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Michael Bonelli, Anastasia Savitskaya, Carl-Walter Steiner, Eva Rath, Josef S. Smolen, and Clemens Scheinecker²

CD4⁺CD25−Foxp3+ regulatory T cells (Treg) that specialize in the suppression of immune responses might be critically involved in the pathogenesis of autoimmune diseases. Recent studies have described increased proportions of CD4⁺Foxp3⁺ T cells that lacked expression of CD25 in systemic lupus erythematosus (SLE) patients but the suppressive capacity of these cells has not been analyzed so far. We therefore performed combined phenotypic and functional analyses of CD4⁺CD25−Foxp3+ T cells in patients with autoimmune diseases and healthy controls (HC). Phenotypic analysis revealed increased proportions of CD4⁺CD25−Foxp3+ T cells in SLE patients as compared with patients with systemic sclerosis, rheumatoid arthritis, (RA), or HC. In addition, increased proportions of CD4⁺CD25−Foxp3+ T cells correlated with the clinical disease activity and the daily cortisone dose. According to phenotypic analysis, CD4⁺CD25−Foxp3+ T cells resembled regulatory T cells rather than activated T cells. For functional analysis, a surrogate surface marker combination to substitute for intracellular Foxp3 was defined: CD4⁺CD25⁺CD127− T cells from SLE patients were isolated by FACS sorting and analyzed for their suppressive capacity in vitro. CD4⁺CD25⁺CD127− T cells, that contained up to 53% Foxp3⁺ T cells, were found to suppress T cell proliferation but not IFN-γ production in vitro. In summary, CD4⁺CD25−Foxp3+ T cells phenotypically and to a certain extent also functionally resemble conventional Treg. Despite increased proportions, however, their selective functional defects might contribute to the failure of Treg to control autoimmune dysregulation in SLE patients. The Journal of Immunology, 2009, 182: 1689–1695.


The CD4⁺CD25⁺ regulatory T cells (Treg)³ constitute on average 1–2% of human PBMC and are characterized by their capacity to actively suppress T cell proliferation in vitro (1). Treg play an important role in T cell homeostasis and are critical regulators of immune tolerance. Quantitative and/or qualitative deficiencies of Treg have been suggested to contribute to the development of autoimmune diseases (2–7).

Treg are characterized by the expression of the IL-2 receptor α-chain (CD25) (3). In contrast to the mouse, the suppressive capacity of human Treg has been shown to be restricted to CD4⁺ T cells with the highest expression levels of CD25, whereas CD4⁺ T cells with intermediate expression of CD25 might also contain activated T cells (1). In addition to CD4⁺CD25⁺ Treg, also other types of T cells with regulatory function including TGF-β-producing Th3 cells, IL-10-producing Tr1 cells, CD8⁺CD28− T cells, γδ T cells, and NKT cells have been described previously (8).

More recently, the forkhead family transcription factor (Foxp3) has been described as a highly specific intracellular marker molecule for Treg. In addition, Foxp3 was found to be necessary for the development and function of naturally occurring Treg (9–11). Mutations in the Foxp3 gene have been shown to result in autoimmune manifestations in the Scurfy mouse and the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans (12). In contrast to the mouse system, transient up-regulation of Foxp3 expression was observed in human T cells upon activation and conflicting data have been published concerning the suppressive capacity of T cells with transient Foxp3 expression (13–18).

We have previously reported increased proportions of CD4⁺Foxp3⁺ T cells but decreased proportions of CD4⁺CD25high T cells in patients with systemic lupus erythematosus (SLE) as compared with healthy controls (HC) (7). Since increased levels of activated T cells due to chronic T cell stimulation can be detected in SLE patients (19), increased proportions of CD4⁺Foxp3⁺ T cells might reflect activated T cells. In line with this, proportions of CD4⁺Foxp3⁺ T cells were found to correlate with clinical scores of disease activity. Interestingly, however, further analyses revealed that a substantial proportion of CD4⁺Foxp3⁺ cells did not express CD25 (CD4⁺CD25−Foxp3+).

