T$_{regs}$. (2) In contrast, patients and unaffected relatives differed sharply in their activated T$_{reg}$ CD25 state: while relatives as control subjects up-regulated CD25 strongly in these cells during differentiation from naive T$_{regs}$, SLE patients specifically failed to do so. This CD25 up-regulation depended upon *IL2RA* genetic variation and was related functionally to the proliferation of activated T$_{regs}$, but not to their circulating numbers. Both effects were found related to T cell IL-2 production. Our results point to (1) a heritable, intrathymic mechanism responsible for reduced CD25 on early T$_{regs}$ and decreased activation capacity in an extended risk population, which can be compensated by (2) functionally independent CD25 up-regulation upon peripheral T$_{reg}$ activation that is selectively deficient in patients. We expect that T$_{reg}$-directed therapies can be monitored more effectively when taking this distinction into account.

Keywords: cytokines, regulatory T cells, systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by combined adaptive and innate-immune aberrations that can affect diverse organs particularly through immune complex-mediated tissue damage. However, the aetiology and pathogenic processes of SLE are not understood fully. Both genetic and environmental factors contribute to immune dysfunction [1]. In the associated breakdown of tolerance, T cells play a key role by amplifying autoimmune responses [2]. Many abnormalities of T lymphocytes have been described in SLE patients, including dysregulated intracellular signal transduction and imbalanced cytokine production [3], which influence B cell function and autoantibody production. While SLE T cells help B cells to generate the pathogenic autoantibodies, they also fail characteristically to produce sufficient amounts of interleukin (IL)-2 [4].

Limited amounts of IL-2 affect primarily forkhead box P3 (FoxP3)$^+$ regulatory T cells (T$_{regs}$), which depend greatly upon this cytokine for their development, survival and function [5]. Recently, the importance of deficient IL-2

production and its effects mediated by T$_{regs}$ was supported strongly by the clinical efficacy of low-dose IL-2 therapy in SLE, shown to boost T$_{reg}$ activity and to re-establish tolerance mechanisms that can counter autoimmunity and improve patients' clinical outcome [6,7]. This is in line with reported T$_{reg}$ aberrations in SLE [8,9], despite some discrepancies in previous studies: while some authors had reported decreased circulating T$_{reg}$ proportions in active disease [10–15], others had found them normal [16–19] or even increased [20–24]. These divergences, however, can be explained largely by a population of aberrant CD4$^+$ T cells expressing FoxP3 but low or no CD25, which is almost non-existent in healthy individuals but present in significant numbers in SLE [19,25–27] where it 'dissociates' FoxP3 from CD25-based T$_{reg}$ quantitation. These CD25-deficient cells have a natural T$_{reg}$ phenotype of deficient functionality [25], and their frequencies correlate with disease activity and (inversely) with T$_{reg}$ suppressive capacity [28]. The hypothesis that they indicate an SLE-specific T$_{reg}$ dysfunction is consistent with earlier reports of functional T$_{reg}$ defects [15,16,29].

Human FoxP3$^+$ cells have been subdivided into three functionally and phenotypically distinct subsets [30]: naive T$_{regs}$ (FoxP3$^+$CD45RA$^+$), short-lived and highly suppressive activated T$_{regs}$ (FoxP3^{high}CD45RA$^-$) and another CD45RA$^-$ population with low FoxP3 expression and deficient or absent functionality (FoxP3^{low}). Activated T$_{regs}$ are derived generally from naive T$_{regs}$ [31], while the FoxP3^{low} subset can, in part, represent transient FoxP3 expression by non-regulatory T helper (Th) cells [30]. While it was speculated that this could also apply to atypical CD25-deficient T$_{regs}$ in SLE [27], it is now clear that it is not the case. In fact, no evidence was found for a role of transient FoxP3 expression in SLE, as not only FoxP3^{high}CD25^{high} but also CD25$^{low/negative}$ activated T$_{regs}$ bear strong markers of bona-fide, probably thymus-derived T$_{regs}$ [7,32], i.e. high levels of Helios expression [33,34], and fully demethylated FoxP3–TSDR [35]. Therefore, T$_{regs}$ in SLE apart from FoxP3^{low} cells can basically be seen as belonging to a lineage derived from naive T$_{regs}$.

In complex diseases, the study of unaffected relatives can reveal shared heritable factors that probably contribute to pathogenesis, and separate them from divergent phenotypes which are secondary to disease manifestation. Unaffected relatives of SLE patients frequently present IgG autoantibodies of SLE-associated specificity [36], although they rarely develop the disease. We have reported previously that these relatives, in contrast to unrelated healthy controls or the patients themselves, showed consistent positive correlations of SLE-associated specific IgG with CD25$^+$ T$_{regs}$ [37]. This suggested a compensatory role of T$_{regs}$ with the capacity to control autoantibody-related pathogenic effects, eventually allowing the unaffected relatives to avoid manifest disease.

In order to elucidate more clearly the SLE T$_{reg}$ phenotype, which also plays a role in intravenous immunoglobulin (i.v.Ig) therapy [38], and the T$_{reg}$-mediated compensation in unaffected relatives, we have now studied the reduced surface CD25 quantitatively in the three classic T$_{reg}$ subsets [30] and tracked it through developmental stages making use of their lineage property. Our results point to two separate contributing effects: (a) a shared, probably intrathymic CD25 reduction that leads to a decreased activation capacity in developmentally early T$_{regs}$, compensated in unaffected relatives but not patients by (b) a functionally independent CD25 up-regulation that occurs upon peripheral T$_{reg}$ activation.

Materials and methods

Patients and sampling

Peripheral blood samples were collected from 102 SLE patients who fulfilled current American College of Rheumatology (ACR) criteria for SLE, 197 first-degree relatives of the patients from a total of 94 families and 141 unrelated healthy controls upon signed informed consent. Relatives were subjected to a survey questionnaire to rule out clinical SLE, and three who were, in fact, diagnosed with SLE either before or subsequent to our collection were excluded from the study that therefore included 194 unaffected relatives at the end. SLE patients underwent a detailed clinical characterization, the results of which are summarized in Table 1. Approval was obtained from the Ethics Committees of Hospital Geral de Santo Antonio, Centro Hospitalar do Porto (Porto), Centro Hospitalar Lisboa Norte/Santa Maria, Centro Hospitalar Lisboa Ocidental and Hospital de Curry Cabral, Centro Hospitalar de Lisboa Central (Lisbon).

Peripheral blood samples (30 ml per individual) were collected into Vacutainer cell preparation tubes with sodium citrate [Becton-Dickinson (BD), Franklin Lakes, NJ, USA], and plasma and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation, according to the manufacturer's protocol.

