The Journal of Immunology



# Regulatory T Cell (Treg) Subsets Return in Patients with Refractory Lupus following Stem Cell Transplantation, and TGF-β -Producing $CD8^+$ Treg Cells Are Associated with Immunological Remission of Lupus

Li Zhang, Anne M. Bertucci, Rosalind Ramsey-Goldman, Richard K. Burt and Syamal K. Datta

*J Immunol* 2009; 183:6346-6358; Prepublished online 19 October 2009;
doi: 10.4049/jimmunol.0901773
http://www.jimmunol.org/content/183/10/6346



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Regulatory T Cell (Treg) Subsets Return in Patients with Refractory Lupus following Stem Cell Transplantation, and TGF-$\beta$-Producing CD8$^+$ Treg Cells Are Associated with Immunological Remission of Lupus[1]

**Li Zhang,* Anne M. Bertucci,* Rosalind Ramsey-Goldman,* Richard K. Burt,† and Syamal K. Datta[2]*‡**

**Compared with conventional drug therapy, autologous hemopoietic stem cell transplantation (HSCT) can induce very-long-term remission in refractory lupus patients. Herein, we show that in posttransplant patients, both CD4$^+$CD25$^{high}$FoxP3$^+$ and an unusual CD8$^+$FoxP3$^+$ Treg subset return to levels seen in normal subjects; accompanied by almost complete inhibition of pathogenic T cell response to critical peptide autoepitopes from histones in nucleosomes, the major lupus autoantigen from apoptotic cells. In addition to a stably sustained elevation of FoxP3, posttransplant CD8 T cells also maintained markedly higher expression levels of latency-associated peptide (LAP), CD103, PD-1, PD-L1, and CTLA-4, as compared with pretransplant CD8 T cells that were identically treated by a one-time activation and rest in short-term culture. The posttransplant CD8 regulatory T cells (Treg) have autoantigen-specific and nonspecific suppressive activity, which is contact independent and predominantly TGF-$\beta$ dependent. By contrast, the pretransplant CD8 T cells have helper activity, which is cell contact dependent. Although CD4$^+$CD25$^{high}$ Treg cells return during clinical remission of conventional drug-treated lupus, the posttransplant patient's CD8 Treg cells are considerably more potent, and they are absent in drug-treated patients in whom CD4 T cell autoreactivity to nucleosomal epitopes persists even during clinical remission. Therefore, unlike conventional drug therapy, hemopoietic stem cell transplantation generates a newly differentiated population of LAP$^{high}$CD103$^{high}$ CD8$^{TGF\text{-}\beta}$ Treg cells, which repairs the Treg deficiency in human lupus to maintain patients in true immunological remission.** ***The Journal of Immunology,*** **2009, 183: 6346–6358.**

Systemic lupus erythematosus (SLE)[3] is characterized by autoantigen-driven interactions between autoimmune Th cells and B cells, leading to production of somatically mutated IgG autoantibodies against apoptotic nuclear Ags (1–6). Nucleosomes and their histone peptides are major lupus immunogens, and five critical autoepitopes that trigger interaction between lupus T and B cells in patients and mice with SLE are in histone (H) regions, H1′$_{22-42}$, H3$_{82-105}$, H3$_{115-135}$, H4$_{16-39}$, and H4$_{71-94}$ (7–11). These histone peptide epitopes accelerate lupus nephritis upon immunization, but they delay or even reverse disease upon tolerization at high or low doses, in mice with spontaneous SLE (10, 11).

Although the immunology of SLE is complex, defect(s) in immunoregulation probably plays a crucial role in expansion of autoimmune cells. Most work has focused on CD4$^+$CD25$^+$ regulatory T (Treg) cells (12–19). However, CD8$^+$ Tregs, originally called T suppressors (20), have not been well defined. CD8$^+$ CD28$^-$ Treg cells that inhibit APCs by contact (21) or by cytokines, IFN-$\gamma$ (22), and/or IL-10 (23) in allogeneic transplant situations, as well as cytotoxic CD8 T cells that are restricted to examples of organ-specific autoimmunity (24), have been reported. Furthermore, TGF-$\beta$ plus IL-2 treatment or TGF-$\beta$-treated APCs can induce CD8 Treg cells, which may produce IL-4, TGF-$\beta$, or IL-10 (19, 25).