This tempted us to address the question of whether CD4⁺CD25−Foxp3⁺ T cells in SLE patients represent activated T cells or Treg. We performed detailed comparative phenotypic analyses of CD4⁺CD25−Foxp3+ and CD4⁺CD25⁺ Foxp3+ from SLE patients and HC and used a combination of surface marker molecules to substitute for Foxp3 which allowed us to isolate these cells for functional studies. Based on our data, we conclude that CD4⁺CD25⁺ Foxp3+ in SLE patients share several properties with conventional Treg but on the other hand are distinct in certain functional qualities.

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³Abbreviations used in this paper: Treg, regulatory T cell; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; SSc, systemic sclerosis; HC, healthy control; SLEDAI, SLE disease activity index; Cy, cyamin; GITR, glucocorticoid-induced TNFR.

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18 U.S.C. Section 1734
Materials and Methods

Patients and controls

Twenty-two SLE patients (mean age, 45 ± 16.8 years) who fulfilled at least four of the revised SLE criteria of the American College of Rheumatology (20, 20 patients with rheumatoid arthritis (RA); mean age, 56 ± 3.2 years), and 5 patients with systemic sclerosis (SSc; mean age, 61 ± 4.1 years) were randomly selected from our outpatient clinic. Healthy volunteers served as an HC (mean age, 32 ± 6.7 years) population (n = 15). The disease activity of SLE patients was assessed using the SLE disease activity index (SLEDAI) (21). Patients were divided into two groups according to their disease activity. A SLEDAI score ≥6 was defined as high disease activity.

Ethical approval for this study was granted by the local ethics committee and informed consent was obtained.

Antibodies

mAb targeting the following molecules were used unlabeled or as FITC, PE, PerCP, allophycocyanin, PE-cyanin (Cy)-5.5, PE-Cy5.5, and PE-cyanin-n 7 (PE-Cy7) conjugates: CD3 (SK7; IOT3), CD4 (SK3), CD25 (2A3), CD62L (SK11), CD71 (M-A712), CD127 (human IL-7R-M21), CTLA-4 (BNI3), and CD69 (CH4), all from BD Biosciences; CD45RO (30-F11) from Serotec; HLA-DR (B8.12.2), CD95 (UB2), CD103 (2G5), and CD14 (RMO52) from Beckman Coulter; mAb against Foxp3 (PCH101) and CD27 (0323) were obtained from eBioscience; and anti-glucocorticoid-induced TNFR (GITR; 110416) was obtained from R&D Systems. In all experiments, control mAb of the respective IgG isotypes were included.

Phenotypic analysis

PBMC were isolated from heparinized blood by Lymphocyte Separation Medium (PAA Laboratories) density gradient centrifugation. PBMC were resuspended in PBS/3% human IgG (Baxter International) to block Fc receptors and prevent nonspecific Ab binding and were incubated for 15 min at 4°C in the dark with combinations of FITC, PE, PE-Cy5.5, PE-Cy7, and allophycocyanin-conjugated mAb. Afterward the cells were washed with PBS/1% BSA. Intracellular staining for Foxp3 was performed according to the instructions of the manufacturer. Background fluorescence was assessed using appropriate isotype- and fluorochrome-matched control mAbs.

Cell cultures

Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 100 U/ml penicillin/100 µg/ml streptomycin, 0.5 mM sodium pyruvate, MEM, 0.05 mM nonessential amino acids, 2 mM l-glutamine, 10 mM HEPES (all from Invitrogen), as well as 10% heat-inactivated FCS (Autogen Bioclear) in 96-well U-bottom plates (Costar) in a humidified CO₂-containing atmosphere at 37°C.

Cell separation

CD4⁺CD25⁺CD127⁻, CD4⁺CD25⁺CD127⁺, and CD4⁺CD25⁻CD127⁺ T cells from five HC and five SLE patients were isolated by flow cytometric cell sorting on a FACS Aria (BD Biosciences). PBMC were stained with mAb against CD4, CD25, CD14, and CD127 for 20 min at 4°C. The sort gates were additionally restricted to a lymphocyte gate as determined by typical forward and side scatter characteristics. CD4⁺CD25⁺CD127⁻, CD4⁺CD25⁺CD127⁺, and CD4⁺CD25⁺CD127⁺ T cells were enriched to a purity of an average over 98%.

