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Deficient CD4⁺CD25high T Regulatory Cell Function in Patients with Active Systemic Lupus Erythematosus

Xavier Valencia,²* Cheryl Yarboro, † Gabor Illei, ‡ and Peter E. Lipsky*  

CD4⁺CD25⁺ T regulatory cells (Tregs) play an essential role in maintaining immunologic homeostasis and preventing autoimmunity. Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of tolerance to nuclear components. We hypothesized that altered function of CD4⁺CD25high Tregs might play a role in the breakdown of immunologic self-tolerance in patients with SLE. In this study, we report a significant decrease in the suppressive function of CD4⁺CD25high Tregs from peripheral blood of patients with active SLE as compared with normal donors and patients with inactive SLE. Notably, CD4⁺CD25high Tregs isolated from patients with active SLE expressed reduced levels of FoxP3 mRNA and protein and poorly suppressed the proliferation and cytokine secretion of CD4⁺ effector T cells in vitro. In contrast, the expression of FoxP3 mRNA and protein and in vitro suppression of the proliferation of CD4⁺ effector T cells by Tregs isolated from inactive SLE patients, was comparable to that of normal individuals. In vitro activation of CD4⁺CD25high Tregs from patients with active SLE increased FoxP3 mRNA and protein expression and restored their suppressive function. These data are the first to demonstrate a reversible defect in CD4⁺CD25high Treg function in patients with active SLE, and suggest that strategies to enhance the function of these cells might benefit patients with this autoimmune disease. The Journal of Immunology, 2007, 178: 2579–2588.

The ability of the immune system to discriminate between self and nonself is controlled by central and peripheral tolerance mechanisms. The former involves deletion of self-reactive T lymphocytes in the thymus at an early stage of development (1, 2). Several mechanisms of peripheral tolerance have also been described, including T cell anergy and ignorance. In addition, studies in the murine system initially provided strong evidence for the existence of a unique CD4⁺ population of naturally occurring regulatory/suppressor T cells that actively prevent both the activation and the effector function of autoreactive T cells that have escaped other mechanisms of tolerance (3–5). Removal of this population from normal rodents leads to the spontaneous development of various autoimmune diseases both organ specific as well as systemic. Recent studies have revealed their presence in human peripheral blood, where they constitute up to 5% of the CD4⁺ T cells (6, 7). These cells require cell-to-cell contact to exert their suppressive effect in vitro. Whether or not a soluble factor is involved depends on the experimental system used (8, 9). Notably, the generation of CD4⁺CD25⁺ regulatory cells (Tregs)³ in the immune system is developmentally and genetically controlled, as recent studies have demonstrated that the transcription factor FoxP3 is essential for their thymic development (10) and is sufficient to activate a program of suppressor function in peripheral CD4⁺CD25⁻ T cells by ectopic expression (11). Genetic defects that primarily affect the development or function of CD4⁺CD25⁻ Tregs can be a primary cause of autoimmune and other inflammatory disorders in humans (12). However, regulation of the suppressive activity of Tregs is more complex because in vitro activation of CD4⁺CD25⁻ T cells results in transient expression of FoxP3 but no regulatory function (13).

Systemic lupus erythematosus (SLE), the prototypical systemic autoimmune disease, is characterized by a wide spectrum of clinical manifestations and abundant production of autoantibodies to nuclear Ags, cell surface molecules, and serum proteins (14, 15). In SLE, it is well recognized that B cells are hyperactive and produce a variety of autoantibodies, resulting in the formation of immune complexes, that play a central role in the effector phase of the disease. Furthermore, it has also become evident that SLE T cells participate in the attack on target cells or tissues through overproduction of proinflammatory cytokines or an increase in cell-to-cell adhesion, ultimately leading to the apoptosis of the target cells (16). One possibility to explain the emergence of autoimmunity in diseases such as SLE could relate to deficient function of Tregs. The deficiency in Treg function could result in increased helper T cell activity or directly in enhanced B cell activity, both of which have been shown to be regulated by Tregs in normal subjects (17, 18). Murine models that lack CD4⁺CD25⁻ Tregs develop a systemic autoimmune disease, characterized by gastritis, oophoritis, arthritis, and thyroiditis (5). Interestingly, some animal models lacking Treg also develop glomerulonephritis and increased titers of anti-dsDNA (5, 19), which are hallmarks of SLE.