The percentage of CD4$^+$CD25$^+$T cells is significantly decreased in patients with lupus (26–28), and in lupus-prone (NZB $\times$ NZW)F$_1$ (BWF$_1$) and (SWR $\times$ NZB)F$_1$ (SNF$_1$) mice (29), but autoantigen specificity of such cells was not determined. Low dose tolerance in SNF$_1$ mice with nucleosomal histone peptide epitope, H4$_{71-94}$, H4$_{16-39}$, or H1′$_{22-42}$ that contain both MHC class II and class I binding motifs, induced CD8$^+$, as well as CD4$^+$CD25$^+$ Treg subsets containing autoantigen-specific Treg cells which suppress responses of pathogenic lupus T cells to nucleosomal epitopes, and reduce autoantibody production by inhibiting the T cell help to nuclear autoantigen-specific B cells (11, 30). High doses of an anti-DNA or other Ig-related peptide (pConsensus or hCDR1) could suppress lupus in BWF$_1$ mice and expand CD4 Treg cells in vivo; pConsensus could also induce human functional CD4$^+$CD25$^{high}$ Treg cells in vitro (31–33). Alternatively, in lupus-susceptible MRL mice, CD4$^+$CD25$^-$ T

*Division of Rheumatology and Division of †Immunotherapy, Department of Medicine, and ‡Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

Received for publication June 3, 2009. Accepted for publication September 10, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grant RO1 AR39157 (to S.K.D.), Lupus Foundation of America (to R.K.B.), and National Institutes of Health Grant P60 AR30692 (to R.R.G.).

[2] Address correspondence and reprint requests to Dr. Syamal K. Datta, Division of Rheumatology, Northwestern University Feinberg School of Medicine, 240 East Huron Street, McGaw No. M300, Chicago, IL 60611. E-mail address: skd257@northwestern.edu

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; Treg, regulatory T cells; HSCT, hemopoietic stem cell transplantation; SLEDAI, SLE disease activity index; CRI, cytokine response index; LAP, latency-associated peptide; CFC, cytokine flow cytometry; aSLE, active SLE; iSLE, inactive SLE; CD62L, L-selectin.



www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901773

cells show reduced sensitivity to suppression by $CD4^+CD25^+$ Treg cells (34), as found in some patients with active lupus (35). $CD4^+CD25^-$ T cells also resist inhibition by $CD4^+CD25^+$ Treg or TGF-$\beta$ in Cbl-b-deficient (Cbl-b-/-) C57BL/6 mice (36). Indeed, human lupus $CD4^+$ T cells are resistant to anergy, associated with reduction of Cbl/Cbl-b (37, 38), and they also resist activation induced apoptosis by increasing cyclooxygenase-2 and c-FLIP expression (39). The $CD8^+$ T cells are also abnormal in lupus patients, being less competent in cytotoxic activity, and they actually help B cells to make autoantibodies (40–42). $CD8^+$ T cells from healthy subjects, derived by culturing with IL-2 and GM-CSF, mediated nonspecific suppression by IFN-$\gamma$ and IL-6, and this Treg function was impaired in active lupus (43). However, pathogenic IgG autoantibodies belong to Th1- or IFN-$\gamma$-dependent subclasses, and IL-6 production is actually increased in lupus, which might contribute to differentiation of autoimmune Th cells into $CD40L^{high}$, IL-17/IL-21 producing inflammatory Th17 and follicular Th cells with concomitant decrease in Treg cells (30, 44–49). Thus, very little is known about how $CD8^+$ Treg cells could control human lupus, although animal models indicate their importance (11, 19, 50). Moreover, it was reported $CD4^+CD25^+$ Treg cells increase after stem cell transplant in patients with juvenile idiopathic arthritis, but no studies on $CD8^+$ Treg were done (51).

