Deficient CD4$^+$CD25^{high} T Regulatory Cell Function in Patients with Active Systemic Lupus Erythematosus

Xavier Valencia, Cheryl Yarboro, Gabor Illei and Peter E. Lipsky

---

References	This article **cites 44 articles**, 17 of which you can access for free at:
http://www.jimmunol.org/content/178/4/2579.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription	Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions	Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Deficient CD4⁺CD25^high T Regulatory Cell Function in Patients with Active Systemic Lupus Erythematosus[1]

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⁺CD25^high 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⁺CD25^high Tregs from peripheral blood of patients with active SLE as compared with normal donors and patients with inactive SLE. Notably, CD4⁺CD25^high 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⁺CD25^high 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⁺CD25^high 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.

T
he 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⁺CD25⁺ 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⁺ T 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.

FIGURE 1. Phenotype of CD4⁺CD25^high Tregs. Purified CD4⁺ T cells from healthy donors and active SLE were stained with anti-CD4 CyChrome and anti-CD25 PE, and the population was sorted into CD4⁺CD25⁻ and CD4⁺CD25^high subsets as indicated in *Materials and Methods*. Staining with the isotype-matched control mAb is indicated by the horizontal bracket. Numbers indicate the percentages of cells in each gate, and those in parentheses show the mean fluorescence intensity of staining. Data are representative of results from >10 different experiments.

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 Treg 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 Institute of Diabetes and Digestive and Kidney 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 μg/ml gentamicin, 1 μg/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 TGFβ1 (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-β2), 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-AlexaF488 (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. Anti-CD3 (64.1; Ref. 26) was used for polyclonal activation of T cells.

Cytokine assays

T cells were stimulated with plate-bound anti-CD3 mAb 64.1 (1 μg/well). Cytokine analysis was conducted after a 72-h incubation by analysis of supernatants with commercially available ELISA kits for human IFN-γ

FIGURE 2. CD4$^+$CD25^{high} Treg from active but not inactive SLE express reduced FoxP3. *A*, Freshly sorted CD4$^+$CD25^{high} 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$^+$CD25^{high} 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.
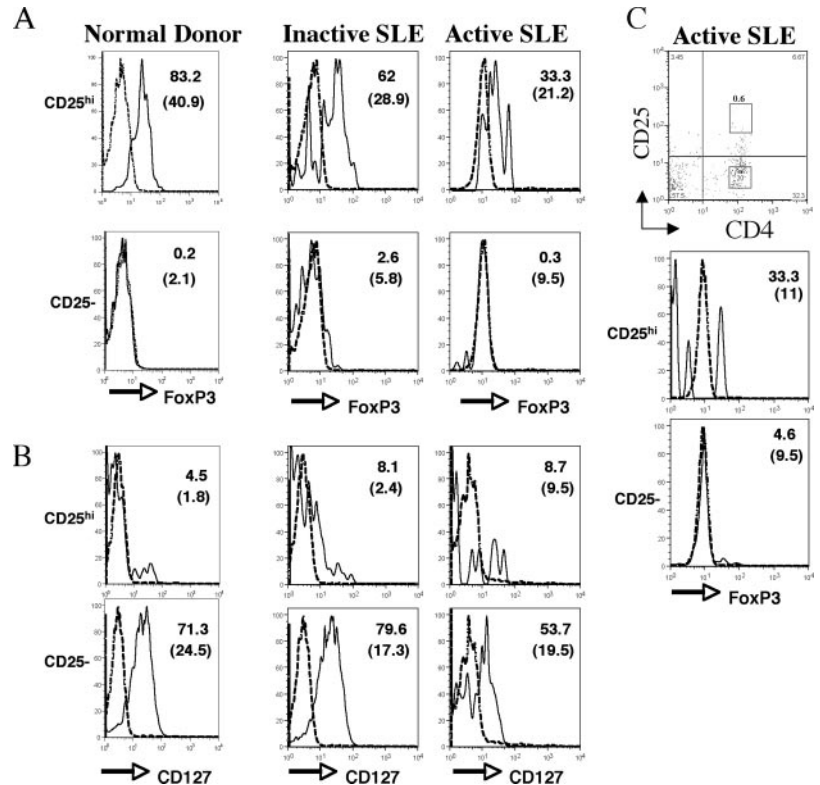
(BD Pharmingen), according to the manufacturer's instructions or by the cytometric bead array kit (BD Biosciences).