Initial studies in SLE suggested there was a decrease in circulating CD4⁺CD25⁻ T cells in patients with active disease (20, 21), and more recently it was claimed that Treg from active SLE were decreased in number during disease flares but displayed normal in vitro suppressive function (22). Therefore, the potential role of Tregs in SLE remains to be fully delineated.

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3 Abbreviations used in this paper: Treg, T regulatory cell; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; GITR, glucocorticoid-induced tumor factor receptor.
We have previously reported a reliable system to assess human Treg function in vitro. Together with flow cytometric analysis of cell surface phenotype and determination of FoxP3 expression (7), this has provided an objective means to assess the presence and function of Tregs in human autoimmune diseases. We, therefore, used these approaches to compare the frequency and function of CD4\(^+\)CD25\(^{high}\) Tregs from a group of SLE patients and with those from age-matched healthy control subjects. In this study, we found that CD4\(^+\)CD25\(^{high}\) Tregs from active but not inactive SLE patients manifest deficient in vitro suppressive activity. Importantly, this defect is associated with a decrease in FoxP3 mRNA and protein that can be restored after in vitro stimulation. A reversible defect in Treg function may contribute to flares of disease activity in patients with SLE.

**Materials and Methods**

**Subjects**

We enrolled 25 patients who were 18 years or older and fulfilled the American College of Rheumatology criteria for the classification of SLE (23, 24), and 40 healthy donors between the ages of 23 and 69 years with no history of autoimmune disease. Disease activity was scored based on the SLE disease activity index (SLEDAI) (25), with one group comprising patients with inactive disease (SLEDAI <3; n = 8) and another group with active SLE (SLEDAI ≥3; n = 17), with or without immunosuppressive treatment. We excluded patients with a history of infection within 3 wk and comorbidities, such as diabetes mellitus. Informed consent was provided according to the declaration of Helsinki. The study was approved by the Institutional Review Board of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health. Data from some normal controls (n = 20) have been previously reported (7).

**Cell culture reagents**

X-VIVO 20 medium (BioWhitaker) supplemented with 1% heat-inactivated normal human serum (BioWhitaker), 20 \(\mu\)g/ml gentamicin, 1 \(\mu\)g/ml Fungizone, and 2 mM glutamine (all obtained from Invitrogen Life Technologies) was used for T cell cultures. FBS was obtained from HyClone.

**Cytokines**

All cytokines used in this study were recombinant human proteins. Final concentrations were as follows: 100 ng/ml GM-CSF, IL-4 and 2 ng/ml TGF\(\beta\)1 (R&D Systems), and 100 U/ml IL-2 (National Cancer Institute, Frederick, MD).

**mAbs**

For immunostaining, mouse PE-, FITC-, and CyChrome-conjugated mAbs against human CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD25 (M-A251), CD45RA (HI 100), CD45RO (UCHL 1), CD62L (DREG-56), CD80 (L307.4), CD83 (HB15e), CD86 (FUN-1), CD122 (MIK-\(\beta\)2), CD127 (hiL7R-M21), CD152 (BNI3.1), HLA-DR (G46-6), CCR4 (1G1), and corresponding mouse isotype controls (all obtained from BD Pharmingen), glucocorticoid-induced tumor factor receptor (GITR)-FITC (110416), TNFRI-FITC (16803), and TNFRII-allophycocyanin (22235.311) (obtained from R&D Systems), and CD25-PE (Beckman Coulter) were used. Cells were stained with FoxP3-allophycocyanin (PCH101; eBioscience) and FoxP3-AlexaFluor 488 (150D; Biolegend) according to the manufacturer’s instructions for fixation and permeabilization, after the cells were stained for surface expression of CD4 and CD25 with CD25-PE and CD4-CyChrome.