Compared with conventional drug treatment, autologous hemopoietic stem cell transplantation (HSCT) can induce very-long-term remission in refractory lupus patients. There are >70 patients who have undergone autologous HSCT at Northwestern University (Chicago, IL) for refractory SLE with promising outcomes in terms of treatment safety and disease remission as reported previously (52–54). Stem cell transplantation for autoimmune disease is a three-step procedure (55, 56): 1) harvesting of autologous hemopoietic/immune stem cells from a patient's blood; 2) ablation or near ablation of a patients immune system (T and B cells) with chemotherapy (cyclophosphamide) and antilymphocyte Abs; and 3) reinfusion of autologous immune stem cells to hasten hemopoietic and immune recovery. The rationale of an autologous hemopoietic (immune cell) transplant is to immune ablate and then immune reset by regenerating T and B cells from the stem cell compartment (55). Importantly, immune ablation is achieved by using a nonmyeloablative regimen that specifically targets only the immune compartment and does not destroy the entire bone marrow compartment, which makes the therapy safer than autologous HSCT performed with myeloablative intent to destroy any leukemia containing bone marrow compartment in leukemic patients. The exact mechanism/s by which self-tolerance returns after HSCT therapy is still undefined.

In this study, we focus on the role of CD8 Treg cells in HSCT therapy. We studied 15 pre- and 15 posttransplant patients with SLE, and some of them were prospectively followed for up to 8 years after receiving HSCT. Our results provide evidence that the TGF-$\beta$-producing $CD8^+LAP^{high}CD103^{high}FoxP3^+$ Treg subset (in which LAP is latency-associated peptide) return in posttransplant patients and play very important role of controlling autoimmune responses in SLE.

## Materials and Methods

### *Subjects*

We enrolled 15 pre- and 15 posttransplant (nonmyeloablative HSCT), as well as 10 conventional drug-treated lupus patients (mean age, 34 years; range, 16–58) who fulfilled the American College of Rheumatology revised criteria for the classification of SLE (57) and 15 healthy donors (age 23–57 years) who had no history of autoimmune disease (normal controls). Disease activity was scored based on the SLE disease activity index (SLEDAI; Ref. 58). Patients with SLEDAI <3 were considered to have inactive disease (remission), and those with SLEDAI ≥3 were considered to have active disease. Clinically relevant demographic profile of the patients is shown in Tables I and II. The study was approved by the Institutional Review Board of Northwestern University.

Table I. *HSCT patient demographic: clinical and treatment status of pre- and posttransplant SLE patients in the study*