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/10^8 cells) for 20 min at 4°C. CD4$^+$CD25$^-$ T cells and CD4$^+$CD25^{high} Tregs were purified using a MoFlo high-speed cell sorter (DakoCytomation) to a purity of >98%. In some experiments, CD4$^+$CD25$^-$ and CD4$^+$CD25^{high} 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$^+$CD25^{high} 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 × 10^4 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 × 10^4 CD4$^+$CD25$^-$ T cells were cultured with plate-bound anti-CD3 in 96-well U-bottom plates. Purified autologous CD4$^+$CD25^{high} 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 [^{3}H]thymidine (37 KBq/well) was added for an additional 16 h to each well. [^{3}H]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$^+$CD25^{high} 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$^+$CD25^{high} cells or percentage of suppression by CD4$^+$CD25^{high} 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$^+$CD25^{high} 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$^+$CD25^{high} Treg subsets among healthy volunteers and patients with active (SLEDAI ≥3; n = 17) SLE was characterized. As shown in Fig. 1, the CD4$^+$CD25^{high} 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$^+$CD25^{high} Treg subset from active SLE (30 ± 12%, mean ± SEM) compared with normal individuals
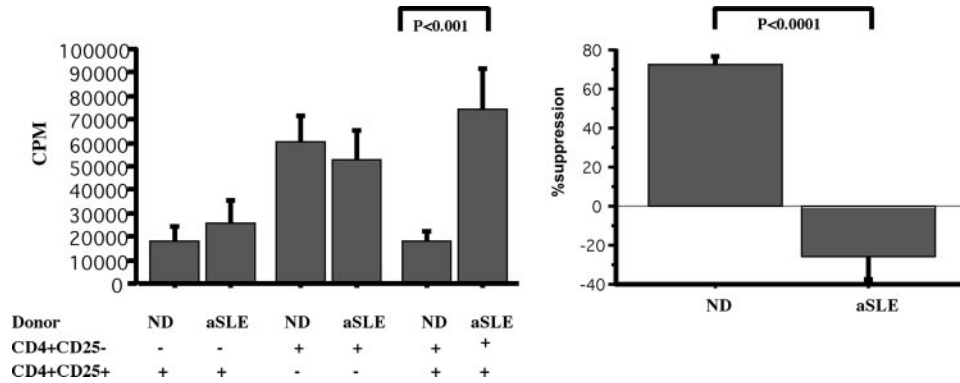
FIGURE 3. CD4$^+$CD25^{high} T cells from patients with active SLE fail to suppress proliferation of CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^-$ responder (5 × 10^4/well) and CD4$^+$CD25^{high} Treg (5 × 10^4/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 h, [^{3}H]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.

(18 ± 5%, mean ± SEM; $p < 0.05$), whereas patients with inactive SLE had similar expression of TNFRII as normal donors ($n = 8$; 25 ± 6%, mean ± SEM; $p = 0.32$). In contrast, TNFRI (CD120a) was practically undetectable in both groups (mean 1.3 ± 0.6%; $n = 10$). No differences were detected in the CD45RO expression on the CD4$^+$CD25^{high} Treg subset from normal individuals (90 ± 5%, mean ± SEM) and active SLE patients (75 ± 8%, mean ± SEM). Additionally, analysis of CD69 expression on the Treg subset (mean 1.0 ± 0.7%; $n = 40$) and in SLE (mean 1.2 ± 0.9%; $n = 20$) confirmed that these cells were not simply contaminated with recently activated CD4$^+$ effector cells, because these would be mainly CD69$^+$. Finally, <25% of CD4$^+$CD25^{high} Tregs in normal donors and patients with active SLE were HLA-DR$^+$ as has been previously reported (27), indicating that CD4$^+$CD25^{high} Tregs in SLE were not enriched in persistently activated T cells. Our data also confirmed previous findings (28) of selective expression of CCR4 on CD4$^+$CD25^{high} Tregs in normal donors (90 ± 7%, mean ± SEM; $n = 40$) and SLE (85 ± 18%, mean ± SEM; $n = 20$) compared with CD4$^+$CD25$^-$ from normal donors (30 ± 10%, mean ± SEM; $n = 40$) and inactive SLE patients (20 ± 8%, mean ± SEM; $n = 8$). These phenotypic characteristics indicate that the cells analyzed were authentic CD4$^+$CD25^{high} Tregs in both normal donors and SLE patients. However, CD4$^+$CD25^{high} Tregs from patients with active SLE exhibited increased expres-

sion of GITR and TNFRII, which has been reported in patients with rheumatoid arthritis and after exposure to TNF (7). By scatter characteristics, CD4$^+$CD25^{high} 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$^+$CD25^{high} Tregs from normal donors uniformly expressed high levels of FoxP3 protein by flow cytometry (mean 85 ± 5%; $n = 40$), whereas CD4$^+$CD25^{high} 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$^-$ effectors from normal donors or 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 CD25^{high} 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 these CD25^{veryhigh}