Functional assays

For the assessment of T cell proliferation, FACS-sorted CD4⁺CD25⁺CD127⁻, CD4⁺CD25⁺CD127⁺, and CD4⁺CD25⁻CD127⁺ from SLE patients were stimulated with soluble anti-CD3 mAb (100 ng/ml) in U-bottom 96-well plates for 72 h. Supernatants were collected and analyzed for IFN-γ, IL-2, IL-10, and TGF-β with commercially available Flow Cytomix Multiplex Kits (Bender MedSystems) according to the manufacturer’s instructions.

Statistical analysis

Values are shown throughout the manuscript as mean ± SEM except of the patients and HC age, which is shown as mean ± SD. Proportions of lymphocyte subpopulations were compared using Student’s t test for normally distributed populations. Relationships between different results were examined using Pearson’s correlation coefficient and Spearman’s rank correlation test. A value of p < 0.05 was considered significant in all statistical tests. The p values were corrected for multiple analyses. All statistical analyses were performed using GraphPad Prism (version 4.0) and SPSS (version 12.0).

Results

Increased proportions of CD4⁺CD25⁺Foxp3⁺ T cells in SLE patients and correlation with disease activity, treatment, and age

Freshly isolated PBMC from HC and patients with RA, SSc, and SLE were analyzed by FACS for proportions of Treg. In addition to CD4⁺CD25⁺Foxp3⁺ T cells, we consistently observed a population of CD4⁺Foxp3⁺ T cells that virtually lacked the expression of CD25 in patients with SLE, but not in HC or in patients with RA or SSc. As shown in Fig. 1, proportions of CD4⁺CD25⁻Foxp3⁺ T cells were significantly increased in patients with SLE (7.5 ± 1%) as compared with HC (1.4 ± 0.4%) or patients with RA (2.2 ± 0.2%) and SSc (2.5 ± 0.7%).

To determine whether the appearance of CD4⁺CD25⁺Foxp3⁺ T cells is linked to a higher clinical disease activity, as we have recently shown for the whole Foxp3⁺ T cell population in SLE (7), patients were divided into two groups according to their disease activity scores. When compared with HC (1.4 ± 0.4%), significantly higher proportions of CD4⁺CD25⁺Foxp3⁺ T cells were observed in PBMC from inactive SLE patients (6.1 ± 0.8%) and even more pronounced from active SLE patients (10.5 ± 5.4%) (Fig. 2a). In line with this finding, a significant correlation was observed for the percentage of CD4⁺CD25⁺Foxp3⁺ T cells with clinical disease activity scores (r = 0.7, p = 0.001; Fig. 2b). In addition, however, we also observed a significant correlation with the daily corticosteroid dose (r = 0.4, p = 0.04; Fig. 2c) but not with other immunomodulatory therapies (data not shown). In contrast, no significant correlation was observed for proportions of CD4⁺CD25⁺Foxp3⁺ T cells with the daily corticosteroid dose in patients with RA (data not shown).

In addition, no significant correlation was observed for proportions of CD4⁺CD25⁺Foxp3⁺ T cells and patient’s age (r = 0.09, p = 0.7; Fig. 2d).

Phenotypic characterization of CD4⁺CD25⁺Foxp3⁺ T cells in SLE patients and HC

CD4⁺CD25⁺Foxp3⁺ T cells in SLE patients might represent Treg without CD25 expression or activated, non-Treg with transient Foxp3 expression, as described previously (22, 23). To address this question, we performed further comparative phenotypic analyses of CD4⁺CD25⁺Foxp3⁺, CD4⁺CD25⁻Foxp3⁺, and CD4⁺CD25⁺Foxp3⁺ T cells from HC and SLE patients (Fig. 3). No significant differences were observed for the expression of early (CD69) and late (CD71, HLA-DR) T cell activation-associated marker molecules between CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺Foxp3⁺ T cells from SLE patients and HC (Table I).
Moreover, a similar expression pattern was observed for both CD4+CD25+Foxp3+ T cells and CD4+CD25+Foxp3+ T cells from SLE patients concerning the expression of several surface and intracellular marker molecules that have been described to be associated with a Treg phenotype, such as CD62L, CD95, GITR, CTLA-4, and CD127 (Table I). In contrast, significant differences were observed between the three cell populations and CD4+CD25+Foxp3+ T cells that presumably contain activated T cells in SLE patients, concerning the expression of Treg-associated marker molecules. In addition, HLA-DR was also found to be differentially expressed in this cell population. In summary, the results support a Treg phenotype of CD4+CD25+Foxp3+ T cells in SLE patients.