**Cytokine assays**

T cells were stimulated with plate-bound anti-CD3 mAb 64.1 (1 \(\mu\)g/well). Cytokine analysis was conducted after a 72-h incubation by analysis of supernatants with commercially available ELISA kits for human IFN-\(\gamma\)
FIGURE 2. CD4+CD25high Treg from active but not inactive SLE express reduced FoxP3. A, Freshly sorted CD4+CD25high Tregs and CD4+CD25+ effector cells were isolated from normal donors and patients with active and inactive SLE, and their expression of FoxP3 was characterized by intracellular staining. Data shown are representative of three different experiments. The isotype staining control is shown by the dotted line, and the staining for FoxP3 is illustrated in black. Numbers in each histogram indicate the percentage of positive cells, and those in parentheses show the mean fluorescence intensity of staining. Cells from these experiments were analyzed for suppressor function and the data is shown in Fig. 3. B, CD127 expression by freshly sorted CD4+CD25high Tregs and CD4+CD25+ effectors from the same donors as in A. The isotype staining control is shown in the dotted line and the staining for CD127 in black. Numbers in each histogram indicate the percentage of positive cells, and those in parentheses show the mean fluorescence intensity of staining. C, Freshly sorted CD25 very high CD4+ T cells (upper 0.6% of CD4+CD25+ T cells) and CD4+CD25 effector cells were isolated from patients with active SLE, and their expression of FoxP3 was characterized by intracellular staining. Data shown are representative of three different experiments. The isotype staining control is shown by the dotted line, and the staining for FoxP3 is illustrated in black. Numbers in each histogram indicate the percentage of positive cells, and those in parentheses show the mean fluorescence intensity of staining.

(BD Pharmingen), according to the manufacturer’s instructions or by the cytometric bead array kit (BD Biosciences).

Cell isolation
CD4+ T cells were enriched from PBMC by negative selection using the AutoMACS (Miltenyi Biotec). Enriched CD4+ T cells were stained with anti-CD4-CyChrome and PE-conjugated anti-CD25 (15 μg/106 cells) for 20 min at 4°C. CD4+CD25+ T cells and CD4+CD25high Tregs were purified using a MoFlo high-speed cell sorter (DakoCytomation) to a purity of >98%. In some experiments, CD4+CD25+ and CD4+CD25high Tregs were stimulated in vitro before analysis. This was accomplished by culturing them for 3 days in microtiter plates coated with anti-CD3 mAb 64.1 (1 μg/well) in medium containing 100 U/ml IL-2.

TNF preincubation experiments
Purified CD4+CD25+ and CD4+CD25high T cells were incubated overnight with TNF at 50 ng/ml in medium supplemented with 1% NHS and 100 U/ml IL-2. Afterward, cells were washed extensively and used in the assays of Treg function.

Flow cytometric analysis
Single-cell suspensions were prepared and stained for 20 min at 4°C with optimal dilutions of each mAb. Expression of cell surface markers was assessed using the flow cytometer (FACSCalibur, BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Proliferation assays
To assess proliferation, 5 × 104 sorted cells were incubated in X-VIVO-20 medium with 10% FBS in 96-well U-bottom plates coated with anti-CD3 (64.1) at 1 μg/well. For assessment of regulatory properties, 5 × 104 CD4+CD25+ T cells were cultured with plate-bound anti-CD3 in 96-well U-bottom plates. Purified autologous CD4+CD25high Tregs were added, usually at a 1:1 ratio if not indicated differently. After 3–4 days of culture, 100 μl of supernatant was removed from each well and used for cytokine detection and 1 μCi of [3H]thymidine (37 KBq/well) was added for an additional 16 h to each well. [3H]Thymidine incorporation was measured using a liquid scintillation counter.

Real-time PCR
Total RNA was isolated from sorted cells using the RNAeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA samples were treated with DNase I to remove contaminating genomic DNA and reverse transcribed with Superscript II (Invitrogen Life Technologies). FoxP3 expression was tested using Assays on Demand reagents from Applied Biosystems (Hs00203958 m1). All reported mRNA levels were normalized to the GAPDH mRNA level, where GAPDH = 1.

Statistical analysis
The mean ± SEM thymidine uptake and mean ± SEM cytokine secretion of triplicate cultures were calculated for each experimental condition. The Mann-Whitney U test was used to evaluate possible differences in the CD4+CD25high function following in vitro and TNF stimulation. Percentage of suppression was determined as 1 – (cpm incorporated in the coculture/cpm of responder population alone) × 100%. Correlations between percentage of FoxP3+CD25high cells or percentage of suppression by CD4+CD25high Tregs and SLEDAI scores were assessed by nonparametric Spearman correlation. All statistical tests were performed using StatView software (SAS Institute).