FIGURE 4. CD4$^+$ effector cells from active SLE patients can be suppressed by CD4$^+$CD25^{high} Tregs from healthy controls. CD4$^+$CD25$^-$ responder (5 × 10^4/well) and CD4$^+$CD25^{high} Tregs (5 × 10^4/well) were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 h, [^{3}H]thymidine incorporation was determined. CD4$^+$CD25$^-$ effectors from active SLE patients were also cocultured with CD4$^+$CD25^{high} Treg from normal individuals. CD4$^+$CD25$^-$ effectors from normal individuals were also cocultured with CD4$^+$CD25^{high} Treg from active SLE patients. Results are the mean ± SEM of three separate experiments.
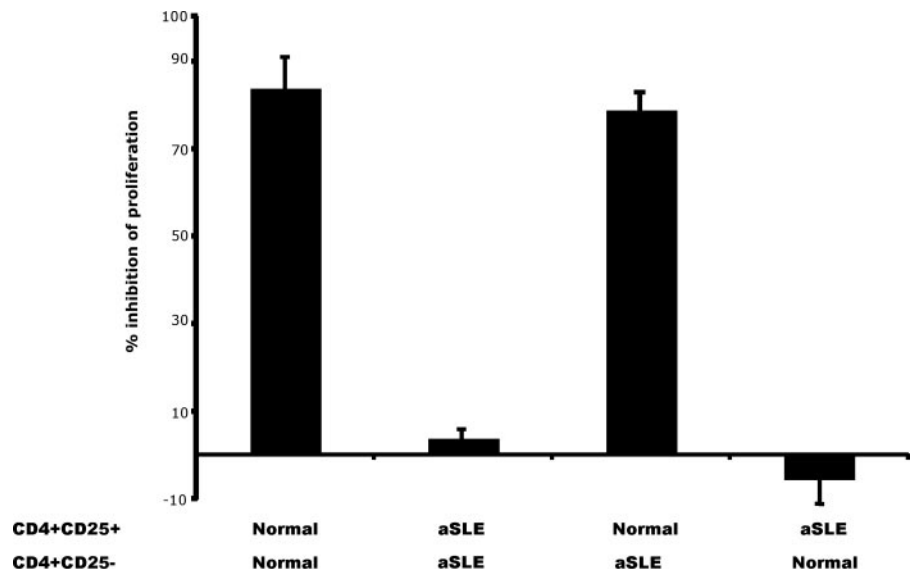
FIGURE 5. CD4$^+$CD25^{high} Treg from subjects with inactive SLE have normal suppressive function. CD4$^+$ CD25$^-$ and CD4$^+$CD25^{high} T cells were sorted as described from normal donors and subjects with inactive SLE. CD4$^+$CD25$^-$ responder and CD4$^+$CD25^{high} Treg were isolated from inactive SLE and normal donors. Cells were cultured with plate-bound anti-CD3 (1 μg/well) either alone or at a 1:1 ratio. After 72 h, [^{3}H]thymidine incorporation was determined. Culture supernatants were harvested and analyzed to determine the IFN-γ content. Data represent the mean $\pm$ SEM of six to eight experiments using cells from subjects with inactive SLE patients.
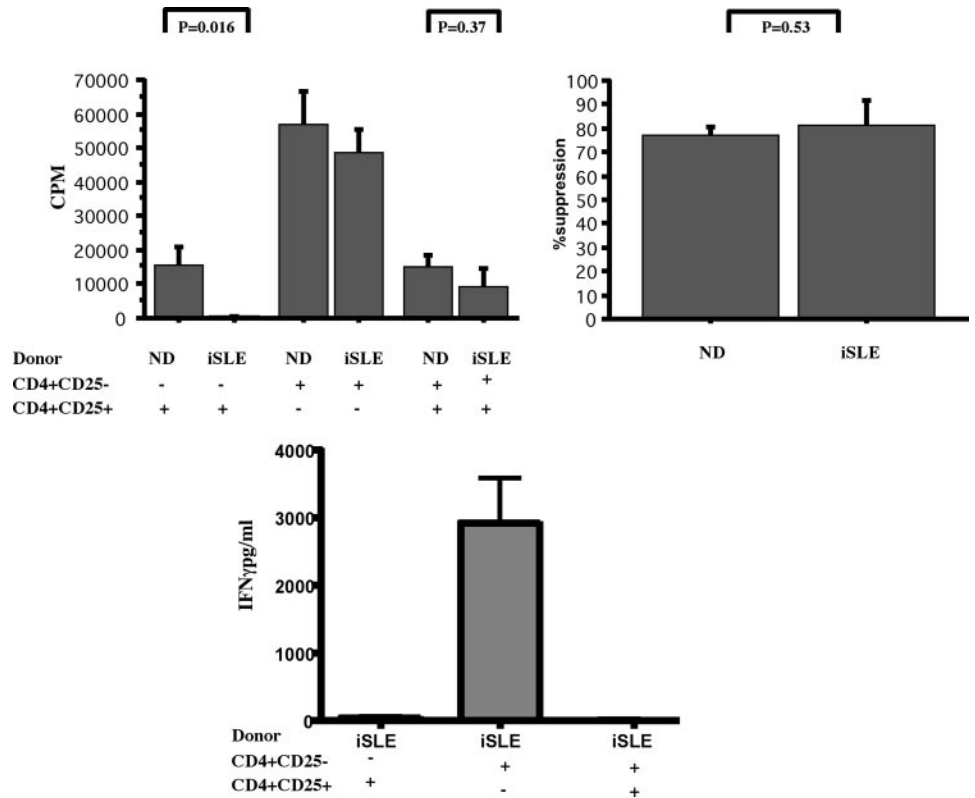

