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Global Natural Regulatory T Cell Depletion in Active Systemic Lupus Erythematosus[1]

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The immune defect that could account for the multisystemic involvement that characterizes systemic lupus erythematosus (SLE) remains unknown. We hypothesized that iterative disease flares correspond to a recurrent defect in the peripheral immune suppression exerted by naturally occurring T regulatory cells (Tregs). Surprisingly, Tregs isolated from lupus patients show the same phenotypic and functional characteristics as corresponding cells found in healthy controls. A decrease in the proportion of circulating Tregs among other CD4$^+$ T cells is nevertheless evidenced in active patients when this group is compared with healthy controls ($0.57 \pm 0.24\%$, $n = 45$ vs $1.29 \pm 0.38\%$, $n = 82$, $p < 0.0001$) or with inactive patients ($1.22 \pm 0.67\%$, $n = 62$, $p < 0.0001$). In contrast, the proportion of Tregs in other systemic autoimmune diseases such as primary Sjögren syndrome and inflammatory myopathy does not significantly differ from controls' values ($1.15 \pm 0.46\%$, $n = 21$, $p = 0.09$ and $1.16 \pm 0.44\%$, $n = 16$, $p = 0.43$, respectively). Lupus Tregs do not accumulate in either the lymph nodes or the diseased kidneys and are not killed by a circulating soluble factor, but demonstrate in vitro a heightened sensitivity to Fas-induced apoptosis. Finally, we show that the extent of Treg depletion correlates with the clinical severity of the flare. SLE flares are therefore associated with a global Treg depletion and not with a phenomenon of tissue redistribution. In summary, we suggest that the physiopathology of SLE could be tied to a defect in the homeostatic control of the Treg subpopulation. *The Journal of Immunology,* **2005, 175: 8392–8400.**

Regulatory T cells play a critical role in the maintenance of peripheral tolerance, preventing the occurrence of autoimmune diseases in murine models (1). At least two types of regulatory cells can be distinguished (2). The first type regulates immune responses via secretion of cytokines and corresponds to IL-10-producing Tr1 cells (3) and TGF-β-producing Th3 cells (4). The second type of regulatory T cell is the naturally occurring, or innate, regulatory T cell (Treg),[4] characterized by constitutive expression of CD25 (1). These cells mediate their suppressive effect through a contact-dependent, but as yet undefined, mechanism to inhibit autologous CD25$^-$ T cell proliferation and Th1 cytokine secretion, and to modulate the Ag-presenting capacity of dendritic cells (5–8).

Since their first description in humans (9–16), Tregs have been the focus of intense research efforts, notably in the context of autoimmune diseases (17–24). It is now clear that the forkhead transcription factor Foxp3 acts as a critical regulator in the development and function of Tregs (25–29). The best evidence for Tregs as being key in the control of self-tolerance in humans therefore comes from the causal association between the rapidly fatal immune dysregulation, polyendocrinopathy, X-linked syndrome, and mutations in *FOXP3* (30, 31). In chronically ill patients, a deficiency in Treg-suppressive function has been observed in vitro and has been suggested to influence the pathogenesis of diabetes (20), multiple sclerosis (21), rheumatoid arthritis (22), type II autoimmune polyendocrinopathy (23), and psoriasis (24). In the latter studies, the underlying defect accounting for the inability of the patient's Tregs to suppress lymphocyte proliferation and/or cytokine production could not be identified. Other authors reported that Tregs isolated from patients affected by various types of rheumatoid disorders (rheumatoid arthritis, spondyloarthropathies, and juvenile idiopathic arthritis) presented no apparent functional deficiency (32–34). Comparison between these studies is complicated by the fact that a means of defining phenotypically Tregs in humans is still lacking. Indeed, live cells cannot be purified on the basis of their Foxp3 expression. Tregs have been shown in humans to be mainly confined to the CD25^{bright} subset of CD4$^+$ cells (13) and were therefore purified according to these criteria (17–24, 32–34). CD25 being also expressed on non-Treg subsets, it is possible that the proportion of actual Tregs purified along with other CD25$^+$ cells would vary between laboratories and/or disease stages, accounting for the apparent discrepancies found in the literature.

Mice depleted in CD4$^+$CD25$^+$ T cells develop a multisystemic autoimmune disease, including gastritis, oophoritis, arthritis, and thyroiditis. Interestingly, some animals also developed glomerulonephritis and activated the production of anti-dsDNA Abs (1). The latter features are obviously reminiscent of systemic lupus

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[4] Abbreviations used in this paper: Treg, naturally occurring regulatory T cell; HCQ, hydroxychloroquine; HPRT, hypoxanthine phosphoribosyltransferase; IM, inflammatory myopathy; LN, lymph node; pSS, primary Sjögren syndrome; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

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erythematosus (SLE), a condition characterized by a multisystemic autoimmune involvement and by the targeting of typical disease-associated Ags such as dsDNA. These findings led us and others (35–37) to envisage that Tregs may play an important role in the pathophysiology of SLE.

Previous studies in SLE patients (35, 36) argued in favor of a decrease in circulating Treg numbers during disease flares, which was suggested to reflect their reallocation to lymphoid organs or disease-affected tissues. It was also reported that the immunosuppressive activity of the latter cells was impaired in patients presenting with active disease (37).

In this study, contrary to what was previously proposed, we show that SLE $CD4^+CD25^{bright}$ T cells are as potent immunoregulators as corresponding control cells. However, levels of circulating Tregs cells are indeed reduced during SLE flares. The contraction of the circulating Treg subset does not seem to reflect its tissue reallocation, as $Foxp3^+$ cells do not accumulate in disease-involved organs or in lymphoid tissues. Finally, a mechanism leading to Treg depletion is proposed as we show that SLE Tregs are more sensitive to Fas-mediated apoptosis than control Tregs (38). Our results argue that inappropriate induction of Treg apoptosis is relevant to SLE pathogenesis.

Materials and Methods

Study patients

One hundred and seven consecutive adult patients (98 women and 9 men, mean age 35.8 ± 14.5 years, range 15–76 years) with a diagnosis of SLE according to the American College of Rheumatology criteria (39, 40) were included in the study. All SLE patients were referred to the internal medicine department at the Hospital Pitié-Salpêtrière (Paris, France). The SLE patients were divided into two groups according to their SLE disease activity index (SLEDAI), a validated index of SLE activity (41), with one group comprising subjects with inactive SLE (SLEDAI ≤3, $n = 62$, mean age 39.5 ± 15.6 years, 56 women and 6 men, mean lymphocyte number 1395 ± 657/mm^3) and a second group comprising patients with active SLE (SLEDAI >3, $n = 45$, mean age 32.5 ± 11.8 years, 42 women and 3 men, mean lymphocyte number 1107 ± 569/mm^3). Blood samples from 82 age- and sex-matched healthy donors (mean lymphocyte number 2117 ± 699/mm^3) were obtained from Etablissement Français du Sang (Hôpital Pitié-Salpêtrière). Treatment regimens in the active SLE group were as follows: hydroxychloroquine (HCQ) alone ($n = 7$), HCQ + prednisone ($n = 22$), HCQ + prednisone + methotrexate ($n = 3$), HCQ + prednisone + cyclophosphamide ($n = 2$). Eleven active patients were untreated at the time of analysis (Table I). Two control autoimmune disease groups consisted of 16 primary Sjögren syndrome (pSS; mean age 50 ± 12.7 years, 15 women and 1 man) and 21 inflammatory myopathy (IM; mean age 48.2 ± 15.5 years, 15 women and 6 men) patients. Clinical features fulfilled Bohan and Peter criteria in IM patients (42) and criteria of the European Community Study group in pSS patients (43).