Enrichment of Foxp3+ T cells in CD4+CD25+CD127+ T cells: isolation and functional characterization

Because CD4+CD25+Foxp3+ resembled Treg rather than activated T cells according to the phenotypic analyses described above, we next sought to determine their suppressive capacity in vitro. Since the intracellular expression of Foxp3 prevents the direct isolation of CD4+CD25+Foxp3+ T cells, we first developed a surrogate surface marker profile for subsequent FACS sorting experiments. When we evaluated various cell populations characterized by cell surface markers for their Foxp3 expression, several Treg-associated surface marker molecules including CD27, CD28, CD30, CD45RO, CD62L, CD95, CD103, and GITR failed to characterize T cells expressing Foxp3. However, in line with the recent results support a Treg phenotype of CD4+CD25+Foxp3+ T cells in SLE patients.

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observation that CD4⁺Foxp3⁺ Treg lack the expression of the IL-7 receptor α-chain CD127 (24, 25), 75 ± 2.5% of CD4⁺CD25⁺CD127⁻ T cells, as opposed to only 2.4 ± 1% of CD4⁺CD25⁺CD127⁺, express Foxp3. Moreover, also among CD4⁺CD25⁺CD127⁻ T cells up to 53% (mean, 35.7 ± 4.8%) expressed Foxp3. Therefore, CD4⁺CD25⁺CD127⁻ T cells were used as a surrogate population for CD4⁺CD25⁺Foxp3⁺ T cells in the subsequent functional experiments. All of these cell populations, CD4⁺CD25⁺CD127⁻ T cells, CD4⁺CD25⁺CD127⁺ T cells serving as a positive control population, and CD4⁺CD25⁻CD127⁺ T cells representing the responder T cell population, were isolated by FACS sorting (Fig. 4b).

CD4⁺CD25⁺CD127⁻ T cells in SLE patients are anergic and suppress T cell proliferation but not IFN-γ production

First, CD4⁺CD25⁺CD127⁻, CD4⁺CD25⁺CD127⁺ and CD4⁺CD25⁻CD127⁺ from SLE were analyzed for their proliferative capacity. As expected, CD4⁺CD25⁺CD127⁻ Treg were found to be anergic as opposed to CD4⁺CD25⁺CD127⁺ T cells. In addition, a comparable low proliferative response was observed for CD4⁺CD25⁺CD127⁻ T cells (see Fig. 5a). For the assessment of their suppressive capacity, CD4⁺CD25⁺CD127⁻ T cells from SLE patients were cocultured with responder T cells (CD4⁺CD25⁺CD127⁺ T cells) at a 1:1 ratio. CD4⁺CD25⁺CD127⁻ served as positive control population and were used at the same ratio. As shown in Fig. 5b, CD4⁺CD25⁺CD127⁻ from SLE patients were capable of suppressing the anti-CD3-induced proliferation of the responder T cell population by 54 ± 13% as opposed to 76 ± 1% in HC. Interestingly, the CD4⁺CD25⁺CD127⁻ T cell population of SLE patients also induced a sizeable suppression of the proliferative activity of responder cells, namely, 41 ± 12%. Thus, when compared with the suppressive capacity of the CD4⁺CD25⁺CD127⁻ cells, the suppression conveyed by CD4⁺CD25⁺CD127⁻ T cells amounted to