cells expressed FoxP3 (Fig. 2*C*). These results confirmed that patients with active SLE have CD25^{high} and CD25^{veryhigh} cells that are deficient in expression of FoxP3.

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

A low proliferative potential is highly characteristic of CD4$^+$ CD25^{high} Tregs both in the murine and human systems. The proliferative capacity of freshly isolated CD4$^+$CD25^{high} Tregs from lupus patients to anti-CD3 stimulation was tested. Freshly isolated CD4$^+$CD25^{high} 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$^+$CD25^{high} 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$^+$CD25^{high} Tregs from healthy volunteers inhibited the proliferation of CD4$^+$CD25$^-$ T cells by a mean of 80 $\pm$ 5% ($n = 40$; Fig. 3). These data indicate that CD4$^+$ CD25^{high} Tregs have a direct suppressive effect on T cells that is independent of APC. However, as shown in Fig. 3, freshly isolated CD4$^+$CD25^{high} 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 $\pm$ 3.3 $\times$ 10, mean $\pm$ SEM; $n = 3$) and also exerted no suppressive function (percentage of suppression = $-93.8 \pm 95\%$, mean $\pm$ 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$^+$CD25^{high} 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 cell from healthy controls, whereas CD4$^+$ CD25^{high} 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$^+$CD25^{high} 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$^+$CD25^{high} Tregs isolated from clinically inactive SLE patients ($n = 8$) were tested for their ability to suppress autologous CD4$^+$ T cell proliferation and cytokine secretion,
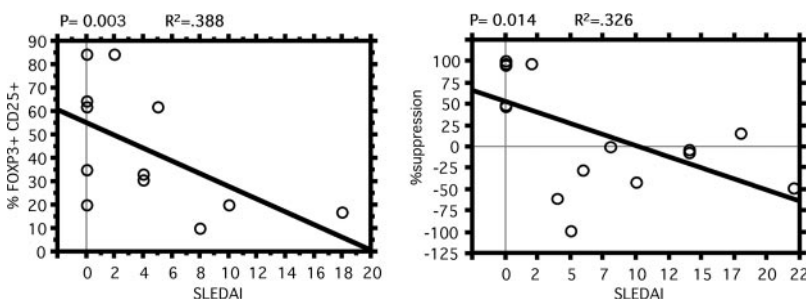