Fresh lymph node (LN) biopsies were obtained from five active SLE patients and two healthy donors undergoing surgery (graft donors).

The study was performed according to the Helsinki declaration. The study protocol was reviewed and approved by the local ethics committee.

Cell isolation and flow cytometry

All blood samples were collected and processed within 2 h. PBMC were purified on Ficoll gradients (Eurobio). LN and kidney samples were disrupted immediately after surgery or biopsy using a scalpel and gently teased in culture medium to obtain a mononuclear cell suspension. Control and active SLE sera were collected and frozen at −20°C until used. Sera were heat inactivated at 56°C for 30 min and centrifuged before use.

Cells were stained with anti-CD4 PerCP or anti-CD4 FITC, anti-CD25 PE or anti-CD25 allophycocyanin, anti-CD95 allophycocyanin mAbs (BD Pharmingen), and anti-CCR4 PE (R&D Systems). For intracellular detection of CTLA-4, surface staining was first performed; cells were then fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and incubated with anti-CD152 PE (BD Biosciences). Samples were analyzed on FACSCalibur equipment (BD Biosciences). A total of 500,000 events was acquired and data were analyzed with WinMDI 2.8 (freeware; J. Trotter; ⟨http://facs.scripps.edu/software.html⟩). $CD4^+$ T cells were FACS sorted according to their CD25 expression level (FACSVantage DIVA; BD Biosciences). $CD25^{bright}$ gate was adjusted to contain $CD4^+$ T cells that express CD25 more brightly than $CD4^-CD25^+$ cells.

Proliferation assays and cytokine detection

Varying numbers of sorted $CD4^+CD25^{bright}$ T cells were cocultured with 2.5 10^3 autologous $CD4^+CD25^-$ responder T cells and 2.5 10^4 allogeneic T cell-depleted PBMCs (irradiated at 5000 rad) in 96 U-bottom well plates coated with 0.5 μg/ml OKT3 (Orthoclone; Orthobiotech). The medium used for culture was based on RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% of FCS (Boehringer Mannheim), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies). On day 5, 1 μCi of [^{3}H]thymidine (Valeant Pharmaceuticals) was added for the final 16 h of culture. Proliferation was determined by scintillation counting on day 6 (PerkinElmer Wallac). IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α levels were measured in supernatants of cell cultures collected on day 5 using a cytometric bead array kit (BD Biosciences), according to the manufacturer's instructions. None of the patients tested for proliferation and cytokine production was on steroids at the time of analysis.

Detection of $Foxp3^+$ T cells in LN

Frozen LN tissues obtained from SLE patients ($n = 5$) and control LN ($n = 2$) were cut 5 μm thick, fixed in acetone, and blocked for endogenous biotin (Vector Laboratories). Biopsy samples were stained with polyclonal goat anti-human Foxp3 (ab2481, 500 μg/ml, IgG, 1/100 dilution; Abcam) and mouse anti-human CD4 (MT310, IgG1, 100 μg/ml, 1/100 dilution; DakoCytomation), followed by FITC-conjugated rat anti-mouse (145-095-166, IgG, 1 mg/ml, 1/100 dilution; Jackson ImmunoResearch Laboratories) and biotinylated rabbit anti-goat (E0466, 1 mg/ml, 1/400 dilution; DakoCytomation), followed by cyanine 3-conjugated streptavidin (PA43001, 1 mg/ml, 1/300 dilution; Amersham Biosciences). Irrelevant isotype-matched Abs (DakoCytomation) were also used as primary Abs in control experiments. Fluorescent images of mounted sections were acquired with an epifluorescent microscope (Axioplan 2; Zeiss) and analyzed with FluoUp image analysis system software (Explora Nova). Density of $Foxp3^+$ T cells was determined as the number of positive cells per mm^2 in relevant LN areas (B cell areas of the LN sections were excluded). The mean size of a single analyzed area was 6.5×10^5 μm^2. Data were collected in three separate areas per section and then averaged. Each biopsy was assessed twice, with result variations always <10%.

Real-time Foxp3 PCR

Real-time PCR was performed with a TaqMan assay on an ABI 7700 system (Applied Biosystems). Total RNA extracted from kidney tissues of patients with lupus nephropathy or healthy controls (graft donors) and from FACS sorted T cells was immediately reverse transcribed in a 50 μl reaction volume (ProSTAR First Strand; Stratagene), according to the manufacturer's instructions. FOXP3 and hypoxanthine phosphoribosyltransferase-1 (HPRT-1) Assays-on-Demand gene expression probes (Hs 00203958 and 99999909, respectively; Applied Biosystems) were used. In each reaction, HPRT-1 was amplified as a housekeeping gene to calculate a standard curve and to correct for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to HPRT-1 by the instrument software.

Fas-induced apoptosis assay

Freshly drawn PBMC (1×10^6) stained with anti-CD4 FITC and anti-CD25 PE were incubated for 12 h in 5 μg/ml plate-bound OKT3 96-well plate, then incubated with medium only or with 5 μg/ml anti-Fas (DX2 clone; BD Biosciences) or with 1% control or active SLE serum for 1 h. Cells were washed with cold PBS. Anti-Fas-induced apoptosis was measured using annexin V allophycocyanin (BD Biosciences), according to the manufacturer's instructions. The 7-aminoactinomycin D staining (BD Biosciences) was used to exclude dead cells. Cells were analyzed on FACSCalibur equipment.

Statistical analysis

Comparisons between active and inactive SLE patients and control subjects were made using the nonparametric Mann-Whitney U test. Comparisons of the rate of circulating $CD25^{bright}CD4^+$ T cells during the evolution of the disease were made using the paired t test. Similar tests were used in apoptosis assays to compare annexin V^+ cell proportions. Correlations were determined by Spearman's ranking. Values of $p < 0.05$ were considered significant.