Results
CD4+CD25+ T cells exhibit phenotypical differences compared with CD4+CD25− T cells
CD4+CD25high Tregs represented ~0.5–3 ± 1% (mean ± SEM) of total CD4+ T cells from healthy donors (n = 40). Because it has been demonstrated that the brightest 2% of the CD25+ population contains most of the Treg (7, 27), the CD25+ brightest subset was studied further. Because we analyzed only the brightest 2% of CD4+CD25+ T cells in both SLE and normal controls, assessment of the comparative number of Tregs could not be undertaken. The surface phenotype of CD4+CD25− and CD4+CD25high Treg subsets among healthy volunteers and patients with active (SLEDAI ≥3; n = 17) SLE was characterized. As shown in Fig. 1, the CD4+CD25high Treg subset from active SLE patients expressed modestly higher but not significantly different levels of GITR (20 ± 8% (mean ± SEM) vs 15 ± 5% (mean ± SEM) in normal volunteers). An increased expression of TNFRII was also observed by the freshly isolated CD4+CD25high Treg subset from active SLE (30 ± 12%, mean ± SEM) compared with normal individuals.
CD4+CD25high T cells from patients with active SLE fail to suppress proliferation of CD4+CD25− T cells. CD4+CD25+ responder (5 × 10⁴/well) and CD4+CD25high Treg (5 × 10⁴/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 h, [³H]thymidine incorporation was determined. Results are the mean ± SEM of 20 separate experiments using individual donors and patients with active SLE. Also shown is the percentage of inhibition of proliferation in these 20 experiments.

Our data also confirmed previous findings (28) of selective expression of CCR4 on CD4+CD25high Tregs in normal donors and patients with active SLE (90 ± 5%, mean ± SEM; n = 40) compared with CD4+CD25− from normal donors (30 ± 10%, mean ± SEM; n = 20). These phenotypic characteristics indicate that the cells analyzed were authentic CD4+CD25high Tregs in both normal donors and SLE patients. However, CD4+CD25high Tregs from patients with active SLE exhibited increased expression of GITR and TNFRII, which has been reported in patients with rheumatoid arthritis and after exposure to TNF (7). By scatter characteristics, CD4+CD25high Tregs were not larger or more complex than CD4+CD25− effector cells in either normal donors or SLE patients (data not shown).

As shown in Fig. 2, CD4+CD25high Tregs from normal donors uniformly expressed high levels of FoxP3 protein by flow cytometry (mean 85 ± 5%; n = 40), whereas CD4+CD25high Tregs from subjects with active SLE expressed significantly less FoxP3 protein (mean 45 ± 10%; n = 10; p = 0.003). Notably, Tregs from patients with inactive SLE expressed increased levels of FoxP3 (mean 64.4 ± 15%; n = 8) that were not significantly different than normal (p = 0.20). Neither CD4+CD25− effector cells nor SLE expressed this transcription factor that governs Treg function (10, 11). Additionally, analysis of CD127 expression, which can be used to discriminate Tregs from effector T cells (29), confirmed that Tregs from normal donors (4.5%), from inactive lupus (8.1%) and active lupus (8.7%) contained few CD127-expressing effector T cells as shown in Fig. 2B. Finally, to ensure that results did not reflect activated effector cells contaminating the CD25high population, even more stringent sorting conditions were used. In these experiments, only the brightest 0.6% of the CD4+CD25− cells from patients with active SLE were sorted. In these additional experiments, only a fraction of the CD25veryhigh T cells from normal donors can be suppressed by CD4+CD25high Tregs from healthy controls. CD4+CD25− responder (5 × 10⁴/well) and CD4+CD25high Tregs (5 × 10⁴/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 h, [³H]thymidine incorporation was determined. CD4+CD25+ effectors from active SLE patients were also cocultured with CD4+CD25high Treg from normal individuals. CD4+CD25− effectors from normal individuals were also cocultured with CD4+CD25high Treg from active SLE patients. Results are the mean ± SEM of three separate experiments.

CD4+CD25+ CD4+CD25−
ND Normal aSLE ND Normal aSLE ND Normal aSLE

P<0.001

P<0.0001
cells expressed FoxP3 (Fig. 2C). These results confirmed that patients with active SLE have CD25high and CD25veryhigh cells that are deficient in expression of FoxP3.