Flow cytometry

For flow cytometric analysis, PBMC were washed twice in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) prior to incubation with antibodies and 10^6 cells were incubated for 30 min on ice with anti-CD4-Pacific Orange (Invitrogen, Carlsbad, CA, USA; clone S3.5), anti-CD31-Pacific Blue (exBio, Vestev, Czech Republic; clone MEM-05) anti-CD127-allophycocyanin (APC)/eFluor780 (eBioscience, San Jose, CA, USA; clone eBioRDR5), anti-CD25-phycoerythrin (PE) (BD; clone 2A3), anti-CD39-PE/cyanin 7 (Cy7) (eBioscience; clone A1) and anti-CD45RO-peridinin chlorophyll (PerCP)

Table 1. General and clinical characteristics

1. SLE patients	Total: 102
Gender	Male: 8%; female: 92%
Age	Median: 40·5 years; range 21–73 years
Time of SLE affection	Median: 10 years; range: 0–46 years
Actual disease activity (SLEDAI-2k)	Median: 2; range: 0–30
Clinical affections	
Malar rashes	59%
Discoid rashes	21%
Photosensitivity	72%
Ulcers	41%
Arthritis	63%
Renal involvement	47%
Lung involvement	18%
Cardiac involvement	16%
Neurological alterations	28%
Haematological alterations	54%
Immunological alterations	86%
Anti-phospholipid syndrome	15%
Secondary Sjögren's syndrome	12%
Therapy	
Glucocorticoids	69%
Glucocorticoid dosages (mg prednisone eq.)	Median: 5·5; range: 1·25–50
Antimalarials	68%
Azathioprine	21%
Methotrexate	3%
Mycophenolate mofetil	13%
Cyclosporin A	1%
Oral steroids	21%
2. Unaffected 1st-degree relatives	Total: 194
Gender	Male: 39%; female: 61%
Age	Median: 48·5 years; range: 18–86 years
3. Unrelated healthy controls	Total: 141
Gender	Male: 48%; female: 52%
Age	Median: 44 years; range: 20–68 years

SLE = systemic lupus erythematosus; SLEDAI = SLE Disease Activity Index.

(Invitrogen; clone UCHL1). For subsequent intracellular staining, cells were washed again, fixed and permeabilized using a fix/perm kit (eBioscience) and stained for intracellular FoxP3 and Ki67 with anti-FoxP3-APC (eBioscience; clone PCH101) and anti-Ki67-fluorescein isothiocyanate (FITC) (BD; clone 35/Ki-67). Fluorescence was assessed using a Cyan ADP flow cytometer and analysed using FlowJo software (TreeStar Inc., Ashland, OR, USA. Adapting the scheme of Miyara *et al.* [30], we distinguished three FoxP3$^+$ subsets: CD45RO$^-$ FoxP3$^+$ naive T$_{regs}$, CD45RO$^+$ FoxP3^{high} activated T$_{regs}$ and CD45RO$^+$ FoxP3^{low} cells (depicted in Fig. 1). To keep subset definitions objective

and unbiased, we used identical absolute distances on the log-fluorescence scale between the lower boundaries of FoxP3^{low} and FoxP3^{high} subset gates in all samples. To measure surface CD25, the PE median fluorescence intensity (MFI) was quantified. Analysis of this property was restricted to samples collected within a defined time-period where parallel assessment of PE-coupled cytometric beads validated the longitudinal commensurateness of MFI measures (45 SLE patients, 103 unaffected relatives and 61 controls). Also, CD127 staining was used principally for quality control (not gating; however, in 10 samples with visible separate CD127^{high} FoxP3$^+$ populations, these were gated out). T$_{reg}$ CD127 MFIs were always below 80% (median = 44%) of the MFIs measured in CD4$^+$ FoxP3$^-$ conventional Th in the same sample with a single exception, where low CD127 as it characterizes T$_{regs}$ was also found on the Th cells.

Cell culture

Isolated 10^6 PBMC were stimulated with phorbol myristate acetate (PMA) (25 ng/ml) and ionomycin (1 µg/ml) for 6 h at 37°C in RPMI-1640 medium containing 10% FCS and 10 µg/ml brefeldin A. Immediately after stimulation, the cells were stained with anti-CD3-APC/Cy7 (BioLegend; clone HIT3a), anti-CD4-PerCP (BioLegend; clone RPA-T4), anti-CD8-PE/Cy7 (eBioscience; clone RPA-T8) and anti-CD45RO-Pacific Blue (BioLegend; clone UCHL1). Subsequently, cells were fixed and permeabilized using Cytofix/Cytoperm (BD), washed and stained for intracellular IL-2 in Perm buffer with anti-IL-2-APC [eBioscience; clone MQ1–17H12, in parallel with interferon (IFN)-γ and tumour necrosis factor (TNF)-α], assessed cytometrically with a fluorescence activated cell sorter (FACS)Canto II cytometer and analysed using FlowJo software (TreeStar, Inc.).

IL2RA (CD25) genotyping

Genomic DNA was extracted from peripheral blood cells by standard salting-out. Genotyping of 25 locus-spanning single nucleotide polymorphisms (SNPs) (Table 2) was performed on the Sequenom^{TM} platform, as described previously [37]. All 25 typed SNPs passed quality control with call rates above 85% and Hardy–Weinberg equilibrium ($P > 0.01$) was fulfilled within the control subjects.