Table I. *Active lupus patients characteristics*

No. Patient	Age (years)	Sex	Treatment	Steroid Dose (mg)	Clinical and Biological Features	SLEDAI[a]	Circulating Tregs	
							% of $CD4^+$ T cells	Absolute numbers ($cells/mm^3$)
1	15	F	HCQ[b]	0	New rash, alopecia, low complement, increased DNA binding, thrombocytopenia, leukopenia	10	0.64	1.68
2	27	F	CT, HCQ	5	Proteinuria	4	1	6.67
3	39	M	None	0	Arthritis, proteinuria, low complement, fever	9	0.51	2.10
4	29	F	CT, HCQ	5	Arthritis, low complement, increased DNA binding	8	0.34	5.72
5	24	F	CT, HCQ	15	Vasculitis, arthritis, proteinuria, new rash, mucosal ulcers, fever, thrombocytopenia, leukopenia	23	0.18	0.52
6	52	F	CT, HCQ, MTX	10	Arthritis, pleurisy	6	0.44	6.66
7	24	M	CT, HCQ	20	Pericarditis, low complement	4	0.72	3.45
8	46	F	None	0	Pleurisy, pericarditis, fever	5	0.75	3.46
9	23	F	CT, HCQ	10	Arthritis, urinary cast, hematuria, proteinuria, low complement, increased DNA binding, thrombocytopenia	21	0.4	1.75
10	16	F	None	0	Hematuria, proteinuria, new rash, low complement, mucosal ulcers, low complement, increased DNA binding, thrombocytopenia, leukopenia	18	0.29	0.97
11	16	F	CT, HCQ	5	Arthritis, new rash, low complement, increased DNA binding, leukopenia	11	1.06	9.55
12	40	M	CT, HCQ	10	New rash, fever, leukopenia	4	0.76	4.04
13	24	F	HCQ	0	Arthritis, pleurisy, low complement, increased DNA binding	10	0.83	7.05
14	44	F	HCQ	0	Pyuria, new rash, alopecia, mucosal ulcers, increased DNA binding	10	0.42	1.35
15	40	F	CT, HCQ	10	Arthritis, pyuria, low complement, increased DNA binding	10	0.6	1.56
16	31	F	HCQ	0	Arthritis, low complement, increased DNA binding	8	0.55	4.25
17	19	F	None	0	New rash, alopecia, low complement, increased DNA binding	8	0.82	6.16
18	39	F	CT, HCQ	5	Hematuria, proteinuria, pyuria, mucosal ulcers, pleurisy, pericarditis, low complement, increased DNA binding	22	0.08	0.53
19	28	F	None	0	Hematuria, proteinuria, pyuria, increased DNA binding, leukopenia	15	0.42	2.99
20	51	F	HCQ	0	Urinary cast, hematuria, proteinuria, low complement, increased DNA binding	20	0.36	0.99
21	32	F	CT, HCQ, MTX	12	Proteinuria, new rash	6	0.55	2.48
22	44	F	None	0	Arthritis, new rash, mucosal ulcers, low complement, increased DNA binding	12	0.55	1.22
23	15	F	CT, HCQ	15	Proteinuria, low complement, increased DNA binding	8	0.77	0.57
24	28	F	CT, HCQ	40	Organic brain syndrome, cerebrovascular accident, proteinuria, leukopenia	21	0.42	1.74
25	30	F	CT, HCQ	16	Hematuria, proteinuria, low complement, increased DNA binding, leukopenia	13	0.8	2.39
26	26	F	None	0	Low complement, thrombocytopenia, leukopenia	4	0.55	5.55
27	24	F	CT, HCQ	15	Hematuria, proteinuria, new rash	10	0.7	0.61
28	27	F	CT, HCQ	13	Arthritis, myositis, pyuria, mucosal ulcers, pleurisy, pericarditis, increased DNA binding	16	0.5	0.31
29	51	F	CT, CPM	35	Visual disturbance, cranial nerve disorder, cerebrovascular accident, arthritis, increased DNA binding, leukopenia	31	0.12	0.47
30	37	F	CT, HCQ	15	Arthritis, new rash, alopecia, mucosal ulcers, pleurisy, low complement, increased DNA binding, leukopenia	17	0.2	1.02
31	47	F	None	0	Arthritis, urinary cast, hematuria, proteinuria, pyuria, low complement, increased DNA binding, fever, thrombocytopenia, leukopenia	27	0.57	1.35
32	32	F	CT, HCQ	15	Lupus headache, arthritis, new rash, alopecia, mucosal ulcers, low complement, increased DNA binding, thrombocytopenia, leukopenia	26	0.28	1.18
33	21	F	None	0	Arthritis, new rash, increased DNA binding, thrombocytopenia	9	0.86	3.84
34	49	F	CT, HCQ	5	Hematuria, proteinuria, pyuria, new rash, low complement, increased DNA binding, fever	19	0.46	2.80
35	29	F	CT, HCQ	7.5	Arthritis, hematuria, proteinuria, new rash, low complement, increased DNA binding	18	0.61	2.29
36	30	F	CT, HCQ, MTX	10	Arthritis, proteinuria	8	0.62	3.24
37	34	F	CT, HCQ	20	Seizure, pericarditis, increased DNA binding, fever	13	0.54	3.57
38	64	F	None	0	Arthritis, new rash, alopecia, increased DNA binding, fever	11	0.67	2.75
39	55	F	HCQ	0	Arthritis	4	1.24	6.54
40	25	F	None	0	Arthritis, hematuria, proteinuria, pyuria, pleurisy, low complement, increased DNA binding, fever, leukopenia	24	0.59	2.65
41	20	F	CT, HCQ	20	Seizure, cerebrovascular accident, proteinuria	20	0.52	2.94
42	37	F	CT, HCQ	15	Alopecia, low complement, thrombocytopenia	5	0.6	2.35
43	29	F	HCQ	0	Organic brain syndrome, cerebrovascular accident, proteinuria, alopecia, low complement, increased DNA binding	26	0.65	1.48
44	32	F	CT, HCQ	15	Proteinuria, increased DNA binding, leukopenia	7	0.82	4.06
45	20	F	CT, CPM	75	Arthritis, myositis, urinary cast, hematuria, proteinuria, pleurisy, pericarditis, low complement, increased DNA binding, fever	28	0.34	1.40

[a] SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.
[b] CT, Corticosteroids; HCQ, hydroxychloroquin; MTX, methotrexate; CPM, cyclophosphamide.

Results

Definition of Tregs in patients and controls

At this time, a clear-cut phenotypic definition of Tregs remains unestablished. Tregs are usually distinguished among $CD4^+$ T cells by their high expression of CD25 and their ability to strongly suppress proliferation of autologous naive $CD25^-CD4^+$ T cells in a contact-dependent manner (13, 44). However, there are no standard criteria to set the minimal CD25 expression level that would define a pure Treg population. We have therefore attempted to base our work on a strictly functional definition of this subpopulation to study its behavior in lupus patients. We FACS sorted $CD4^+$ T cells above various thresholds of CD25 expression and tested each subset for its ability to suppress autologous T cell proliferation and cytokine secretion. As shown, subtle contamination of the $CD4^+CD25^{bright}$ subpopulation by $CD4^+CD25^{low}$ cells greatly impacts analytic evaluation of immunosuppressive function (Fig. 1*a*). According to our comparative functional assessment of $CD4^+CD25^+$ fractions of increasing size, a minimal CD25 threshold for Treg definition corresponds in active patients and controls to the highest expression level of the same marker in $CD4^-$ T cells (Fig. 1*a*).