**Freshly isolated CD4+CD25+ T cells from active lupus patients are functionally defective**

A low proliferative potential is highly characteristic of CD4+CD25high Tregs both in the murine and human systems. The proliferative capacity of freshly isolated CD4+CD25high Tregs from lupus patients to anti-CD3 stimulation was tested. Freshly isolated CD4+CD25high Tregs from active lupus patients showed a somewhat increased proliferative response to immobilized anti-CD3, compared with normal donors, but this increase was not significant (p = 0.27) (Fig. 3). The regulatory properties of CD4+CD25high T cells were investigated by testing their ability to suppress the proliferative responses of CD4+CD25− T cells to immobilized anti-CD3. At a ratio of 1:1, CD4+CD25high Tregs from healthy volunteers inhibited the proliferation of CD4+CD25− T cells by a mean of 80 ± 5% (n = 40; Fig. 3). These data indicate that CD4+CD25high Tregs have a direct suppressive effect on T cells that is independent of APC. However, as shown in Fig. 3, freshly isolated CD4+CD25high Tregs from patients with active SLE exhibited significantly less suppressive activity than those from normal donors (p < 0.005; n = 18). Notably, the functional activity of Tregs was also assessed in the 0.5% of CD4 cells expressing the very brightest levels of CD25. In three individuals with active SLE studied, these cells were hyporesponsive to anti-CD3 stimulation (cpm = 11.0 ± 3.3 × 10, mean ± SEM; n = 3) and also exerted no suppressive function (percentage of suppression = −93.8 ± 95%, mean ± SEM; n = 3). To determine whether the loss of regulatory function in active SLE was explained by a decrease in the intrinsic function of CD4+CD25high Tregs or an increase in the resistance of CD4−CD25− effector T cells to inhibition, we conducted mixing experiments with cells from patients with active SLE and normal controls. Tregs from patients with active SLE failed to suppress the proliferation of autologous CD4−CD25− effector T cells as well as CD4−CD25− effector T cells from healthy controls, whereas CD4−CD25high Tregs from healthy controls readily suppressed the proliferative response of CD4−CD25− effectors from SLE patients (Fig. 4). These data clearly indicate that the primary regulatory defect is in the function of CD4+CD25high Tregs isolated from the circulation of patients with active SLE, and not a resistance of lupus CD4−CD25− effector cells to suppression.

Notably, when CD4+CD25high Tregs isolated from clinically inactive SLE patients (n = 8) were tested for their ability to suppress autologous CD4+ T cell proliferation and cytokine secretion,
we found no statistically significant difference ($p = 0.37$) compared with normal donors (Fig. 5). Moreover, when suppressive function of CD4$^+$CD25$^{high}$ Tregs from active and inactive SLE patients was compared (Fig. 3 vs Fig. 5), a significant difference was noted ($p < 0.0001$). Of note, Tregs from quiescent SLE displayed a somewhat more hyporesponsive phenotype than normal donor Tregs ($p = 0.016$) or Tregs from patients with active SLE ($p = 0.028$).

Correlations were assessed to determine the relationship between lupus disease activity and Treg function. We found significant inverse correlations between the expression of FoxP3 in Tregs from patients with SLE and the SLEDAI score ($p < 0.003$;}

*FIGURE 7.* CD4$^+$CD25$^{high}$ Tregs from active SLE express reduced FoxP3 mRNA and protein and it increases after in vitro stimulation. CD4$^+$CD25$^-$ and CD4$^+$CD25$^{high}$ T cells were sorted as described from normal donors (A) and from subjects with active SLE (A and B). Freshly sorted CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^{high}$ Tregs were assessed immediately ex vivo or were incubated with anti-CD3 mAb at 1 $\mu$g/ml in medium supplemented with IL-2 at 100 U/ml. A. After a 3-day incubation RNA was isolated. Real-time PCR was conducted in triplicate for FoxP3 mRNA and relative fold changes were normalized to GAPDH. Data are representative of three different experiments. B. After a 3-day incubation, FoxP3 protein was assessed by flow cytometry.

*FIGURE 8.* CD4$^+$CD25$^{high}$ Tregs from active SLE recover their suppressive function after in vitro activation. CD4$^+$CD25$^-$ and CD4$^+$CD25$^{high}$ T cells were sorted as described from normal donors and from subjects with active SLE. Freshly sorted CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^{high}$ Tregs from active lupus were incubated with anti-CD3 mAb at 1 $\mu$g/ml in medium supplemented with IL-2 at 100 U/ml for 3 days. After that, cells were washed and assessed for suppressive activity. After 72 h, [3H]thymidine incorporation was determined. Data are the mean ± SEM of six independent experiments.
$r^2 = 0.388$) as well as between the percentage of suppression in the in vitro regulatory assay and the SLEDAI score ($p = 0.014; r^2 = 0.326$) (Fig. 6). Importantly, analysis of the possible relationship between glucocorticoid dose and Treg function did not show a significant correlation ($p = 0.33$), indicating that the decrease in Treg function was more likely to be related to disease activity than therapy.