Data analysis

Data were analysed with Igor Pro software (WaveMetrics, Lake Oswego, OR, USA) and a statistical package that we have developed for it (also see [36–38]). Pairwise group comparisons of continuous variables were performed by the distribution-independent Mann–Whitney test. Our statistical approach was to compare patients and unaffected relatives separately to a control group, followed by within-group analysis. We did not make all possible groupwise comparisons,
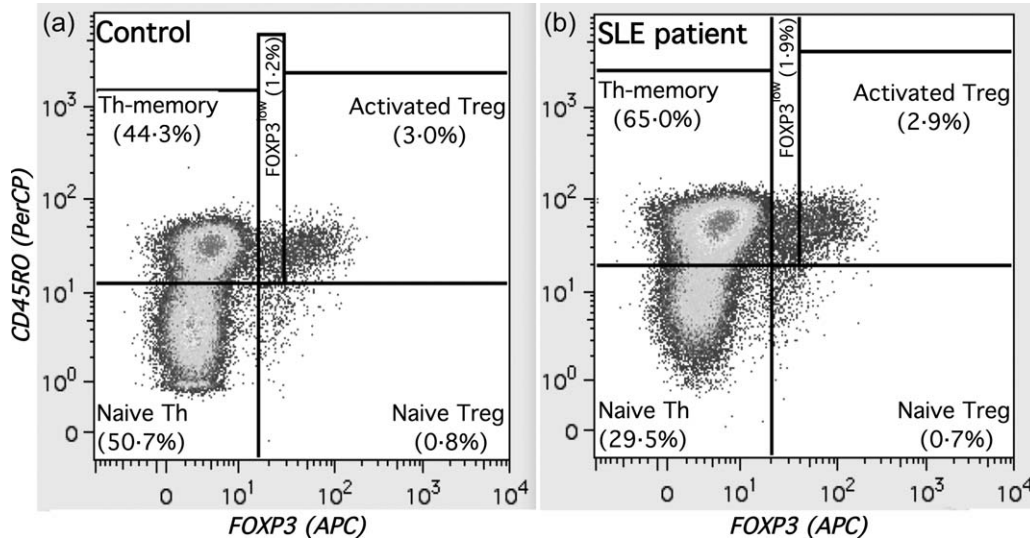
Fig. 1. Regulatory T cell (T$_{regs}$) subset gating. The figure shows our cytometric gating of CD4$^+$ T cell subsets. Apart from the conventional forkhead box P3 (FoxP3)$^-$ (naive and memory) T helper (Th), FoxP3$^+$ T$_{regs}$ were divided into three subsets using FoxP3 and CD45RO staining. Two examples are depicted with their T helper (Th) and T$_{regs}$ subset frequencies within CD4$^+$ cells: (a) a healthy control subject; (b) a systemic lupus erythematosus (SLE) patient. Irrespective of differences in absolute staining intensities, the distance between the lower boundaries of FoxP3^{low} and activated T$_{regs}$ was kept constant throughout all samples. Parallel CD127 staining (not shown) was not used for gating but for quality control.
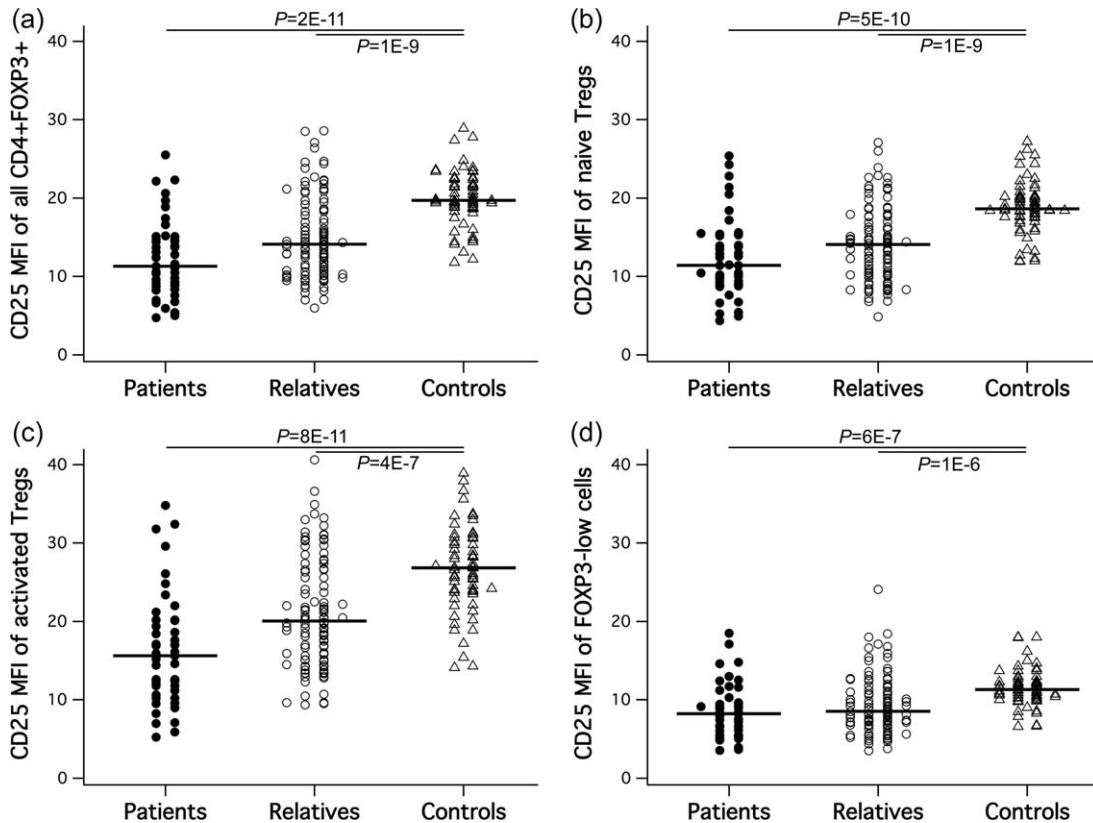

Fig. 2. Surface CD25 in T$_{reg}$ subsets. Quantitative surface CD25 distributions and group differences for (a) all CD4$^+$forkhead box P3 (FoxP3)$^+$ cells, (b) naive regulatory T cells (T$_{regs}$), (b) activated T$_{regs}$ and (d) FoxP3^{low} cells. Significant P-values are shown; n.s. = non-significant.
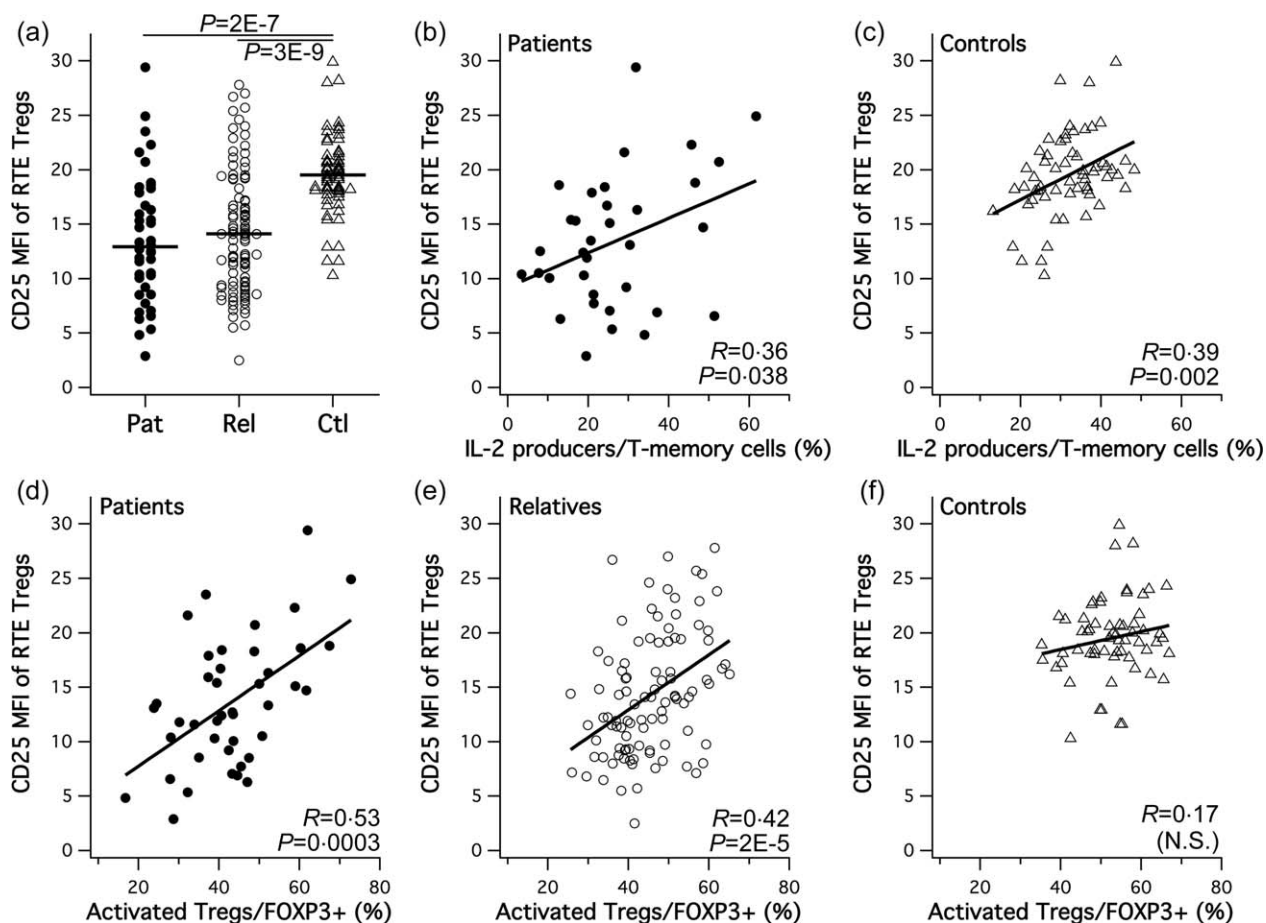
Fig. 3. Characteristics of surface CD25 levels of recent thymic emigrant (RTE) T$_{regs}$. (a) Reduction of RTE regulatory T cells (T$_{regs}$) CD25 in systemic lupus erythematosus (SLE) patients and in unaffected relatives *versus* control subjects. (b,c) Significant correlation of RTE T$_{reg}$ CD25 with proportions of interleukin (IL)-2 producers among phorbol myristate acetate (PMA)/I-stimulated memory T cells in SLE patients and controls (while insignificant in unaffected relatives, not shown). (d–f) Significant correlations of RTE T$_{reg}$ CD25 with activated T$_{reg}$ frequencies in patients and relatives, but not control subjects. Linear correlation coefficients (*R*) and significant *P*-values are shown; n.s. = non-significant.