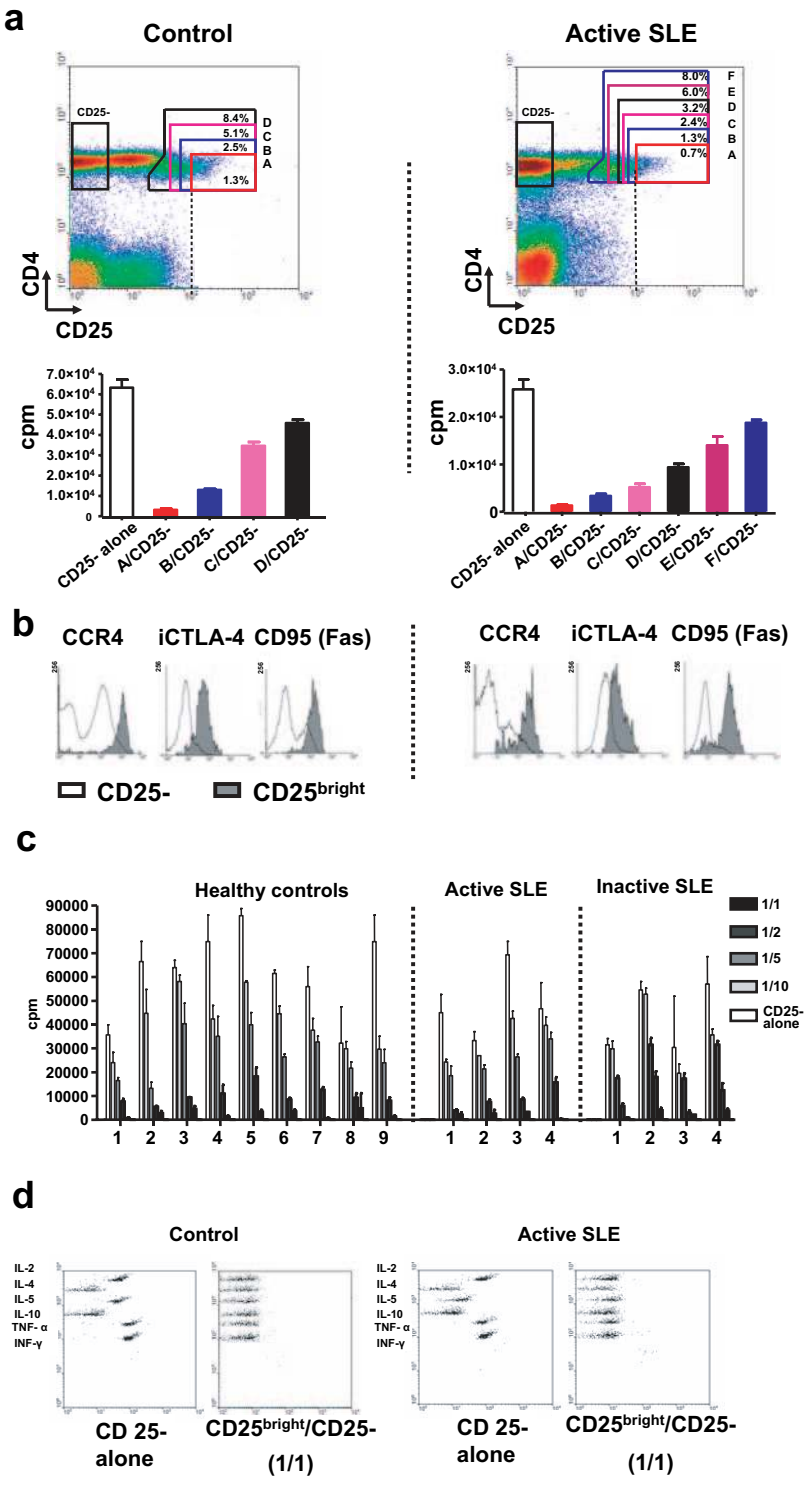

FIGURE 1. Phenotype and function of natural Tregs in patients and controls. *a*, Representative cytofluorometric and functional analysis of healthy donor PBMCs (*left panels*) and active SLE PBMCs (*right panels*). Indicated sorting gates were used (*top panels*). Sorted cells were stimulated alone ($CD25^-$) or in the presence of $CD4^+CD25^+$ autologous T cells (1:1 $CD25^+$:25^- ratio). Only cells purified using A gates suppress >95% of baseline $CD4^+CD25^-$ cell proliferation (*low panels*). As indicated by the dashed line, the A gate contains $CD4^+$ T cells that express CD25 more brightly than $CD4^-CD25^+$ cells. One healthy control of nine and one SLE patient of eight analyzed for natural Treg activity are presented (see summary of all results in *c* below). *b*, $CD4^+CD25^-$ (empty histograms) and $CD4^+CD25^{bright}$ cells (sorted using gate A; shaded histograms) from the same representative control (*left*) and patient (*right*) as above were simultaneously analyzed for their expression of membrane CCR4, CD95 (Fas), and intracellular CTLA-4 (iCTLA-4) using multicolor cytofluorometry. *c*, $CD4^+CD25^-$ cells were stimulated alone (□) or in the presence of different proportions of $CD4^+CD25^{bright}$ autologous T cells ($CD25^{bright}$:25^- ratios are indicated). As shown, nine healthy controls, four active SLE patients, and four inactive SLE were tested for natural Treg activity. *d*, $CD4^+CD25^{bright}$ cells from a representative control (*left*, $n = 9$) and from a representative active patient (*right*, $n = 4$) completely block secretion of IL-2, IL-5, IFN-γ, and TNF-α by autologous $CD4^+CD25^-$ T cells.

In SLE patients, $CD4^+CD25^{bright}$ T cells defined according to this criterium express high levels of other markers previously associated with human Tregs such as CCR-4 (45, 46), intracellular CTLA-4 (47), and CD95 (10, 48) (Fig. 1*b*).

Treg-suppressive function is not qualitatively impaired in SLE

$CD4^+CD25^{bright}$ T cells from healthy controls ($n = 9$), active SLE patients ($n = 4$), and inactive patients ($n = 4$) were FACS sorted, as described above, and tested for their ability to suppress autologous T cell proliferation and cytokine secretion. In all cases, purified cells inhibited at least 95% of baseline proliferation of autologous T lymphocytes (Fig. 1*c*) and abolished Th1 and Th2 cytokine secretion (Fig. 1*d*).

Contraction of the circulating Treg subset is correlated to disease activity

The percentage of circulating $CD4^+CD25^{bright}$ T cells among $CD4^+$ T cells was measured in healthy controls and SLE patients using cytofluorometry. As shown in Fig. 2*a*, a significant decrease in the mean value for patients with active SLE ($0.57 \pm 0.24\%$, $n = 45$, $p < 0.0001$) is evidenced when this group is compared with healthy controls ($1.29 \pm 0.38\%$, $n = 82$) or with inactive patients ($1.22 \pm 0.67\%$, $n = 62$, $p < 0.0001$). Within the active group, we found no significant difference in terms of Treg proportion between treated ($n = 34$) and untreated patients ($n = 11$; data not shown). No significant difference was observed between inactive SLE and healthy controls ($p = 0.07$). (See Fig. 1*a* for a representative staining pattern.) Off note, the decrease in Treg proportion among $CD4^+$ T cells that we observe indeed corresponds to a significant decrease in absolute numbers of such cells in active SLE patients. Treg absolute numbers are significantly decreased in active SLE patients when compared with controls (2.97 ± 2.1 cells/mm^3 vs 13.51 ± 5.3, $p < 0.0001$) or inactive SLE patients (7.33 ± 5.7 cells/mm^3, $p < 0.001$). We also found that the absolute number of Tregs in inactive SLE patients was lower than in controls ($p < 0.0001$). Because mild lymphopenia can often persist in inactive SLE (mean lymphocyte counts in our inactive patients: 1395 ± 657/mm^3 vs 2117 ± 699/mm^3 in controls), the absolute numbers of Tregs and of cells of other CD4 subsets are reduced in a parallel way, leading to a normal Treg proportion in inactive patients.

To determine whether such a contraction of the Treg subset is also found in other types of systemic autoimmune disorders, we then studied control groups including IM and pSS patients. The proportion of circulating $CD4^+CD25^{bright}$ T cells among $CD4^+$ T cells was not significantly different between IM patients ($1.15 \pm 0.46\%$, $n = 21$, $p = 0.09$) or pSS patients ($1.16 \pm 0.44\%$, $n = 16$, $p = 0.43$) and controls (Fig. 2*a*).

We next questioned whether Treg subset size would vary within the same individual in relation to disease status. In 10 individuals tested longitudinally, there was a significant increase in the latter subset size, which returned to control levels upon resolution of the flare ($0.39 \pm 0.20\%$ vs $1.28 \pm 0.39\%$, $p = 0.0005$; Fig. 2*b*). Moreover, we found a strong negative correlation between Treg subset size and the SLEDAI ($p = 0.0002$, $p = -0.58$; Fig. 2*c*).

These results argue strongly in favor of a Treg involvement in the disease process.

Tregs do not accumulate in SLE-involved organs or in lymphoid tissues

One possible explanation for the observed reduction in circulating Treg numbers during SLE flares is recruitment of these cells to lymphoid tissues and/or compartments with disease activity.