FIGURE 6. Inverse correlation between FoxP3 expression and suppressive activity of CD4$^+$CD25^{high} Tregs and SLEDAI score in subjects with SLE. Spearman correlation analysis of results from lupus patients shows a significant correlation between FoxP3 expression and suppressive activity of CD4$^+$CD25^{high} Treg and the SLEDAI score; data are from 23 subjects with SLE.
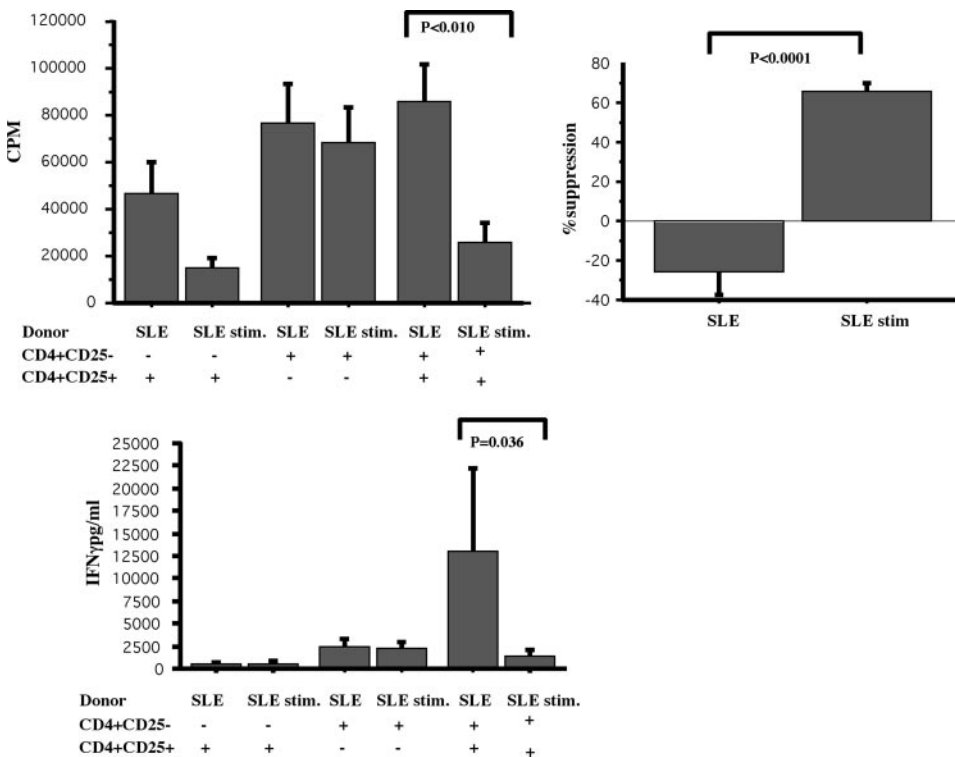
FIGURE 7. CD4+CD25high Tregs from active SLE express reduced FoxP3 mRNA and protein and it increases after in vitro stimulation. CD4+CD25− and CD4+CD25high T cells were sorted as described from normal donors (*A*) and from subjects with active SLE (*A* and *B*). Freshly sorted CD4+CD25− T cells and CD4+CD25high Tregs were assessed immediately ex vivo or were incubated with anti-CD3 mAb at 1 μg/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.
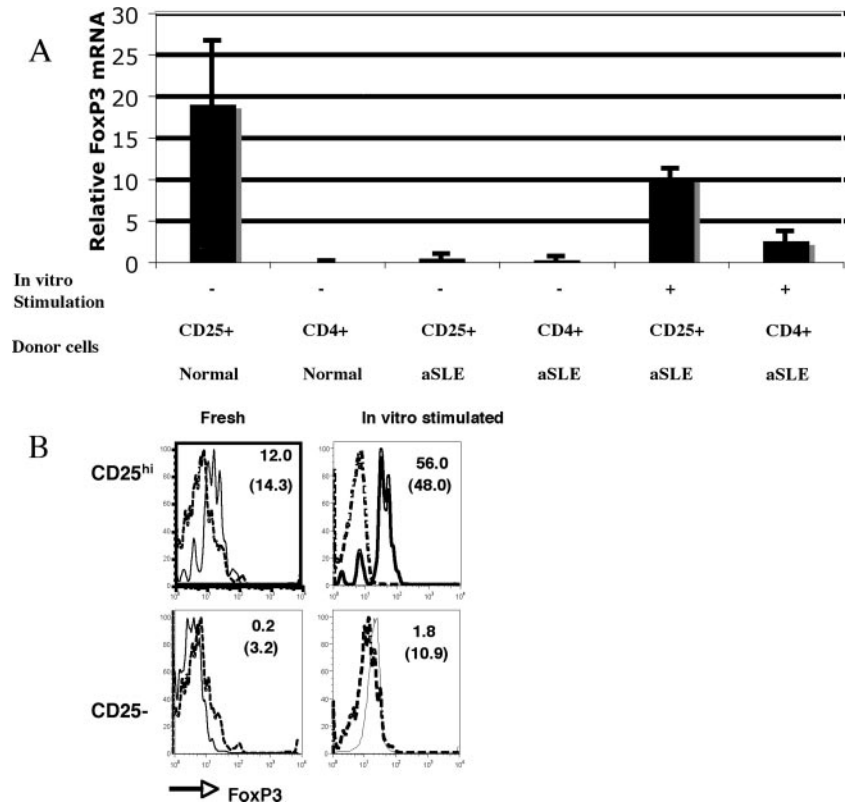
we found no statistically significant difference ($p = 0.37$) compared with normal donors (Fig. 5). Moreover, when suppressive function of CD4+CD25high Tregs from active and inactive SLE patients was compared (Fig. 3 vs Fig. 5), a significant difference was noted ($p < 0.0001$). Of note, Tregs from quiescent 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 8. CD4+CD25high Tregs from active SLE recover their suppressive function after in vitro activation. CD4+CD25− and CD4+ CD25high T cells were sorted as described from normal donors and from subjects with active SLE. Freshly sorted CD4+CD25− T cells and CD4+CD25high Tregs from active lupus were incubated with anti-CD3 mAb at 1 μg/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.
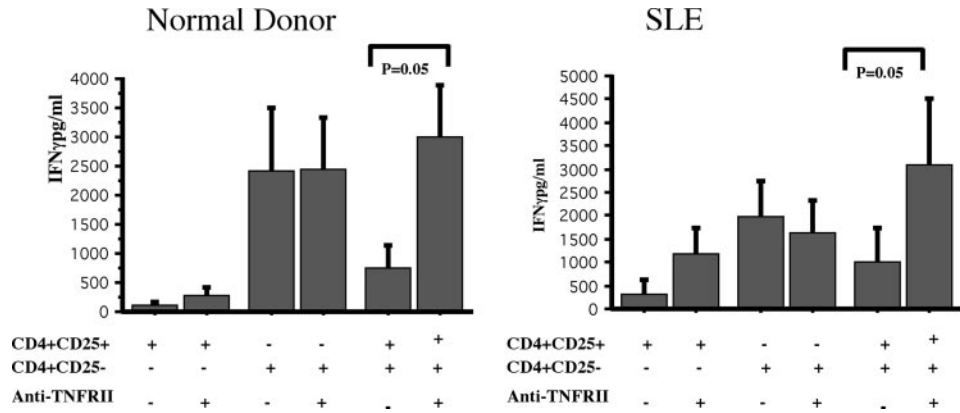
FIGURE 9. Inhibition of IFN-γ secretion by CD4$^+$CD25^{high} Treg is abrogated by cross-linking TNFRII. Previously activated T cells that had up-regulated surface TNFRII expression were used for in vitro regulatory assays. CD4$^+$CD25$^-$ T cells (5 × 10^4/well) or CD4$^+$CD25^{high} Tregs (5 × 10^4/well) 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.