**FoxP3 expression is reversibly decreased in CD4$^+$CD25$^{high}$ Tregs from patients with active SLE**

Constitutive expression of the transcriptional repressor, FoxP3, is characteristic of CD4$^+$CD25$^{high}$ Tregs. As shown in Fig. 7, FoxP3 mRNA levels were significantly diminished in CD4$^+$CD25$^{high}$ Tregs from patients with active SLE. Of note, FoxP3 mRNA increased after in vitro stimulation of Tregs from patients with active SLE. A modest increase in FoxP3 mRNA was also noted in activated CD4$^+$CD25$^-$ effector T cells. As noted with the mRNA analysis, the expression of FoxP3 protein also increased in Tregs from patients with active SLE after in vitro activation (up to 60 ± 10%, mean ± SEM). However, we did not note a uniform increase in FoxP3 expression in CD4$^+$CD25$^-$ effector cells. In only one of six experiments was a significant increase in FoxP3 expression noted after in vitro stimulation of CD4$^+$CD25$^-$ effector cells.

**Reversibility of defective Treg function in patients with active SLE by in vitro activation**

It was next determined whether CD4$^+$CD25$^{high}$ Tregs from active SLE patients could become suppressive after in vitro activation. Notably, in vitro activation of CD4$^+$CD25$^{high}$ Tregs from active SLE patients restored the capacity of these cells to suppress both proliferation and IFN-γ secretion ($p = 0.036$; Fig. 8). Of note, in vitro activation of CD4$^+$CD25$^{high}$ Tregs from lupus patients also increased their hypersensitivity to in vitro stimulation ($p = 0.05$). In vitro activation of CD4$^+$CD25$^-$ effector cells did not lead to induction of suppressive activity (data not shown).

**TNF and signaling through TNFRII block suppressive activity of CD4$^+$CD25$^+$ Tregs**

We have previously reported the effect of signaling through TNFRII in downmodulating the suppressive function of CD4$^+$CD25$^{high}$ Treg. Because CD4$^+$CD25$^{high}$ Tregs from active SLE patients failed to display suppressive function ex vivo and expressed increased TNFRII, we therefore examined whether TNF could modulate the action of CD4$^+$CD25$^{high}$ Tregs from patients with active SLE, as we had previously reported with normal donors (7). Whereas activated CD4$^+$CD25$^{high}$ Tregs from healthy donors were able to inhibit the proliferation of CD4$^+$CD25$^-$ T cells, the same ratio of Treg to effector cells was unable to induce suppression after incuba-

*FIGURE 9. Inhibition of IFN-γ secretion by CD4$^+$CD25$^{high}$ Treg is abrogated by cross-linking TNFRII. Previously activated T cells that had up-regulated surface TNFRII expression were used for in vitro regulatory assays. CD4$^+$CD25$^-$ T cells (5 × 10$^5$/well) or CD4$^+$CD25$^{high}$ Tregs (5 × 10$^5$/well) alone or mixed together at a ratio of 1:1 were stimulated with plate-bound anti-CD3 (1 μg/well), with or without anti-TNFRII. After 72 h, culture supernatants were harvested and analyzed to determine the IFN-γ content. Data are the mean ± SEM of five independent experiments.*

*FIGURE 10. Incubation with TNF suppresses the subsequent ability of CD4$^+$CD25$^{high}$ Tregs to inhibit the proliferation of CD4$^+$CD25$^-$ T cells. Previously activated CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^{high}$ Tregs that had up-regulated surface TNFRII expression were used for in vitro regulatory assays. CD4$^+$CD25$^-$ T cells (5 × 10$^5$/well) or CD4$^+$CD25$^{high}$ Treg (5 × 10$^5$/well) alone or mixed together at a ratio of 1:1 were stimulated with plate-bound anti-CD3 (1 μg/well). Recombinant TNF was added at the beginning of the culture at 50 ng/ml as indicated. After 72 h, [3H]thymidine incorporation was determined. Data are the mean ± SEM of six independent experiments.*
and in vitro-activated active SLE subjects were able to suppress the proliferation of CD4⁺CD25⁻ T cells, they completely lost their regulatory activity when TNFRII was cross-linked (Fig. 9). In contrast, cross-linking TNFRII did not provoke the loss of anergic phenotype of CD4⁺CD25<sup>high</sup> Tregs. It was next determined whether the regulatory function of CD4⁺CD25<sup>high</sup> Tregs could also be blocked by soluble TNF instead of anti-TNFRII mAb. Again, the addition of soluble TNF to the regulatory assay completely reversed the suppression of the proliferation of CD4⁺CD25⁻ T cells without influencing the anergic phenotype of CD4⁺CD25<sup>high</sup> Tregs (Fig. 10).