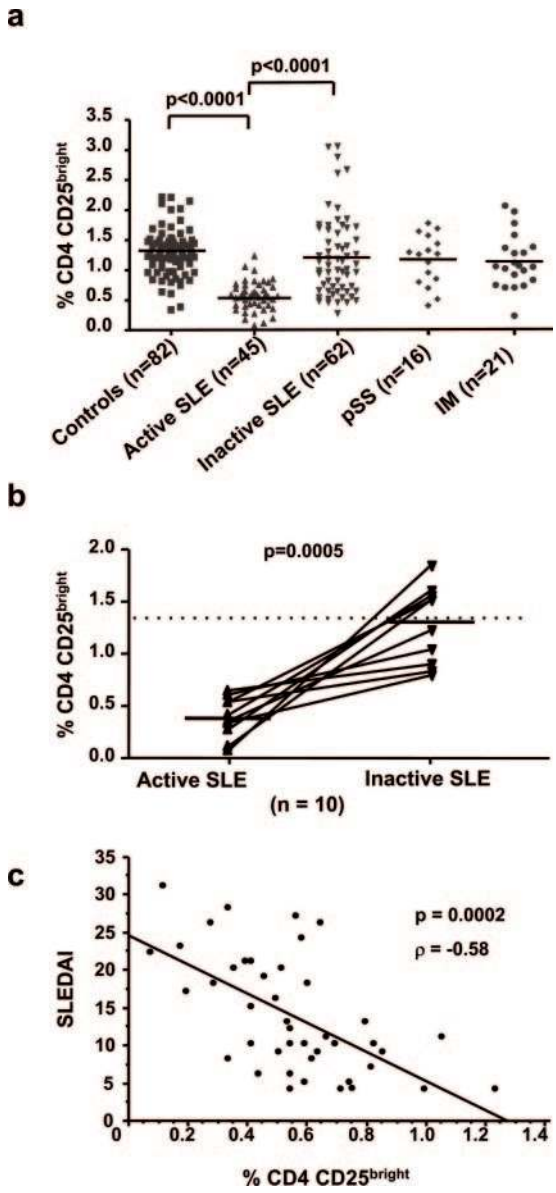

FIGURE 2. Contraction of the circulating Treg subset during disease flares. *a*, Transversal cytofluorometric analysis. Percentage of peripheral blood $CD4^+CD25^{bright}$ T cells in, as indicated, controls ($n = 82$), active SLE patients ($n = 45$), inactive SLE patients ($n = 62$), pSS patients ($n = 16$), and IM patients ($n = 21$). Horizontal lines represent mean levels for each group. *b*, Longitudinal monitoring of $CD4^+CD25^{bright}$ T cells in 10 patients. Percentage of peripheral blood $CD4^+CD25^{bright}$ cells was measured initially during SLE flare and following resolution (mean time between two measures: 8 mo $\pm$ 3.9). The horizontal dashed line represents the average percentage of $CD4^+CD25^{bright}$ T cells in healthy controls ($n = 82$). *c*, Negative correlation between proportion of $CD4^+CD25^{bright}$ cells among circulating $CD4^+$ T cells and clinical severity of the flare, scored using the SLEDAI ($n = 45$).

We first evaluated whether the decrease in circulating Tregs could be correlated with signs related to a particular organ involvement. We compared the proportion of $CD4^+CD25^{bright}$ cells in active SLE patients with kidney disease and in active SLE patients without kidney involvement. There is no significant difference between these two groups (kidney disease group, $0.533 \pm 0.213\%$, $n = 23$ and no kidney disease group, 0.61 ± 0.267, $n = 22$, $p = 0.335$). We also compared the proportion of Tregs in active SLE patients with and without

skin involvement. Neither was there a significant difference between the later (skin disease group, 0.581 ± 0.232%, $n = 17$ and no skin disease group, 0.565 ± 0.251, $n = 28$, $p = 0.66$). Finally, we compared the proportion of Tregs in active SLE patients with and without arthritis. Again, we did not find any statistically significant difference between these two groups (arthritis group, 0.586 ± 0.256, $n = 21$ and no arthritis group, 0.559 ± 0.233, $n = 24$, $p = 0.91$).

We next attempted to directly localize Tregs in tissues using sensitive techniques. Foxp3 is a transcription factor critical to the development of Tregs (25, 26) and to date considered the best marker available to define innate Tregs. We used bicolor microscopic analysis to localize $CD4^+Foxp3^+$ cells in tissues. We show that there are significantly less Tregs in SLE LN ($n = 5$) than in control LN (9.63 ± 3.74 cells/square millimeter vs 94.00 ± 39.20 cells/square millimeter, $n = 2$; $p < 0.0001$, Fig. 3*a*). We also studied kidney biopsies obtained from patients with lupus nephritis. Using real-time PCR, we found that *FOXP3* expression is not significantly increased in SLE kidneys ($n = 5$, Fig. 3*b*) compared with control kidney tissue ($n = 1$, Fig. 3*b*). Finally, FACS analysis of cells isolated from a spleen removed for refractory lupus thrombocytopenia evidenced only 0.63% of $CD4^+CD25^{bright}$ T cells among $CD4^+$ T cell splenocytes (data not shown).

Because we neither found any correlation between Treg decrease and signs of organ involvement nor any direct evidence for an accumulation of Tregs in disease-involved organs or in lymphoid tissues from active patients, we conclude that these cells are globally depleted during SLE flares.

Tregs of SLE patients are sensitive to Fas-dependent apoptosis

We hypothesized that a dysfunction in the control of Treg survival could lead to their depletion in vivo. It was shown previously that Fas (CD95) is constitutively expressed on Tregs (48). As shown above (Fig. 1*b*), we confirmed that SLE $CD4^+CD25^{bright}$ T cells also express high levels of Fas. We assessed the susceptibility of Tregs from active or inactive SLE patients and healthy controls to Fas-mediated apoptosis. Total PBMCs were labeled with anti-CD4 and anti-CD25 Abs and incubated for a short time period (12 h) in the presence of immobilized anti-CD3 Abs. Apoptosis was then induced by adding an anti-Fas mAb to the cultures for 1 h. Cells were then washed and $CD4^+CD25^{bright}$ T cells monitored for annexin V binding. We determined in preliminary experiments that, although fluorescence intensities declined, $CD4^+CD25^{bright}$ T cells remain detectable and that $CD4^+CD25^{bright}/CD4^+CD25^-$ ratios remain stable up until 16 h in culture medium (data not shown). In healthy controls, Tregs activated by anti-CD3 are less prone to Fas-dependent apoptosis than $CD4^+CD25^-$ T cells (2.04 ± 0.4% vs 5.05 ± 0.4%, $n = 24$, $p = 0.0003$, Fig. 4). In contrast, SLE Tregs are more susceptible to Fas-mediated apoptosis than autologous $CD4^+CD25^-$ T cells (6.52 ± 1.15%