$r^2 = 0.388$) 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 hyporesponsiveness 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
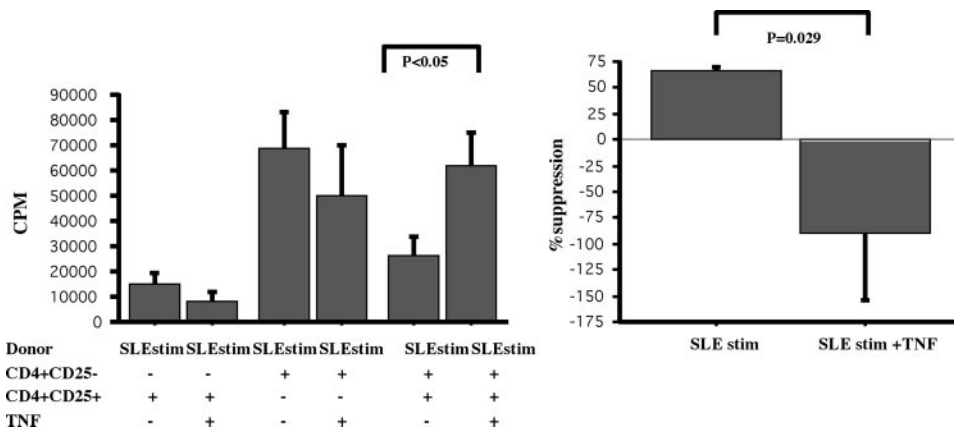
FIGURE 10. Incubation with TNF suppresses the subsequent ability of CD4$^+$CD25^{high} Tregs to inhibit the proliferation of CD4$^+$CD25$^-$ T cells. Previously activated CD4$^+$CD25$^-$ T cells and CD4$^+$CD25^{high} Tregs that had up-regulated surface TNFRII expression were used for in vitro regulatory assays. CD4$^+$CD25$^-$ T cells (5 × 10^4/well) or CD4$^+$CD25^{high} Treg (5 × 10^4/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, [^{3}H]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^{high} Tregs. It was next determined whether the regulatory function of CD4$^+$CD25^{high} 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^{high} 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^{high} 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^{high} 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^{high} 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^{high} Tregs. More recently, a study reported a numeric decrease but preserved in vitro function of CD4$^+$CD25^{high} 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^{high} 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^{high} Tregs from SLE could become sup-

pressive 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^{high} 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^{high} 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^{high} 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^{high} Treg.