and particularly did not directly compare patients and relatives, which were drawn from the same families and cannot be compared irrespective of their familial relations with adequate statistical power. Relations between continuous variables (including genotypes) were characterized by linear correlation coefficients (sometimes partial or semipartial) and tested by linear regression analysis. Multiple linear regression was used to consider covariates. For genetic association, univariate *P*-values were corrected for multiple comparisons (i.e. the 25 SNPs tested) with the Benjamini–Hochberg false discovery rate (FDR) method. *P*-values below 0·05 were considered statistically significant.

Results

CD25 reduction on SLE T$_{regs}$ is shared by the patients' first-degree relatives

Comparing quantitative CD25 surface staining intensities in 45 SLE patients, 103 unaffected relatives and 61

unrelated healthy controls, we found CD25 MFI impressively reduced in patients *versus* controls not only when considering all FoxP3$^+$ cells (Fig. 2a), but also within each of their three subsets (depicted in Fig. 1) defined by Miyara *et al.* [30]: naive (Fig. 2b) and activated (Fig. 2c) T$_{regs}$, as well as FoxP3^{low} cells (Fig. 2d). Patients' median CD25 was reduced 1·7-fold in total FoxP3$^+$, 1·6 and 1·7-fold in naive and activated T$_{regs}$, respectively, and 1·4-fold in FoxP3^{low} cells. This subset-overarching CD25 deficiency was shared remarkably by the unaffected first-degree relatives, where CD25 densities were also significantly lower in all CD4$^+$FoxP3$^+$ subpopulations than in controls (1·4-fold in total FoxP3$^+$ and 1·3-fold in all three subsets), while still higher than in the patients. The intermediate position of the unaffected relatives points to shared heritable factors and therefore supports T$_{reg}$ CD25 as a pathogenesis-related biomarker. Approximations of cell proportions lacking surface CD25 (although based on an arbitrary threshold definition) mirror the MFI characteristics (Supporting information, Fig. S1).
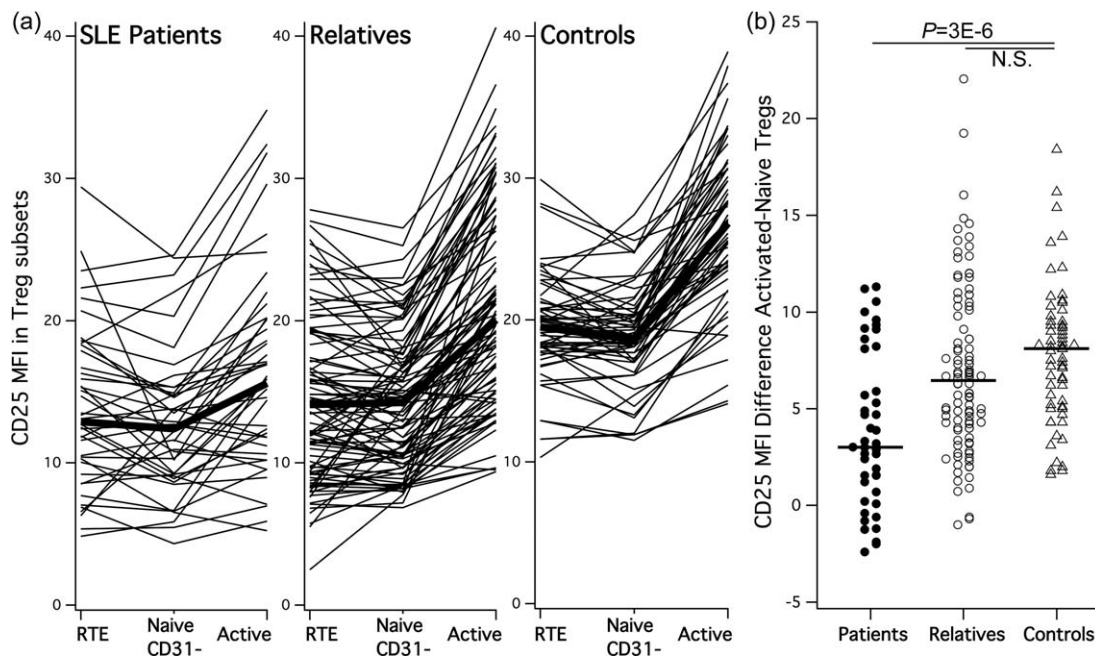
Fig. 4. CD25 up-regulation in activated regulatory T cells (T$_{regs}$) is selectively impaired in systemic lupus erythematosus (SLE) patients. (a) Individual trajectories of CD25 levels through the three T$_{reg}$ differentiation stages (thin lines) and their groupwise averages (thick lines). Reduced CD25 in naive T$_{reg}$ subsets appears 'compensated' in unaffected relatives but not in SLE patients by a subset-specific up-regulation in activated T$_{regs}$. (b) Quantified as CD25 mean fluorescence intensity (MFI) difference between naive and activated T$_{regs}$; this up-regulation was equal in relatives and controls, but significantly lower in the SLE patients. The P-value is shown; n.s. = non-significant.