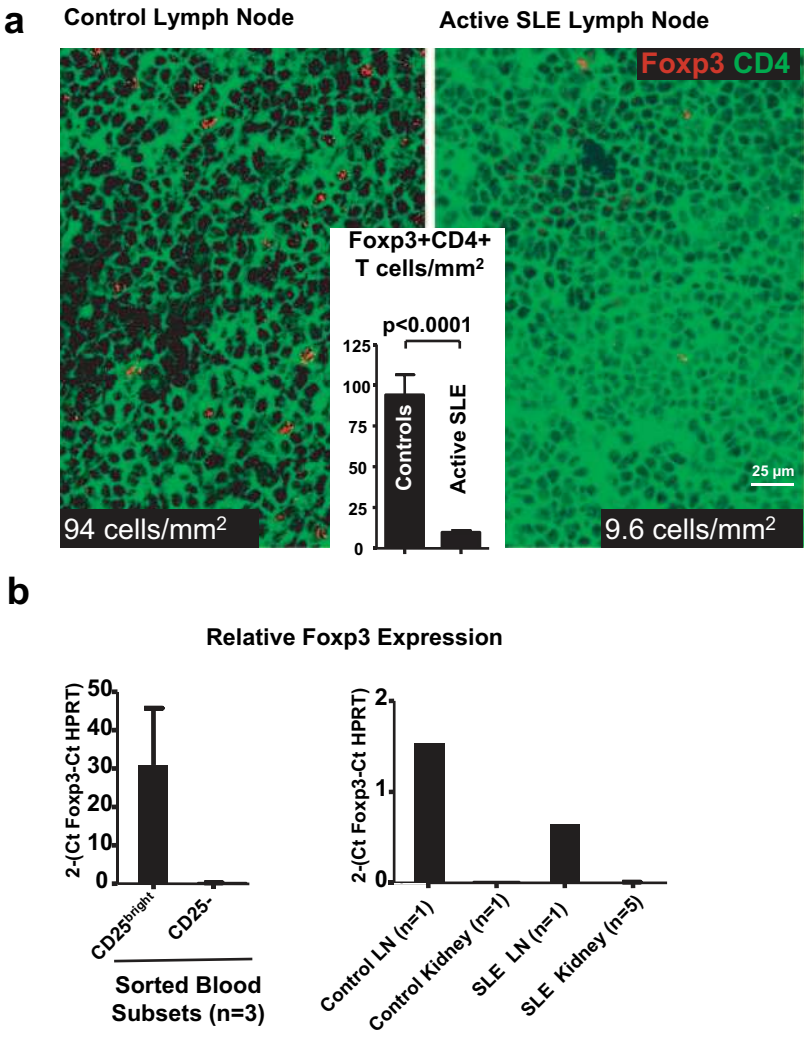

FIGURE 3. $FoxP3^+$ cells in tissues. *a*, Immunohistochemistry. Detection of $Foxp3^+CD4^+$ cells in LN samples taken from a representative control (of two analyzed) or from an active patient (of five analyzed) (magnification, ×250). The density of $Foxp3^+$ cells is indicated. For statistical analysis, $Foxp3^+$ cells were enumerated in three independent areas in each sample. *b*, Mean relative Foxp3 mRNA levels in indicated $CD4^+$ T cell subsets sorted from three healthy controls (*left panels*) and, as indicated, LN or kidney samples (*right panels*) from controls and patients. cDNA samples were subjected to real-time quantitative PCR analyses using primers and an internal fluorescent probe specific for Foxp3 or HPRT.

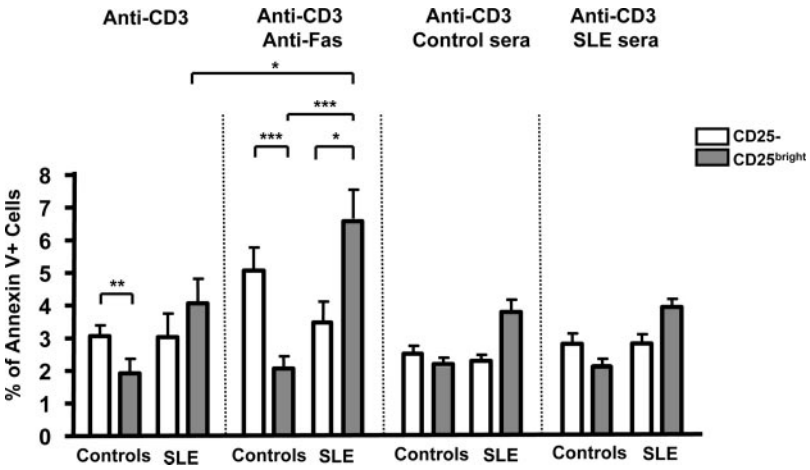

FIGURE 4. SLE Tregs are more sensitive to pas-mediated apoptosis than control Tregs. Sensitivity of indicated $CD4^+$ T cell subsets to activation-induced apotosis (*left panel*) and to Fas-mediated activation-induced apotosis (*right panel*). Freshly drawn PBMCs stained with anti-CD4 FITC and anti-CD25 PE were stimulated for 12 h with anti-CD3, then incubated with medium alone or with anti-Fas or with 1% control or active SLE sera for 1 h. Percentages of annexin V-binding cells in gated subsets were then measured using cytofluorometry (means ± SEM values obtained from healthy controls and SLE patients). Values of p were as follows: ***, ≤0.0005; **, <0.005; *, <0.05.

vs 3.56 ± 0.50%, $p = 0.013$, $n = 10$). Patients with inactive ($n = 5$) or active disease ($n = 5$) were both included in the test. Tregs of SLE patients are also more prone to Fas-mediated apoptosis than those of controls (6.52 ± 1.15% vs 2.04 ± 0.4%, $p = 0.0005$). In the absence of Fas stimulation, SLE Tregs do not engage significantly faster in activation-induced apoptosis than autologous $CD4^+CD25^-$ T cells.

It was recently demonstrated that SLE patients can produce anti-T cell Abs that could possibly affect T cell metabolism (49). To determine whether Treg depletion could result from the effect of circulating autoantibodies, we conducted additional apoptosis assays using control or active SLE serum. Under these conditions, Treg apoptosis was not enhanced, neither in controls (2.07 ± 0.27%, $n = 6$ with SLE sera vs 1.93 ± 0.42%, $n = 24$ without serum, $p = 0.13$) nor in patients (3.95 ± 0.2%, $n = 9$ with SLE sera vs 4.06 ± 0.75%, $n = 10$ without serum, $p = 0.51$).

These results suggest that exacerbated Fas-mediated apoptosis susceptibility could lead to Treg depletion during SLE flares independently of a soluble factor.

Discussion

The pathogenesis of SLE is largely unknown, and a common mechanism that would be responsible for the combined tissue injuries that occur periodically in these patients has not been elucidated. In this study, we report that all active patients studied ($n = 45$) presented not only with a decreased proportion of Tregs among $CD4^+$ T cells, but also with decreased absolute number of the later cells, as compared with mean values obtained from healthy controls. The decrease in circulating Treg numbers corresponds to a true global depletion, as these cells were not found redistributed to sites of disease activity or to lymphoid organs. We further linked this anomaly to clinical outcomes by demonstrating that Treg subset size varies within the same individual in relation to disease activity. More importantly, we demonstrate an inverse correlation between the proportion of circulating Tregs among other $CD4^+$ T cells and severity of the disease flare, independently of any particular organ involvement.

Although the probable implication of Tregs in diverse pathologies has generated enormous interest (17–24, 32–34), it remains difficult to purify them in humans, and even more so in leukopenic subjects such as SLE patients. Tregs are relatively easy to characterize in mice given that they correspond to the $CD4^+$ T lymphocytes that constitutively express CD25. However, in humans, a large fraction of $CD25^+$ cells are activated cells, and the majority of these are nonregulatory. Previous publications have reported the existence of quantitative anomalies (35, 36) within the Treg subpopulation over the course of SLE. These studies could only give rise to speculative conclusions, as they were based on peripheral blood phenotypic analysis, omitting functional assays and the study of other tissues. Another group reported that Tregs would be functionally impaired during SLE flares (37). The latter study used for reference surprisingly elevated numbers of Tregs in control subjects (up to 10% of $CD4^+$ T cells), raising the possibility that the studied subsets could have been highly contaminated with $CD25^+$ non-Tregs. In the adult, the Treg subset represents a mere 1.5% of the total $CD4^+$ T lymphocyte population (13). Any study using for reference significantly more elevated numbers (20, 23, 24, 50) should be interpreted cautiously. It is shown in this study how Tregs could appear poorly functional in vitro if they are contaminated by $CD4^+CD25^{low}$ lymphocytes. Therefore, if Treg purification is not as stringent as possible, the latter can be erroneously evaluated as being less functional. For the purposes of this study, analysis and sorting gates were defined to select only those cells that express CD25 most strongly. In control experiments, we used a single cell PCR approach to calculate precisely the fraction of $Foxp3^+$ cells present among cells sorted according to criteria presented in this work (Fig. 1). Although virtually no $CD4^+CD25^-$ cells expressed Foxp3, we determined by this approach that, on average, 86.5% of the sorted $CD4^+CD25^{bright}$ cells were $Foxp3^+$ (M. Miyara, Z. Amoura, and G. Gorochov, submitted for publication).