It was important to document the fact that the CD4$^+$CD25^{high} 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^{high} Tregs in patients with active SLE were activated. Thirdly, the CD4$^+$CD25^{high} 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^{high} T cells from SLE patients was significantly less than that of CD4$^+$CD25$^-$ effector cells. The degree of responsiveness noted by all CD4$^+$CD25^{high} 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^{high} 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^{high} 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$^+$CD25^{high} 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$^+$CD25^{high} Treg from patients with SLE. Similarly, high concentrations of TNF inhibited the function of CD4$^+$ CD25^{high} Tregs, consistent with an action mediated by TNFRII (39–41). These results clearly show that CD4$^+$CD25^{high} 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$^+$CD25^{high} Tregs correlated with disease activity as measured by SLEDAI in lupus patients. Moreover, FoxP3 expression was up-regulated in CD4$^+$ CD25^{high} Tregs 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 CD25^{high}CD4$^+$ T cells, such as B lymphocyte-induced maturation protein 1 (Blimp-1) (42) or gene related in anergy lymphocytes (GRAIL) (43) or an as yet unidentified molecule along with FoxP3 may be essential for their suppressive function.

Finally, we should note that in vitro activation of CD4$^+$CD25$^-$ effector cells did not routinely lead to up-regulation of FoxP3 or suppressive activity. Previous investigators have noted that in vitro stimulation can lead to up-regulation of FoxP3 expression (44) but differ as to whether suppressive activity can be induced (13). This may relate to the reagents used to detect FoxP3 because we noted that there may be some possible FoxP3 detected with some available mAb but not others. In addition, aspects of the culture system, such as the mode of stimulation, presence of APCs, or levels of contaminating TGFβ may alter the results. In the current results, in vitro stimulation of CD4$^+$CD25$^-$ effectors did not routinely up-regulate FoxP3 expression or confer suppressive activity. However, the same mode of stimulation clearly increased the regulatory function of CD4$^+$CD25^{high} Tregs from patients with active SLE, consistent with the reversible nature of the regulatory defect in these patients.

In summary, we have provided evidence for a reversible functional defect in the CD4$^+$CD25^{high} Treg in SLE. Excessive TNF production and diminished IL-2 production may contribute to this defect. Based on these results, it becomes conceivable to design strategies to amplify the decreased function of Tregs in patients with active SLE by specific therapeutic interventions currently available, such as low-dose IL-2 or TNF blocking agents.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Van Parijs, L., and A. K. Abbas. 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 280: 243–248.

2. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273–280.

3. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101: 455–458.

4. Shevach, E. M. 2002. CD4$^+$CD25$^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.

5. Sakaguchi, S. 2004. Naturally arising CD4$^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.

6. Godfrey, W. R., Y. G. Ge, D. J. Spoden, B. L. Levine, C. H. June, B. R. Blazar, and S. B. Porter. 2004. In vitro-expanded human CD4$^+$CD25$^+$ T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. *Blood* 104: 453–461.

7. Valencia, X., G. Stephens, R. Goldbach-Mansky, M. Wilson, E. M. Shevach, and P. E. Lipsky. 2006. TNF down-modulates the function of human CD4$^+$CD25^{high} T regulatory cells. *Blood* 108: 253–261.

8. Jonuleit, H., E. Schmitt, H. Kakirman, M. Stassen, J. Knop, and A. H. Enk. 2002. Infectious tolerance: human CD25$^+$ regulatory T cells convey suppressor activity to conventional CD4$^+$ T helper cells. *J. Exp. Med.* 196: 255–260.

9. Levings, M. K., R. Sangregorio, C. Sartirana, A. L. Moschin, M. Battaglia, P. C. Orban, and M. G. Roncarolo. 2002. Human CD25$^+$CD4$^+$ T suppressor cell clones produce transforming growth factor β, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J. Exp. Med.* 196: 1335–1346.

10. 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.

11. 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.

12. 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.

13. Gavin, M. A., T. R. Torgerson, E. Houston, P. DeRoos, W. Y. Ho, A. Stray-Pedersen, E. L. Ocheltree, P. D. Greenberg, H. D. Ochs, and A. Y. Rudensky. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc. Natl. Acad. Sci. USA* 103: 6659–6664.