Subsetwise CD25 levels were particularly interesting. We found that surface CD25 MFIs of the naive and activated T$_{reg}$ subsets were statistically independent from other measures obtained for the respective subset, i.e. CD25 was never significantly correlated with the same subset's circulating numbers, frequencies within total FoxP3$^+$ or CD4$^+$ cells, or Ki67$^+$ proliferating fractions in any of our three study groups (not shown). There were also no significant relations to patients' SLE Disease Activity Index (SLEDAI)-2k disease activity, other clinical characteristics or treatments (Table 1) or to gender or age, except for a marginal $R = 0.2/P = 0.046$ age correlation with naive T$_{reg}$ CD25 only within unaffected relatives.

Although uncorrelated with surface CD25, circulating T$_{reg}$ numbers were also decreased both in patients and relatives, but less significantly (Supporting information, Fig. S2). In terms of T$_{reg}$ subset numbers per blood volume this was, however, restricted to the activated T$_{regs}$, which alone accounted for the differences in total FoxP3$^+$ cells while naive T$_{reg}$ and FoxP3^{low} numbers did not differ between groups (Supporting information, Fig. S2, first column). Relatively counted within total FoxP3$^+$ cells (Supporting information, Fig. S2, second column), FoxP3^{low} cells replaced the activated T$_{regs}$. Only when calculated within total CD4$^+$ cells (Supporting information, Fig. S2, third column), T$_{reg}$ subset characteristics were no longer shared by patients and relatives – an effect that can, in fact, be explained by a relative over-representation of T$_{regs}$ in SLE

patients with lymphopenia and that disappeared when only non-lymphopenic subjects were considered (Supporting information, Fig. S3).

Effect 1: characteristic and functionally relevant CD25 reduction already in CD31$^+$ recent thymic emigrant T$_{regs}$

Like naive Th cells, naive T$_{regs}$ also contain a subpopulation of recent thymic emigrants (RTE), which can be distinguished as the developmentally earliest T$_{regs}$ by their CD31 expression [39]. We studied RTE T$_{regs}$ with specific emphasis and found that their surface CD25 was already reduced strongly, actually showing almost identically low levels in SLE patients and unaffected relatives (1.5 and 1.4-fold reduced, respectively) compared to healthy controls (Fig. 3a, mirrored by CD25$^-$ approximations within RTE T$_{regs}$; see Supporting information, Fig. S1).

As human T$_{reg}$ development involves IL-2-driven intrathymic CD25 induction [40], we asked whether CD25 expression by RTE T$_{regs}$ depended upon the individual capacity to produce IL-2. The CD25 MFI of RTE T$_{regs}$ was indeed found to be correlated positively with the capacity of cultured CD3$^+$CD45RO$^+$ T memory cells to produce IL-2 upon PMA/ionomycin stimulation in SLE patients and also in controls (Fig. 3b,c; for IL-2 stainings, for example, see Supporting information, Fig. S4). By contrast, RTE T$_{reg}$ CD25 levels were unrelated to patients' SLEDAI-2k disease
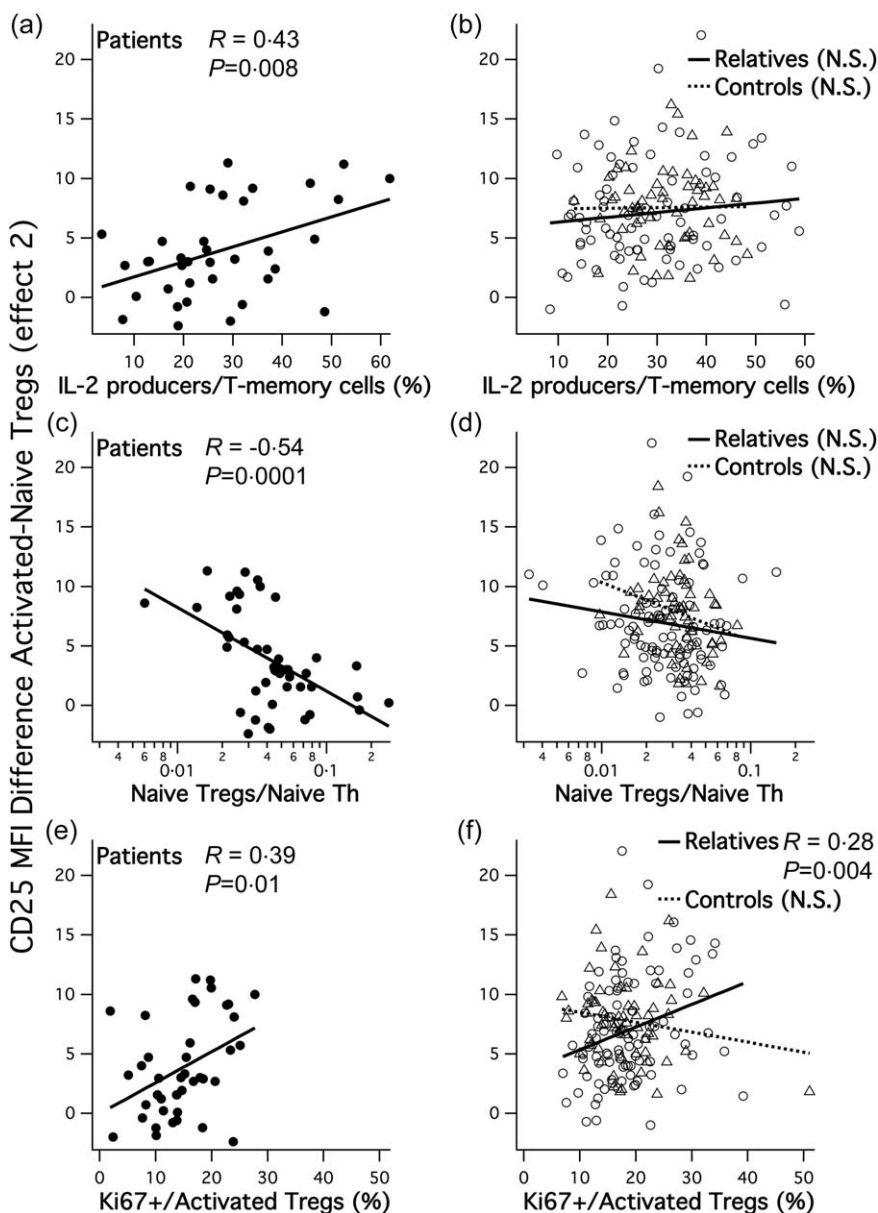
Fig. 5. Characteristics of surface CD25 up-regulation levels by activated regulatory T cells (T_{regs}). (a,b) CD25 up-regulation correlates with proportions of interleukin (IL)-2-producing T memory cells upon phorbol myristate acetate/ ionomycin (PMA/I) stimulation in systemic lupus erythematosus (SLE) patients but not in relatives or controls. (c,d) CD25 up-regulation correlates with reduced naive T_{regs} relative to naive conventional T helper (Th) cells in SLE patients but not in relatives or controls. (e,f) CD25 up-regulation correlates with Ki67^{+} proliferating fractions of activated T_{regs} in SLE patients and relatives but not in controls. (b,d,f) Circles and full regression lines indicate unaffected relatives; triangles and pointed lines indicate unrelated controls. Linear correlation coefficients (R) and significant P-values are shown; n.s. = non-significant.

activity, other clinical characteristics and treatment (Table 1) and to gender and age in all groups, except for a marginal increase with age within unaffected relatives, $R = 0.25/P = 0.02$ (not shown).