The hallmark of SLE is the production of anti-dsDNA Abs (51). Anti-dsDNA are found not only in the serum, but also in the diseased kidneys of SLE patients, suggesting a pathogenic role for these autoantibodies. Although there is now ample evidence that Tregs can regulate T cell-mediated responses, their role on the humoral response has been less explored. In a recent study (52), it was shown that a depletion or a lack of recruitment of Tregs to B cells and APCs resulted in a deregulated humoral response. A direct role of Tregs on B cells was evidenced in the same study because the authors further showed that Tregs can suppress LPS-induced B cell activation. More recently, it was shown in a mouse model that the anergy of anti-dsDNA B cells is reverted when T cell help is provided in the absence of Tregs (53). In light of these studies, our own data strongly suggest that the loss of Tregs during SLE flares could contribute to a lack of control on autoreactive B cells and therefore to the pathogenesis of SLE. Our findings are also in good agreement with the initial description of Sakaguchi et al. (1), in which mice depleted in $CD25^+CD4^+$ T cells developed multisystemic autoimmune

features, including arthritis, glomerulonephritis, and anti-dsDNA Abs. Finally, it is proposed that depleting Tregs would represent a potential strategy for treating human cancers (45, 54, 55). Our data indirectly support this assertion, but also indicate that such potential therapies might be at the origin of adverse autoimmune side effects.

The specificity of Tregs remains unknown, but it is probable that they express TCRs with relatively high affinity for self Ags (56). SLE has been shown in numerous studies to be associated with reduced elimination of apoptotic cells (57, 58). It is proposed that dysfunction of rapid dead cell clearance leads to a superexposure of self Ags and to the expansion of a subpopulation of self-directed effector cells, the resulting imbalance ultimately leading to a loss of immune tolerance. To check the expansion of self-directed effector cells, it appears important that the immune system could maintain, or if necessary, amplify the level of the Treg subpopulation. Over the course of a graft-vs-host reaction, for example, patients appear capable of amplifying their Treg subpopulation in an appropriate manner (59). In the case of SLE, in contrast, the homeostatic control mechanisms of the Treg population appear to be profoundly perturbed.

We have attempted to explore at least two mechanisms that could affect Treg survival in SLE patients. We found no evidence for the presence of autoantibodies that would directly target Tregs, and therefore focused on the study of Tregs' programmed cell death. Murine Tregs are relatively resistant to apoptosis (38). DNA array analyses identified in this subset a moderate up-regulation of several genes linked to cell survival (60) that could explain this behavior. In agreement with our own data, Taams et al. (10) reported that although human Tregs expressed high amounts of CD95, they were not particularly susceptible to CD95-mediated activation-induced cell death. The same authors reported that Tregs were more sensitive to cytokine deprivation-induced cell death (10), but this result was not confirmed by others (23). In this study, to avoid introducing bias such as cell proliferation and cell lysis, we only monitored early apoptotic events taking place after 13 h of culture (only 1 h in the presence of anti-Fas). We confirm that control human Tregs are not more prone to CD95-mediated activation-induced cell death than $CD25^+CD4^+$ T cells. In contrast, we show that lupus Tregs are hypersensitive to Fas-mediated cell death. We propose, therefore, that Tregs would be eliminated in vivo via inappropriate induction of apoptosis following massive exposure to self Ags arising during the course of lupus flares.

It is impossible to conclude at present whether the observed global depletion of Tregs is in fact the cause or consequence of the flare. In either case, this depletion could only exacerbate the extent of tissue damage. It would be of interest to determine whether the Tregs isolated from lupus patients express abnormal levels of the antiapoptotic factors already described (60). It would be equally interesting to determine whether this abnormal propensity for Fas-induced apoptosis can be reversed after in vivo expansion. Such manipulations could very well offer new therapeutic perspectives for the management of patients suffering from SLE (61).

We have previously described that active sarcoidosis (M. Miyara, Z. Amoura, and G. Gorochov, submitted for publication) is associated with a very significant Treg expansion, not only in blood, but also in involved organs. In this study, we conclude that the same subset is globally depleted during lupus flares. It is interesting to note that these two clinical entities, presenting with opposite immunoregulatory features (anergy vs systemic autoimmunity), rarely occur together (62).

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Disclosures

The authors have no financial conflict of interest.

References

1. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
2. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
3. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
4. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol. Rev.* 182: 207–214.
5. Shevach, E. M. 2002. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
6. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2: 816–822.
7. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
8. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431–440.
9. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. *J. Exp. Med.* 193: 1303–1310.
10. Taams, L. S., J. Smith, M. H. Rustin, M. Salmon, L. W. Poulter, and A. N. Akbar. 2001. Human anergic/suppressive $CD4^+CD25^+$ T cells: a highly differentiated and apoptosis-prone population. *Eur. J. Immunol.* 31: 1122–1131.
11. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.
12. Ng, W. F., P. J. Duggan, F. Ponchel, G. Matarese, G. Lombardi, A. D. Edwards, J. D. Isaacs, and R. I. Lechler. 2001. Human $CD4^+CD25^+$ cells: a naturally occurring population of regulatory T cells. *Blood* 98: 2736–2744.
13. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. *J. Immunol.* 167: 1245–1253.
14. Annunziato, F., L. Cosmi, F. Liotta, E. Lazzeri, R. Manetti, V. Vanini, P. Romagnani, E. Maggi, and S. Romagnani. 2002. Phenotype, localization, and mechanism of suppression of $CD4^+CD25^+$ human thymocytes. *J. Exp. Med.* 196: 379–387.
15. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* 193: 1295–1302.
16. Stephens, L. A., C. Mottet, D. Mason, and F. Powrie. 2001. Human $CD4^+CD25^+$ thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur. J. Immunol.* 31: 1247–1254.
17. Huang, Y., R. Pirskanen, R. Ciscombe, H. Link, and A. K. Lefvert. 2003. Circulating $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in myasthenia gravis. *Ann. NY Acad. Sci.* 998: 318–319.
18. Sun, Y., J. Qiao, C. Z. Lu, C. B. Zhao, X. M. Zhu, and B. G. Xiao. 2004. Increase of circulating $CD4^+CD25^+$ T cells in myasthenia gravis patients with stability and thymectomy. *Clin. Immunol.* 112: 284–289.
19. Salama, A. D., A. N. Chaudhry, K. A. Holthaus, K. Mosley, R. Kalluri, M. H. Sayegh, R. I. Lechler, C. D. Pusey, and L. Lightstone. 2003. Regulation by $CD25^+$ lymphocytes of autoantigen-specific T-cell responses in Goodpasture's (anti-GBM) disease. *Kidney Int.* 64: 1685–1694.
20. Lindley, S., C. M. Dayan, A. Bishop, B. O. Roep, M. Peakman, and T. I. Tree. 2005. Defective suppressor function in $CD4^+CD25^+$ T-cells from patients with type 1 diabetes. *Diabetes* 54: 92–99.
21. Viglietta, V., C. Baecher-Allan, H. L. Weiner, and D. A. Hafler. 2004. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* 199: 971–979.
22. Ehrenstein, M. R., J. G. Evans, A. Singh, S. Moore, G. Warnes, D. A. Isenberg, and C. Mauri. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. *J. Exp. Med.* 200: 277–285.