| Patients Code | Status | Age/Sex/Race | SLEDAI | Current Treatment |
|---|---|---|---|---|
| 1 | Pre-t | 24/F/W | 28 | Pred.,[a] CTX, MMF, HCQ |
| 1-B | Post-t 3 mo | | 0[b] | Pred., MMF |
| 2 | Pre-t | 40/F/W | 12 | Pred., CTX, HCQ |
| 2-B | Post-t 3 yr | | 1 | Pred., MMF |
| 3 | Pre-t | 26/F/W | 6 | MMF |
| 3-B | Post-t 2 yr | | 0 | None |
| 4 | Pre-t | 51/F/W | 26 | Pred., MMF |
| 4-B | Post-t 2 yr | | 0 | HCQ |
| 5 | Pre-t | 42/F/W | 35 | Pred., MMF |
| 5-B | Post-t 2 yr | | 4 | Pred., MMF |
| 6 | Pre-t | 40/F/H | 24 | MMF |
| 6-B | Post-t 2 yr | | 0 | MMF |
| 7 | Pre-t | 31/F/W | 18 | CTX |
| 7-B | Post-t 1 yr | | 0 | None |
| 8 | Pre-t | 47/F/W | 13 | Pred., MMF |
| 8-B | Post-t 6 mo | | 0 | Pred., |
| 9 | Pre-t | 20/F/H | 38 | Pred., CTX, MMF |
| 9-B | Post-t 1 yr | | 0 | None |
| 10 | Pre-t | 21/F/W | 11 | Pred., CTX, MMF, RTX |
| 10-B | Post-t 1 yr | | 0 | None |
| 11 | Pre-t | 40/F/W | 23 | Pred., MMF, RTX |
| 12 | Pre-t | 27/F/ | 8 | Pred., CTX, |
| 13 | Pre-t | 16/F/W | 15 | Pred., AZA, CTX, RTX |
| 14 | Pre-t | 54/F/W | 26 | Pred., MMF, CTX |
| 15 | Pre-t | 22/M | 11 | Pred., MMF, CTX, RTX |
| 16 | Post-t 5 yr | 27/F | 0* | None |
| 17 | Post-t 8 yr | 48/F/W | 1 | MMF, HCQ |
| 18 | Post-t 5 yr | 51/F | 0 | MMF |
| 19 | Post-t 2 yr | 46/F | 0 | Pred. |
| 20 | Post-t 8 yr | 34/F/W | 0 | None |

[a] Pre-t, Pretransplant; Post-t, posttransplant; W, white/Caucasian; H, Hispanic; Pred., prednisone or steroids; HCQ, hydroxychloroquine (Plaquenil); MMF, mycophenolate Mofetil (CellCept); AZA, azathioprine (Imuran); CTX, cyclophosphamide (Cytoxan); RTX, rituximab.
[b] No score compiled, but clinically in remission.

### *mAbs and cytokines*

For immunostaining, mouse PE-, FITC-, PerCP-, and allophycocyanin-conjugated mAbs against human CD3 (UCHT1), CD28 (L293), CD4 (SK3), CD8 (SK1), CD25 (M-A053), CD103 (Ber-ACT8), CD56 (B159), CD27 (M-T271), L-selectin (CD62L; Dreg56), CTLA-4 (BNI3), IFN-$\gamma$ (25723.11), IL-13 (JES10-5A2), CD127 (HIL-7R-M21), and corresponding mouse isotype controls (obtained from BD Biosciences or BD Pharmingen) were used. FITC-conjugated PD-1 (MIH4) and allophycocyanain conjugate PD-L1 (MIH1) as well as corresponding isotype control were purchased from eBioscience. PE-conjugated LAP and FITC-conjugated IL-10, with corresponding isotype controls, and IL-2, IL-7, and IL-15 were purchased from R&D Systems for cell culture. Anti-TGF-$\beta$, anti-IL-13,

Table II. *Conventionally treated patient demographic: clinical and treatment status of conventional drug-treated SLE patients in the study*

| Patient Code | Age/Sex | SLEDAI Score | Current Treatment |
|---|---|---|---|
| 1 | 42/F | 0 | HCQ[a] |
| 2 | 36/F | 0 | MTX, HCQ |
| 3 | 53/F | 0 | HCQ |
| 4 | 58/F | 0 | Pred., HCQ |
| 5 | 54/F | 0 | Pred., HCQ |
| 6 | 38/F | 0 | None |
| 7 | 47/F | 2 | AZA, LEF |
| 8 | 51/F | 0 | None |
| 9 | 53/F | 6 | HCQ |
| 10 | 34/F | 2 | None |

[a] HCQ, hydroxychloroquine (Plaquenil); Pred., prednisone or steroids; AZA, azathioprine (Imuran); MTX, methotrexate; LEF, leflunomide (Arava).

and anti-IL-10 (R&D Systems) neutralization Abs were used to test for blocking suppression.