**Discussion**

It is now well accepted that a small population of CD4⁺ T cells, identified by the coexpression of CD25, has the ability to regulate immune responses. These Tregs have been found and characterized in humans and rodents. Furthermore, studies in both humans and mice have demonstrated that defective regulatory T cell function contributes to autoimmune diseases in both animal models and human disease (4, 30). Importantly, Treg function in humans largely resides in the fraction of CD4⁺CD25⁻ T cells that express the highest density of CD25 (7, 27). Therefore, we sought to determine whether a defect in CD4⁺CD25<sup>high</sup> Tregs occurs in patients with SLE. We observed a significant reversible reduction in the suppressive function of Tregs in subjects with active SLE, associated with loss of FoxP3 expression, compared with healthy donors and inactive SLE patients. Our data are the first clear demonstration of the functional defect in CD4⁺CD25<sup>high</sup> Tregs in active SLE as opposed to alterations in the frequency of CD4⁺CD25⁻ T cells in these patients (21). Although we only analyzed the CD25 brightest T cell subset defined as the 2% of cells with the greatest expression of CD25, the finding that this population was deficient in regulatory function is consistent with previous results that the number of functionally active Tregs is decreased in patients with active SLE.

In this previous report, it was found that SLE patients have significantly lower levels of CD4⁺CD25⁻ T cells and CD4⁺CD25<sup>high</sup> Tregs compared with normal donors (21). Notably, in a subset of patients, no correlation between the SLEDAI score and the percentage or number of CD4⁺CD25⁻ T cells was found, although this analysis was not conducted with CD4⁺CD25<sup>high</sup> Tregs. More recently, a study reported a numeric decrease but preserved in vitro function of CD4⁺CD25<sup>high</sup> Tregs in active lupus patients (22). A potential explanation to account for the discrepancy between this study and our results is that these investigators analyzed Treg function after activation by allogeneic stimulator cells. It has been reported that after allogeneic stimulation, even inefficient suppressor cells can adequately suppress the proliferation of CD4⁺CD25⁻ effectors (30, 31). In a very recent study, defective regulatory function of CD4⁺CD25⁻ Tregs was observed in patients with active SLE (32). In this study, the potent mitogen, PHA, was used to assess Treg function. In the current study, the anti-CD3 mAb 64.1, a very robust activation stimulus, was used. Suppression in this model requires fully functional Tregs. Therefore, the current and previous results are consistent with the conclusion that patients with active SLE do not manifest maximal function of Tregs, although some residual activity may persist. Importantly, we documented markedly diminished FoxP3 mRNA and protein in Tregs from subjects with active SLE, consistent with their decreased function.

We found that freshly isolated CD4⁺CD25<sup>high</sup> Treg from subjects with active SLE failed to suppress the proliferation of autologous CD4⁺CD25⁻, whereas patient’s CD4⁺CD25⁻ effectors were suppressed by normal donor Treg. We next sought to determine whether CD4⁺CD25<sup>high</sup> Tregs from SLE could become suppressive after in vitro activation, because in the murine system this leads to the development of the most potent suppressors (33). Notably, after in vitro activation of Tregs from SLE subjects by culturing them for 3 days with plate-bound anti-CD3 and high doses of IL-2 their suppressive function was restored, because they were able to suppress the proliferation of autologous CD4⁺CD25⁻ T cells by nearly 85%. In addition, in vitro-activated CD4⁺CD25<sup>high</sup> Tregs suppressed the production of IFN-γ and IL-2 (data not shown) by CD4⁺CD25⁻ effectors activated with anti-CD3 mAb and their FoxP3 expression increased. Our findings on the restoration of suppressor function of CD4⁺CD25<sup>high</sup> Tregs in SLE by in vitro activation with anti-CD3 in the presence of IL-2 is consistent with the fundamental role of IL-2 in maintaining the fitness of CD4⁺CD25<sup>high</sup> Tregs in the periphery as has been recently demonstrated (34), although in our system we demonstrated an increase in Treg function not maintenance. We have noted that Treg function can be maintained by IL-2 in vitro (our unpublished data), but IL-2 is not sufficient to induce Treg function (35). The combination of anti-CD3 and IL-2, therefore, may be necessary for up-regulation of Treg function, whereas IL-2 alone may be sufficient to maintain them. Thus, in human SLE, reduced IL-2 generation, as has been previously reported (36), may be a key factor underlying reduced CD4⁺CD25<sup>high</sup> Treg.