23. Kriegel, M. A., T. Lohmann, C. Gabler, N. Blank, J. R. Kalden, and H. M. Lorenz. 2004. Defective suppressor function of human $CD4^+CD25^+$ regulatory T cells in autoimmune polyglandular syndrome type II. *J. Exp. Med.* 199: 1285–1291.
24. Sugiyama, H., R. Gyulai, E. Toichi, E. Garaczi, S. Shimada, S. R. Stevens, T. S. McCormick, and K. D. Cooper. 2005. Dysfunctional blood and target tissue $CD4^+CD25^{high}$ regulatory T cells in psoriasis: mechanism underlying unrestrained pathogenic effector T cell proliferation. *J. Immunol.* 174: 164–173.
25. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
26. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
27. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for scurfin in $CD4^+CD25^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
28. Fontenot, J. D., and A. Y. Rudensky. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat. Immunol.* 6: 331–337.
29. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
30. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27: 20–21.
31. Wildin, R. S., S. Smyk-Pearson, and A. H. Filipovich. 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J. Med. Genet.* 39: 537–545.
32. De Kleer, I. M., L. R. Wedderburn, L. S. Taams, A. Patel, H. Varsani, M. Klein, W. de Jager, G. Pugayung, F. Giannoni, G. Rijkers, et al. 2004. $CD4^+CD25^{bright}$ regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J. Immunol.* 172: 6435–6443.
33. Cao, D., V. Malmstrom, C. Baecher-Allan, D. Hafler, L. Klareskog, and C. Trollmo. 2003. Isolation and functional characterization of regulatory $CD25^{bright}CD4^+$ T cells from the target organ of patients with rheumatoid arthritis. *Eur. J. Immunol.* 33: 215–223.
34. Cao, D., R. van Vollenhoven, L. Klareskog, C. Trollmo, and V. Malmstrom. 2004. $CD25^{bright}CD4^+$ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. *Arthritis Res. Ther.* 6: R335–R346.
35. Crispin, J. C., A. Martinez, and J. Alcocer-Varela. 2003. Quantification of regulatory T cells in patients with systemic lupus erythematosus. *J. Autoimmun.* 21: 273–276.
36. Liu, M. F., C. R. Wang, L. L. Fung, and C. R. Wu. 2004. Decreased $CD4^+CD25^+$ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand. J. Immunol.* 59: 198–202.
37. Valencia, X., L. S. He, G. Illei, and P. Lipsky. 2002. $CD4^+CD25^+$ T regulatory cells in systemic lupus erythematosus. *Arthritis Rheum.* 46: 3404 (Abstr. SY3409).
38. Banz, A., C. Pontoux, and M. Papiernik. 2002. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. *J. Immunol.* 169: 750–757.
39. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25: 1271–1277.
40. Hochberg, M. C. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 40: 1725.
41. Bombardier, C., D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang. 1992. Derivation of the SLEDAI: a disease activity index for lupus patients: The Committee on Prognosis Studies in SLE. *Arthritis Rheum.* 35: 630–640.
42. Bohan, A., J. B. Peter, R. L. Bowman, and C. M. Pearson. 1977. Computer-assisted analysis of 153 patients with polymyositis and dermatomyositis. *Medicine* 56: 255–286.
43. Vitali, C., S. Bombardieri, R. Jonsson, H. M. Moutsopoulos, E. L. Alexander, S. E. Carsons, T. E. Daniels, P. C. Fox, R. I. Fox, S. S. Kassan, et al. 2002. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann. Rheum. Dis.* 61: 554–558.
44. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188: 287–296.
45. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
46. Iellem, A., L. Colantonio, and D. D'Ambrosio. 2003. Skin-versus gut-skewed homing receptor expression and intrinsic CCR4 expression on human peripheral blood $CD4^+CD25^+$ suppressor T cells. *Eur. J. Immunol.* 33: 1488–1496.
47. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303–310.
48. Wing, K., A. Ekmark, H. Karlsson, A. Rudin, and E. Suri-Payer. 2002. Characterization of human $CD25^+$ $CD4^+$ T cells in thymus, cord and adult blood. *Immunology* 106: 190–199.
49. Juang, Y. T., Y. Wang, E. E. Solomou, Y. Li, C. Mawrin, K. Tenbrock, V. C. Kyttaris, and G. C. Tsokos. 2005. Systemic lupus erythematosus serum IgG increases CREM binding to the IL-2 promoter and suppresses IL-2 production through CaMKIV. *J. Clin. Invest.* 115: 996–1005.
50. Boyer, O., D. Saadoun, J. Abriol, M. Dodille, J. C. Piette, P. Cacoub, and D. Klatzmann. 2004. $CD4^+CD25^+$ regulatory T-cell deficiency in patients with hepatitis C-mixed cryoglobulinemia vasculitis. *Blood* 103: 3428–3430.
51. Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44: 93–151.
52. Bystry, R. S., V. Aluvihare, K. A. Welch, M. Kallikourdis, and A. G. Betz. 2001. B cells and professional APCs recruit regulatory T cells via CCL4. *Nat. Immunol.* 2: 1126–1132.
53. Seo, S. J., M. L. Fields, J. L. Buckler, A. J. Reed, L. Mandik-Nayak, S. A. Nish, R. J. Noelle, L. A. Turka, F. D. Finkelman, A. J. Caton, and J. Erikson. 2002. The impact of T helper and T regulatory cells on the regulation of anti-double-stranded DNA B cells. *Immunity* 16: 535–546.
54. Steitz, J., J. Bruck, J. Lenz, J. Knop, and T. Tuting. 2001. Depletion of $CD25^+$ $CD4^+$ T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon α-induced, $CD8^+$ T-cell-dependent immune defense of B16 melanoma. *Cancer Res.* 61: 8643–8646.
55. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163: 5211–5218.
56. Schwartz, R. H. 2005. Natural regulatory T cells and self-tolerance. *Nat. Immunol.* 6: 327–330.
57. Herrmann, M., R. E. Voll, O. M. Zoller, M. Hagenhofer, B. B. Ponner, and J. R. Kalden. 1998. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum.* 41: 1241–1250.
58. Perniok, A., F. Wedekind, M. Herrmann, C. Specker, and M. Schneider. 1998. High levels of circulating early apoptotic peripheral blood mononuclear cells in systemic lupus erythematosus. *Lupus* 7: 113–118.
59. Clark, F. J., R. Gregg, K. Piper, D. Dunnion, L. Freeman, M. Griffiths, G. Begum, P. Mahendra, C. Craddock, P. Moss, and R. Chakraverty. 2004. Chronic graft-versus-host disease is associated with increased numbers of peripheral blood $CD4^+CD25^{high}$ regulatory T cells. *Blood* 103: 2410–2416.
60. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells in vivo. *Nat. Immunol.* 3: 33–41.
61. Vigouroux, S., E. Yvon, E. Biagi, and M. K. Brenner. 2004. Antigen-induced regulatory T cells. *Blood* 104: 26–33.
62. Wallace, D. 2002. Differential diagnosis and disease association. In *Dubois' Lupus Erythematosus*, 6th Ed. B. Hahn, ed. Lippincott Williams & Wilkins, Philadelphia, pp. 959–983.