RTE T_{reg} CD25 levels were also completely unrelated to their highly age-dependent and barely group-characteristic circulating numbers and frequencies (Supporting information, Fig. S5), as well as to Ki67^{+} proliferating fractions (not shown). Instead, we detected a remarkably strong correlation to proportions of activated among total T_{regs} in patients ($R = 0.53/P = 3E$-4) as well as in unaffected relatives ($R = 0.42/P = 2E$-5) (Fig. 3d–f). These activated T_{reg} proportions were found to be correlated far less with CD25 levels when measured on all naive T_{regs} [patients: $R = 0.29/$ not significant (n.s.); relatives: $R = 0.32/P = 0.001$], and

completely unrelated to CD25 levels of the activated T_{regs} themselves. This suggests that the RTE T_{reg} surface CD25 levels uniquely indicate the conditioning effect that the state of early T_{regs} appears to have on the individual capacity in our SLE risk population to produce high numbers of fully activated peripheral T_{regs}.

Effect 2: CD25 up-regulation in activated *versus* naive T_{regs} is specifically deficient in manifest SLE

As activated T_{regs} are derived largely from naive T_{regs} [30,31] but generally show higher surface CD25 levels, we addressed specifically their capacity to up-regulate CD25 when differentiating from naive T_{regs}, measured as the MFI difference between both subsets in the same subject. While

there was no significant difference between unaffected relatives and controls, CD25 up-regulation was significantly lower and almost absent in SLE patients (Fig. 4). Thus, unaffected relatives of SLE patients appeared able to up-regulate CD25 in activated T_{regs} to the same extent as healthy controls, while SLE patients had a specific deficiency in this respect.

The SLE patients' CD25 up-regulation in activated *versus* naive T_{regs} was also found to be correlated positively with the individual capacity of their T memory cells to produce IL-2 upon PMA/ionomycin stimulation (Fig. 5a), as was CD25 in RTE T_{regs}. In contrast, no such correlation was found in unaffected relatives or controls (Fig. 5b), indicating that the IL-2 dependency of CD25 up-regulation was limited to SLE patients where it was also particularly defective.

CD25 up-regulation in activated T_{regs} was uncorrelated with RTE T_{reg} CD25 levels (effect 1) in relatives and in control subjects, and a positive correlation of both within patients was due entirely to their shared IL-2 dependency (Supporting information, Fig. S6). We also detected no significant relations to age, gender, SLEDAI-2k disease activity, clinical characteristics or treatment (not shown). Thus, CD25 up-regulation fulfills all criteria of being an

independent second effect contributing to the SLE-associated T_{reg} defect, reflecting peripheral but not thymic influences on T_{reg} CD25 expression.

While CD25 up-regulation in activated T_{regs} was basically uncorrelated with numbers and frequencies of activated T_{regs}, there were negative correlations with naive T_{regs} (Supporting information, Fig. S7). Interpreted as precursor consumption accompanying T_{reg} activation, this could particularly explain that among patients, those with virtually absent CD25 up-regulation were paradoxically the ones with most naive T_{regs} also in relation to naive $FoxP3^-$ T helpers (Fig. 5c,d). Furthermore, in the patients and relatives groups, CD25 up-regulation was correlated positively with $Ki67^+$ fractions within activated T_{regs} (Fig. 5e,f) suggesting that, apart from recruitment, their proliferation also influences this property.

Effect 1 shows the strongest heritability, and effect 2 is associated to *IL2RA* (CD25) genetic variants

In order to estimate overall genetic effects on the two described effects contributing to T_{reg} CD25 deficiency in SLE, we addressed their probable heritability within our affected families by assessing familial correlations. Both

Table 2. Genetic associations with polymorphisms in the *IL2RA* locus in control subjects

SNP	Position (Chr.10)	Alleles	Association with effect 1			Association with effect 2			Allele assoc. with low CD25	Allele assoc. with T1D§
			R*	P†	P_FDR‡	R*	P†	P_FDR‡		
rs12359875	6009144	CT	0·04	0·7438		0·32	**0·0129**	0·0647		
rs12244380	6011411	AG	−0·01	0·9204		−0·02	0·8863			
rs9663421	6013641	CT	0·16	0·2213		0·21	0·1030			
rs2076846	6021290	AG	−0·18	0·1736		0·03	0·8338			
rs11256369	6024237	CG	−0·18	0·1654		−0·10	0·4211			
rs7072398	6037883	AG	−0·19	0·1534		−0·20	0·1189			
rs4749924	6040433	AC	0·08	0·5316		0·20	0·1275			
rs706781	6044422	CT	0·10	0·4586		−0·12	0·3728			
rs11256497	6045831	AG	−0·14	0·2860		−0·28	**0·0283**	0·1010		
rs791587	6046736	AG	−0·04	0·7653		−0·09	0·4823			
rs791589	6047608	AG	0·04	0·7891		−0·26	0·0506			
rs10905669	6050130	CT	0·02	0·8787		−0·01	0·9635			
rs2256774	6055202	CT	−0·30	**0·0191**	0·2384	−0·34	**0·0079**	0·0656		
rs706779	6056861	CT	−0·35	**0·0056**	0·1409	−0·33	**0·0112**	0·0701		
rs706778	6056986	CT	−0·14	0·2805		−0·20	0·1375			
rs2104286	6057082	CT	0·06	0·6458		0·13	0·3368			
rs7072793	6064303	CT	0·14	0·2743		0·19	0·1443			
rs7073236	6064589	CT	0·14	0·2743		0·19	0·1443			
rs11597367	6065571	AG	0·11	0·3804		0·37	**0·0035**	**0·0434**	A	A
rs10795791	6066377	AG	−0·14	0·2743		−0·19	0·1443			
rs4147359	6066476	AG	0·01	0·9360		0·07	0·5913			
rs7090530	6068912	AC	0·19	0·1322		0·29	**0·0228**	0·0948		
rs41295061	6072697	AC	−0·01	0·9393		0·02	0·8867			
rs11594656	6080046	AT	−0·11	0·3804		−0·37	**0·0035**	**0·0434**	T	T
rs12251307	6081532	CT	0·05	0·7050		−0·01	0·9630			

*Linear correlation coefficient. †P-values from univariate regression (significant values < 0·05 in bold type). ‡P-values false-positive discovery rate (FDR)-corrected for multiple comparisons (significant values < 0·05 in bold type). §According to Lowe *et al.* [41].

parental and sibling correlations were indeed found highly significant for effect 1, RTE T_{reg} CD25 ($\rho_P = 0.73/P = 2E\text{-}12$; $\rho_S = 0.70/P = 5E\text{-}8$), indicating a high heritability. For effect 2, CD25 MFI up-regulation, familial correlations were clearly lower but still significant ($\rho_P = 0.44/P = 1E\text{-}5$; $\rho_S = 0.27/P = 0.032$).