It was important to document the fact that the CD4⁺CD25<sup>high</sup> Tregs in patients with active SLE were not diluted with activated CD25⁻ effector T cells. Although it is difficult to rule out this possibility completely, the approaches taken and the results obtained make this explanation quite unlikely. First, only the brightest 2% of the CD25⁻ cells were analyzed to exclude activated T cells that are usually intermediate in their CD25 expression (7, 27). Moreover, in additional studies, analysis of the 0.6% of cells with the very brightest expression of CD25 also showed they were deficient in both FoxP3 expression and suppressive activity. Secondly, by size or phenotype, we could find no evidence that the CD4⁺CD25<sup>high</sup> Tregs in patients with active SLE were activated. Thirdly, the CD4⁺CD25<sup>high</sup> Tregs were clearly anergic and failed to produce effector cytokines. Although the level of anergy varied somewhat between Tregs from patients with active and inactive SLE, in all experiments proliferation and IFN-γ production by CD4⁺CD25<sup>high</sup> T cells from SLE patients was significantly less than that of CD4⁺CD25⁻ effector cells. The degree of responsiveness noted by all CD4⁺CD25<sup>high</sup> T cells may relate to the extremely potent stimulus used in this analysis. Finally, upon in vitro stimulation, these cells up-regulated expression of FoxP3 and became suppressive. Although FoxP3 can be up-regulated after in vitro activation of CD4⁺ effector cells, these activated effector cells do not uniformly become suppressive (13). The fact that CD4⁺ CD25<sup>high</sup> Tregs from active SLE patients reacquire suppressive function following in vitro activation is consistent with the conclusion that they were indeed Tregs that had become functionally inactivated in vivo.

We conducted a series of experiments aimed to unravel the additional mechanisms of impaired Treg function in active SLE. Because the cells were present in the blood but were functionally impaired and their function could be regained after in vitro culture and during disease quiescence, we reasoned that a soluble factor might contribute to the impaired suppressive function. Among the panoply of altered cytokines in SLE, TNF is known to be secreted in excess in human lupus (37), and in different murine models it can contribute to or ameliorate lupus (38). Importantly, we had previously found that TNF can down modulate CD4⁺CD25<sup>high</sup> Treg function (7). Therefore, we explored the possibility that TNF might impair Treg function in SLE.
As in rheumatoid arthritis, our results showed an increased constitutive expression of TNFRII in CD4+CD25high Tregs from healthy volunteers and SLE patients. Importantly, patients with active but not inactive SLE exhibited increased expression of TNFRII, consistent with in vivo exposure to TNF. Similar to our findings in RA, we demonstrated that cross-linking TNFRII completely abrogated the suppression exerted by fresh or activated CD4+CD25high Treg from patients with SLE. Similarly, high concentrations of TNF inhibited the function of CD4+CD25high Tregs, consistent with an action mediated by TNFRII (39–41). These results clearly show that CD4+CD25high Tregs from patients with SLE are sensitive to the modulatory influences of TNF and are consistent with the conclusion that overproduction of TNF may contribute to the defective Treg function in patients with active SLE.

We next sought to determine whether FoxP3 expression correlated with Treg function in SLE. Expression of FoxP3 mRNA and protein was clearly diminished in patients with active but not inactive SLE. In addition, appearance of FoxP3+CD25high Tregs correlated with disease activity as measured by SLEDAI in lupus patients. Moreover, FoxP3 expression was up-regulated in CD4+CD25high T cells of active SLE patients after in vitro activation, unlike the results noted with CD4+CD25− T cells in which in vivo stimulation up-regulates FoxP3 expression but not suppressive function (13). In CD4+ T cells from SLE patients initially identified by the bright expression of CD25, suppressive function clearly associated with FoxP3 expression. This suggests that a cofactor in CD25highCD4+ T cells, such as B lymphocyte-induced maturation protein 1 (Blimp-1) (42) or gene related in anergy lymphocytes function (13). In CD4+ stimulation up-regulates FoxP3 expression but not suppressive function (13). FoxP3 expression was up-regulated in CD4+CD25high T regulatory cells. Blood 108: 253–261.