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<th>CD4⁺CD25⁺Foxp3⁺ HC</th>
<th>CD4⁺CD25⁺Foxp3⁺ SLE</th>
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<tr>
<td>% CD69</td>
<td>5.7 ± 1.9</td>
<td>3.1 ± 0.18</td>
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<td>% CD71</td>
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<td>21.1 ± 2.3</td>
<td>17 ± 2.2</td>
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<td>% HLA-DR</td>
<td>17.7 ± 4.3</td>
<td>11.4 ± 1.6</td>
<td>16.7 ± 2*</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>% CD27</td>
<td>90.3 ± 2.2</td>
<td>89.3 ± 2.5</td>
<td>80 ± 3.1</td>
<td>81.6 ± 2.4</td>
</tr>
<tr>
<td>% CD45RO</td>
<td>62.8 ± 5.9</td>
<td>91 ± 4.5*</td>
<td>76.8 ± 3</td>
<td>58.6 ± 6.3</td>
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<tr>
<td>% CD62L</td>
<td>91.6 ± 1.6</td>
<td>93.9 ± 1.2*</td>
<td>90.3 ± 1.3</td>
<td>83.2 ± 2.6</td>
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<tr>
<td>% CD95</td>
<td>91.8 ± 1.7</td>
<td>90.4 ± 3.6*</td>
<td>80.1 ± 5.3</td>
<td>67.7 ± 5.2</td>
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<tr>
<td>% CD103</td>
<td>27 ± 12</td>
<td>5.9 ± 0.9*</td>
<td>6.5 ± 1.7</td>
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<tr>
<td>% GITR</td>
<td>13.8 ± 6.4</td>
<td>24.4 ± 2.9*</td>
<td>24.1 ± 3.3*</td>
<td>2.3 ± 0.7</td>
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<tr>
<td>% CTLA-4</td>
<td>33.3 ± 6.5*</td>
<td>25.1 ± 4.1*</td>
<td>26.7 ± 3.7*</td>
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<td>% CD127</td>
<td>13.4 ± 6*</td>
<td>9.6 ± 1.6*</td>
<td>15 ± 3.5*</td>
<td>63.9 ± 5.9</td>
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* Summarized data from comparative phenotypic analysis of CD4⁺CD25⁺Foxp3⁺ T cells in HC and of CD4⁺CD25⁺Foxp3⁺ T cells, CD4⁺CD25⁻Foxp3⁻ T cells and CD4⁺CD25⁺Foxp3⁻ T cells in SLE patients. Data are shown as mean values ± SEM.

Significant differences in percent positive cells between the first three Foxp3⁺ T cell populations as compared to the Foxp3⁻ T cell population are indicated.
CD4+/T cells were analyzed for their capacity to suppress IFN-γ. Patients suppressed the proliferation of responder T cells. c. Supernatants from cocultures were analyzed for IFN-γ concentrations. Bars represent mean values ± SEM (n = 5).

Discussion

In this study, we show that T cells from patients with SLE contain ~75% of that seen by the former population, in line with the lower proportion of Foxp3 expression (see above). Next, CD4+CD25−CD127+ and CD4+CD25+CD127− T cells from SLE patients were analyzed for their capacity to suppress IFN-γ production (see Fig. 5c). Whereas a significant suppression of IFN-γ production was observed in cocultures of responder T cells with CD4+CD25+CD127− cells, no suppression was observed in cocultures with CD4+CD25−CD127+ T cells. Finally, cells were analyzed for their capacity of cytokine production upon stimulation. Stimulation of responder T cells led to the production of IFN-γ (383 ± 148 pg/ml) and IL-10 (39 ± 16 pg/ml), whereas no substantial amounts of IFN-γ and IL-10 were detected in culture supernatants of CD4+CD25+CD127− cells and CD4+CD25−CD127− T cells. None of the three T cell populations produced detectable amounts of TGF-β upon stimulation.

Comparative phenotypic analysis of CD4+CD25 Foxp3+ and CD4+CD25 Foxp3+ T cells in SLE patients and HC revealed a similar expression pattern of activation-associated marker molecules and of molecules that have been reported to characterize a Treg phenotype, such as CD62L, CD95, GITR, CTLA-4, and CD127 (2, 39–41). Nevertheless, according to the phenotypic analysis, these findings suggested that CD4+CD25 Foxp3+ resembled regulatory rather than activated T cells which also can express some of these markers, such as CTLA-4 and GITR (42, 43). Interestingly, the levels of these CD4+CD25 Foxp3+ correlated highly with the clinical disease activity but not with the patient’s age. This is in contrast to murine studies where at least in healthy animals an aging-dependent increase of CD4+CD25 Foxp3+ T cells has been described (44–46).