In our control group, where subjects were unrelated, we finally studied whether the two component effects were associated with genetic variation in the *IL2RA* locus that encodes CD25. For effect 1, RTE T_{reg} CD25, association with two of 25 typed SNPs was univariately significant but did not retain significance upon FDR correction for multiple comparisons (Table 2). In contrast, *IL2RA* SNP association with effect 2, CD25 MFI up-regulation, was significant also when FDR-corrected. The associated SNPs were located in the *IL2RA* 5' portion (Table 2), corresponding to the region where genetic association primarily with type 1 diabetes (T1D) was also reported. In fact, the two SNP alleles that we found associated significantly with reduced CD25 up-regulation were also described as T1D risk alleles [41].

Discussion

In SLE T_{regs}, a particular surface CD25 reduction 'dissociated' from FoxP3 expression was described previously [25], while the details and origins of this reduction have not been assessed thoroughly. Studying CD25 in T_{reg} subsets, we could discriminate two independent effects contributing to the reduced surface CD25 in SLE that were both related to individual IL-2 production:

- effect 1, surface CD25 of the developmentally earliest RTE T_{regs}, was reduced in both SLE patients and unaffected relatives in a largely shared manner; and
- effect 2, the degree to which T_{regs} up-regulated CD25 upon activation, was only deficient in manifest SLE but not in unaffected relatives. This CD25 up-regulation was associated (in controls) with *IL2RA* genetic variants.

Effect 1 – RTE T_{reg} surface CD25 – likely reflects intrathymic T_{reg} development [40]. It neither depended upon disease manifestation nor did it correlate with disease activity. It was also unrelated to the age-dependent RTE T_{reg} numbers and frequencies, but highly heritable, as suggested by familial correlations. Thus, RTE T_{reg} CD25 clearly does not represent an effect secondary to clinical disease, treatment or age, but rather a primary, largely genetically determined and individually variable 'baseline' CD25 that seems to be set intrathymically, as it is best visible in the developmentally earliest T_{regs}. Most interestingly, RTE T_{reg} CD25 (unlike surface CD25 of activated or overall naive T_{regs}) was correlated uniquely positively, thus predictive for peripheral proportions of fully differentiated activated T_{regs}

in the individual patients and relatives. This suggests that the RTE T_{reg} functional state reflected by their surface CD25 level is crucial for the capacity of subjects at risk for SLE to produce high proportions of activated peripheral T_{regs}.

Effect 2 – CD25 up-regulation in activated versus naïve T_{regs} – demonstrates a specific failure of SLE T_{regs} to increase CD25 expression when differentiating into activated CD45RO$^+$ T_{regs} in the periphery. This failure was not seen in first-degree unaffected relatives and therefore appears secondary to SLE manifestation (although we observed no relation to clinical features or treatment). Coincident with our earlier report pointing to a compensatory T cell regulation [37], it can be said that unaffected relatives 'compensate' their shared baseline CD25 reduction in early non-activated T_{regs} (effect 1) by activation-dependent up-regulation – a mechanism that could be important for subjects at risk to avoid clinical disease. Unlike effect 1, effect 2 was not associated with increased activated T_{reg} quantities in circulation, while it was related to naive T_{reg} consumption and to activated T_{reg} proliferation. This can be explained by the short-lived nature of activated T_{regs} [30].

Technically, our quantitation of CD25 up-regulation (effect 2) is based upon the assumption of a common T_{reg} lineage, i.e. that transient FoxP3 expression and peripherally induced T_{regs} do not need to be considered. This seems reasonable to us in this context, as SLE Foxp3$^+$ T_{regs}, including CD25^{low} and CD25$^-$ cells, have shown extraordinarily high levels of Helios expression and FoxP3–TSDR demethylation [7,32]. While Helios expression indicates a thymic origin [33,34], cytosine–phosphate–guanine (CpG) demethylation of the FoxP3–TSDR region [35] confirms sustained bona-fide FoxP3 expression. Both markers do not occur in transiently FoxP3-expressing T helpers or induced T_{regs}. Moreover, FoxP3^{low} cells where transient expression may occur were excluded thoroughly from our cytometric definition of activated T_{regs}.

Our findings add to a series of papers which showed neatly that surface CD25 (but not FoxP3) is the only marker that can reflect the SLE-associated phenotypical [21,28] and functional [25,28] T_{reg} impairment appropriately. The two effects acting on reduced T_{reg} CD25 expression described by us here were both related to the capacity of effector cells to produce its ligand, IL-2, which is recognized widely as the most important factor for T_{reg} homeostasis, since the time when it was first demonstrated that IL-2-deficient mice harbour very few T_{regs} with low surface CD25 [42]. The crucial role of IL-2, particularly for peripheral T_{reg} maintenance and expansion, is also generally accepted for the human system [43], and we have recently described its effects particularly in respect to SLE-associated autoantibody profiles [37]. More recently, IL-2 was shown to be similarly important (besides IL-15) for the thymic generation of human T_{regs} [40]. Particularly in

SLE, T cells obviously produce insufficient amounts of IL-2 due to altered transcriptional regulation [4]. Accordingly, a recent report demonstrates that the CD25 deficiency of SLE T$_{regs}$ could be reversed efficiently by IL-2 in cell cultures, as well as in patients undergoing low-dose IL-2 therapy [7]. This corresponds with findings that restoring IL-2 production in lupus-prone mice enhanced the generation of CD25$^+$FoxP3$^+$ T$_{regs}$ [44,45].

Our results suggest that deficient IL-2 in SLE affects surface CD25 in two separate ways in early and in late T$_{regs}$. Both effects appear genetically influenced. While the early effect 1 showed indirect evidence of (unspecified) genetic factors, the patient-characteristic late effect 2 was found associated with genetic variants in the region of the CD25-encoding locus *IL2RA* that also conferred T1D risk [41]. Located 5' upstream of the *IL2RA* locus, the effect linked to these variants is unlikely to alter CD25 molecular properties, but rather transcriptional control by (not well known) transcription factors [46]. Among the different haplotypes that have meanwhile been identified in this region [41,47], our findings point mainly to a functionally unexplored rs11594656 haplotype where, conversely, T1D risk variants protected from multiple sclerosis (MS) [48]. For the better-studied rs2104286 haplotype in the same region, healthy individuals bearing the risk variant for both diseases with low IL-2 receptor signalling [49] also had fewer T$_{regs}$ with reduced suppressive function and less stable FoxP3 expression under limiting IL-2 concentrations [50]. Loss of FoxP3 expression by T$_{regs}$ in IL-2 insufficient conditions is also well documented from mouse experiments [51,52] and explained by IL-2 mediated transcriptional stabilization [53]. Ex-T$_{regs}$ convert typically into Th17 cells, can easily become pathogenic and cause lung and liver inflammation [51], arthritis [54] and autoantigen-driven encephalitis [55]. Also in human MS, IL-2–IL2R pathway alterations were linked to decreased FoxP3 expression [56], and T$_{regs}$ converting to Th17 cells probably contribute to human inflammatory bowel diseases [57], rheumatoid arthritis [58] and psoriasis [59] by shifting the paradigmatic balance between these two developmentally related cell types [60]. As the relevance of this for SLE remains somewhat unclear, our discrimination of two separate component effects may help to characterize further how the probably multiple associated mechanisms affect each type of autoimmunity.