14. Cohen, P. L. T- and B-cell abnormalities in systemic lupus. 1993. *J. Invest. Dermatol.* 100: S69–S72.

15. Mills, J. A. 1994. Systemic lupus erythematosus. *N. Engl. J. Med.* 330: 1871–1879.

16. Kevil, C. G., and D. C. Bullard. 1999. Roles of leukocyte/endothelial cell adhesion molecules in the pathogenesis of vasculitis. *Am. J. Med.* 106: 677–687.

17. Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim. 2005. Cutting edge: direct suppression of B cells by CD4$^+$CD25$^+$ regulatory T cells. *J. Immunol.* 175: 4180–4183.

18. Lim, H. W., P. Hillsamer, and C. H. Kim. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J. Clin. Invest.* 114: 1640–1649.

19. Koonpaew, S., S. Shen, L. Flowers, and W. Zhang. 2005. LAT-mediated signaling in CD4$^+$CD25$^+$ regulatory T cell development. *J. Exp. Med.* 203: 119–129.

20. 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.

21. 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.

22. Miyara, M., Z. Amoura, C. Parizot, C. Badoual, K. Dorgham, S. Trad, D. Nochy, P. Debre, J. C. Piette, and G. Gorochov. 2005. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J. Immunol.* 175: 8392–8400.

23. 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.

24. Hochberg, M. C. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 40: 1725.

25. 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.

26. Geppert, T. D., and P. E. Lipsky. 1988. Activation of T lymphocytes by immobilized monoclonal antibodies to CD3: regulatory influences of monoclonal antibodies to additional T cell surface determinants. *J. Clin. Invest.* 81: 1497–1505.

27. 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.

28. Iellem, A., M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4$^+$CD25$^+$ regulatory T cells. *J. Exp. Med.* 194: 847–853.

29. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. F. de St Groth, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ T reg cells. *J. Exp. Med.* 203: 1701–1711.

30. 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.

31. Baecher-Allan, C., V. Viglietta, and D. A. Hafler. 2002. Inhibition of human CD4⁺CD25⁺ʰⁱᵍʰ regulatory T cell function. *J. Immunol.* 169: 6210–6217.

32. Alvarado-Sanchez, B., B. Hernandez-Castro, D. Portales-Perez, L. Baranda, E. Layseca-Espinosa, C. Abud-Mendoza, A. C. Cubillas-Tejeda, and R. Gonzalez-Amaro. 2006. Regulatory T cells in patients with systemic lupus erythematosus. *J. Autoimmun.* 27: 110–118.

33. 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.

34. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.

35. Skapenko, A., J. R. Kalden, P. E. Lipsky, and H. Schulze-Koops. 2005. The IL-4 receptor α-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25⁺CD4⁺ regulatory T cells from CD25⁻CD4⁺ precursors. *J. Immunol.* 175: 6107–6116.

36. Tsokos, G. C., J. P. Mitchell, and Y. T. Juang. 2003. T cell abnormalities in human and mouse lupus: intrinsic and extrinsic. *Curr. Opin. Rheumatol.* 15: 542–547.

37. Aringer, M., and J. S. Smolen. 2004. Tumour necrosis factor and other proin-flammatory cytokines in systemic lupus erythematosus: a rationale for therapeutic intervention. *Lupus* 13: 344–347.

38. Jacob, C. O., Z. Fronek, G. D. Lewis, M. Koo, J. A. Hansen, and H. O. McDevitt. 1990. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor α: relevance to genetic predisposition to systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA* 87: 1233–1237.

39. Decoster, E., B. Vanhaesebroeck, P. Vandenabeele, J. Grooten, and W. Fiers. 1995. Generation and biological characterization of membrane-bound, uncleavable murine tumor necrosis factor. *J. Biol. Chem.* 270: 18473–18478.

40. Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich. 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83: 793–802.

41. Grell, M., H. Wajant, G. Zimmermann, and P. Scheurich. 1998. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* 95: 570–575.

42. Martins, G. A., L. Cimmino, M. Shapiro-Shelef, M. Szabolcs, A. Herron, E. Magnusdottir, and K. Calame. 2006. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat. Immunol.* 7: 457–465.

43. Soares, L., C. Seroogy, H. Skrenta, N. Anandasabapathy, P. Lovelace, C. D. Chung, E. Engleman, and C. G. Fathman. 2004. Two isoforms of otubain 1 regulate T cell anergy via GRAIL. *Nat. Immunol.* 5: 45–54.

44. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J. Clin. Invest.* 112: 1437–1443.