Although, in line with our data, an increase of CD4+CD25 Foxp3+ T cells in SLE patients has recently been described (22, 23), functional analyses of these cells have not been performed and, therefore, it remains unclear whether these cells constitute a subpopulation of Treg or of activated T cells.

Staining for Foxp3 requires fixation and permeabilization of cells which precludes functional studies. In search of a surrogate surface marker molecule for Foxp3, however, we observed that CD4+CD25 CD127− T cells contained up to 53% of Foxp3+ T cells, demonstrating that CD4+CD25 Foxp3+ T cells are at least enriched in this population. Data obtained in mice, humans, and macaques have previously suggested that the CD127low phenotype...
is an intrinsic quality of Treg and allows the isolation and functional characterization of these cells, which also comprise a sub-population of CD25- T cells (24, 25, 47).

CD4+CD25-CD127+ and to a slightly lesser extent CD4+CD25+CD127+ T cells but in contrast to CD4+CD25+CD127+ responder T cells did not respond to in vitro stimulation in line with previously described functional properties of Treg (48). In addition, CD4+CD25+CD127+ T cells were capable of suppressing the proliferation of responder T cells. Their lesser suppressive capacity as well as their slightly detectable proliferation compared with their CD4+CD25+CD127+ counterpart, however, is consistent with their lower contingent of Foxp3+ T cells. On the other hand and in contrast to CD4+CD25+CD127+ T cells, CD4+CD25+CD127- T cells did not suppress the production of IFN-γ. Interestingly, a suppressive defect as well as an insufficient suppression of IFN-γ secretion, despite an undisturbed phenotype has been reported for Treg in MRL/lpr lupus-prone mice (49). In addition, Treg from patients with multiple sclerosis as well as with RA have also been described as functionally defective in terms of their ability to suppress proliferation and IFN-γ production by activated T cells (50, 51). Finally, we did not detect substantial amounts or IL-10 or TGF-β in culture supernatants, suggesting that the suppressive capacity of CD4+CD25 Foxp3+ T cells is not cytokine mediated.

Thus, the results from our phenotypic and functional analyses suggest that CD4+CD25+Foxp3+ T cells represent Treg rather than activated T cells. Nevertheless, CD4+CD25+Foxp3+ T cells were found to be only partially functional and their capacity to suppress T cell proliferation as well as the suppressive capacity of CD4+CD25+CD127+ T cells was found to be significantly reduced as compared with CD4+CD25+CD127+ T cells of HC. In line with this, a diminished Treg-mediated suppression of T cell proliferation has been described in SLE patients (29, 33–35).

One also has to point out that our results have to be viewed with some caution since the surface marker combination we chose to substitute for Foxp3 expression allowed only for the enrichment of CD4+CD25+Foxp3+ T cells and a sizeable fraction of the CD4+CD25-CD127+ cells did not express Foxp3. Moreover, despite their suppressive function in vitro their functional role in vivo is not entirely clear.

The source of the CD4+CD25+Foxp3+ T cells and the reasons for their increase in SLE patients, however, still remains unknown. Our efforts to generate this population in vitro (including culture conditions with various cytokines such as IL-2, IL-10, TGF-β, IFN-γ, lupus serum, or corticosteroids) failed to generate this population from PBMC so far (data not shown). Nevertheless, it is conceivable that they arise under certain autoimmune or inflammatory conditions from a preexisting pool following autoantigen stimulation. Alternatively, they might represent an intrinsic aberrant Treg population in SLE patients with phenotypic and partially functional deficiencies. In either case, the presence of CD4+CD25+Foxp3+ T cells in SLE patients might represent an, albeit ineffective, effort of the immune system in SLE patients to combat autoimmunity dysregulation. Their functional defects, however, might contribute to pathogenic mechanisms in these patients that are finally responsible for overt autoimmunity.

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Disclosures
The authors have no financial conflict of interest.

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