In SLE patients, defective CD25 expression by T$_{regs}$ was found completely reversible under low-dose IL-2 therapy [7]. In our context, this corresponds most probably to a boost of effect 2, most affected in SLE patients and in fact showing less overall genetic determination (i.e. heritability), thus a more 'acquired' character, than effect 1. However, low-dose IL-2 therapy increased not only CD25 expression but even more impressively the numbers of circulating FoxP3$^+$ T$_{regs}$. Interpreted in the context of our results, this also points to the involvement of IL-2 effects on non-activated T$_{regs}$, possibly even inside the thymus analogously to our effect 1, in addition to peripheral stimulation (effect 2). It will be extremely interesting to determine this in future clinical studies, as well as how the clinical outcome relates to the two effect types, in order to identify optimal therapy schemes for IL-2 and possibly other therapies seeking to improve T$_{reg}$ function.

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Author contributions

C. F., M. L. and B. M. designed research; N. C., O. M., S. I. G., C. C., B. L., A. M. F. and C. F. performed experiments; O.M., C. V., A. M., M.F. M. F., A. G. C., C. P., R. C. M., T. C., A. R. M. and J. F. V. collected data; N. C., O. M., S. I. G. and C. F. analysed and interpreted data; N. C. and C. F. wrote the manuscript.

Disclosure

The authors declare no competing financial interests.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Approximation of regulatory T cell (T$_{reg}$) proportions lacking surface CD25. Although we are cautious about threshold-defined CD25 gating and do not use it for our own quantitative analysis, we have approximated proportions of CD25$^-$ cells within T$_{regs}$ by a plausible but still arbitrary threshold criterion (fluorescence intensity < 5) for comparability with other published work. (a,b) CD25$^-$ cells within naive T$_{regs}$, separately for CD31$^+$ (recent thymic emigrants) and CD31$^-$, (c) within activated T$_{regs}$ and (d) within all CD4$^+$ forkhead box P3 (FoxP3)$^+$ T cells. Group differences of patients or unaffected relatives, respectively, to healthy unrelated control subjects were tested. Significant *P*-values are shown.

Fig. S2. Group differences between regulatory T cell (T$_{reg}$) numbers and frequencies depend strongly upon the type of measure. The three common measures of cell abundance are shown comparatively in columns: circulating cell numbers per blood volume (first column), frequencies within total forkhead box P3 (FoxP3)$^+$ cells (second column) and frequencies within total CD4$^+$ T cells (third column). Rows represent the three T$_{reg}$ subsets and total FoxP3$^+$. For each measure, group differences of patients or unaffected relatives, respectively, to healthy unrelated control subjects were tested. Significant *P*-values are shown; n.s. = non-significant.

Fig. S3. Lymphopenia effect on regulatory T cell (T$_{reg}$) frequencies when measured within CD4$^+$ cells. (a) Circulating lymphocyte numbers per group demonstrate that many systemic lupus erythematosus (SLE) patients (but not unaffected relatives) have low lymphocyte numbers. (b) forkhead box P3 (FoxP3)$^+$ frequencies within total CD4$^+$ cells correlated strongly with lymphocyte numbers, showing their overrepresentation particularly in lymphopenic SLE patients. This over-representation also caused a distribution bias of T$_{reg}$ subset measures within CD4$^+$ cells: when only non-lymphopenic subjects were considered, the previously detected increase in patients' naive T$_{regs}$/CD4$^+$ was abrogated (c), and activated T$_{reg}$ differences corresponded to those found for absolute cell numbers (d). Linear correlation coefficients (*R*) and significant *P*-values are shown; n.s. = non-significant.

Fig. S4. Intracellular interleukin (IL)-2 staining examples. Gated on CD3$^+$CD45RO$^+$ memory T cells, representative cytograms for intracellular IL-2 [vertically *versus* horizontal interferon (IFN)-γ] staining are shown for (a) a systemic lupus erythematosus (SLE) patient, (b) a first-degree relative, (c) an unrelated healthy control and (d) without anti-IL-2 antibody. Percentages of IL-2 positive cells are annotated.

Fig. S5. Characteristics of different measures of recent thymic emigrants (RTE) regulatory T cell (T$_{reg}$) numbers and frequencies. (a–d) Four different measures of RTE T$_{regs}$ were analysed for their relation to age and groups. All measures showed a significant decrease with age in all three groups. Accordingly, group comparisons were calculated with age as a covariate. None of the four measures showed a significant difference between relatives and controls. Patients had significant reductions in absolute numbers (a) and frequencies within naive T$_{regs}$ (d) but not when measured within total forkhead box P3 (FoxP3)$^+$ (b) or CD4$^+$ (b). (e,f) Relations of RTE T$_{reg}$ measures to RTE T$_{reg}$ surface CD25. No significant relations were found, as all groupwise direct or semipartial correlations (numbers/frequencies regressed against age) were found univariately insignificant.

Fig. S6. The correlation between recent thymic emigrants (RTE) regulatory T cell (T$_{reg}$) CD25 and CD25 up-regulation in activated T$_{regs}$ is due entirely to their shared interleukin (IL)-2 dependency. (a) The direct correlation between the two CD25 component effects was found significant only for patients but not relatives or controls. (b)

As both CD25 component effects depended upon IL-2 producers in patients, we further calculated their partial correlation (i.e. of both regressed against our measures of IL-2 producer proportions). The absence of significance demonstrates that the previously detected correlation was due to an IL-2 effect. Linear correlation coefficients (R) and significant P-values are shown; n.s. = non-significant.

Fig. S7. Relations of CD25 up-regulation in activated regulatory T cells (T$_{regs}$) (effect 2) to T$_{reg}$ subset measures. We studied the possible relation of CD25 up-regulation in activated T$_{regs}$ (effect 2) to three common measures of naive and activated T$_{reg}$ numbers and frequencies, respectively (analogously to Fig. S2). (a–c) None of the activated T$_{reg}$ measures showed a significant relation when assessed by multiple regression, differentiating the three subject groups by two contrast covariates. Univariately, only activated T$_{regs}$/CD4$^+$ measured within patients showed a significant correlation (c) which was, however, ascribable to lymphopenia bias as abrogated when considering only non-lymphopenic subjects analogously to Fig. S3. (d–f) In contrast, all three measures of naive recent thymic emigrants (RTE) regulatory T cell (T$_{reg}$) numbers or frequencies were found related significantly to decreasing CD25 up-regulation in activated T$_{regs}$ (effect 2) using the same multiple regression approach. Univariate group-wise correlation (although not significant throughout) also remained significant with naive T$_{regs}$/CD4$^+$ in patients when considering only non-lymphopenic patients (f). Linear correlation coefficients (R) and significant P-values are shown; n.s. = non-significant.