### *Peptides and nucleosomes*

All peptides were synthesized by F-moc chemistry and their purity was checked by amino acid analysis by the manufacturers (Chiron Mimotopes and New England Peptide). Nucleosomes were prepared as described (6, 7).

### *Cell preparation*

PBMCs from patients or healthy donors were isolated as described (7, 9). Aliquots of these cells were used to make short-term T cell lines, and other cells were used directly for stimulation with Ags. After an initial period of rest and recovery with IL-2 (10 U/ml), short-term CD4 and CD8 T cell lines were derived from PBMCs of lupus and normal subjects under identical conditions by one-time stimulation with anti-CD3 and anti-CD28 Abs, with IL-2, IL-7, and IL-15 in culture, and then resting for 10 days to remove any confounding effects of cytokines, anti-T cell autoantibodies, autoantigenic stimulation, and drugs (5, 9, 37, 39). The short-term line T cells from lupus patients retain autoimmune function and other immune abnormalities characteristic of lupus (9, 37, 39, 41). To obtain $CD4^+CD25^-$ T cells, CD4 T cells were stained with anti-CD4-PerCP and anti-CD25-PE or the isotype control Ab conjugated with PE for 30 min at 4°C, and $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were purified using a MoFlo high-speed cell sorter (DakoCytomation) to a purity of >98%. In some experiments, $CD4^+CD25^{high}$ T cells, or $CD4^+CD127^-CD25^{high}$ T cells were purified from PBMCs or short-term CD4 lines by cell sorting using published methods (59).

### *Flow cytometry*

T cells from patients and healthy donors were stained with CD4-PerCP plus CD25-FITC, or CD8-allophycocyanin plus CD28-FITC and PE-conjugated anti-CD103, anti-CD56, anti-CD27, anti-CD62L, anti-PD-1, and anti-PD-L1 at 4°C for 30 min in the dark. Matched PE-conjugated IgG isotype controls were used. To stain for PD-1, PD-L1, and CTLA-4, T cells were stimulated with anti-CD3/anti-CD28 for 24 h in the presence of 20 U/ml IL-2. For maximal (surface and intracellular) staining of CTLA-4, T cells were cultured with 0.1 mM pervanadate (phosphatase inhibitor) for 15 min at room temperature in the dark (37), washed once in complete RPMI, and then stained first for surface Ags. Next, they were fixed, permeabilized, and then incubated with anti-CTLA-4 or the isotype control Ab at 37°C for 1 h. To detect intracellular levels of FoxP3 in the CD4 and CD8 T cells, the cells were first stained for surface markers by anti-CD4-PerCP, CD8-allophycocyanin, CD25-FITC, or CD28-FITC, and then the cells were stained with FoxP3-PE or the isotype control (PCH101; eBioscience) according to the manufacturers after fixation and permeabilization. Data for 20,000 cells were collected using FACSCalibur or LSR II flow cytometer (BD Biosciences), and analyzed by BD CellQuest or Tree Star FlowJo.

### *Detection of CD4 T cells response to autoepitopes*

Fresh PBMC samples were cultured with nucleosomal histone peptide epitopes ($H1'_{22-42}$, $H3_{82-105}$, $H3_{115-135}$, $H4_{16-39}$, $H4_{71-94}$) or whole nucleosomes in the presence of IL-7, IL-15, and anti-CD28/CD49d (BD Biosciences) for 3 days; golgistop brefeldin A (final concentration, 1 $\mu$g/ml; Sigma-Aldrich) was added to the wells for the last 17 h of incubation, and then surface-stained with anti-CD4, anti-CD8 and intracellularly with anti-IFN-$\gamma$ or IL-13. Cytokine response index (CRI) ratios were calculated by dividing values for corresponding staining of resting control (without peptide or nucleosome stimulation). CRI <2 is considered to be at background level (9). Viable cells gated for being $CD4^+$ or $CD8^+$ were analyzed for IFN-$\gamma$ or IL-13 production by cytokine flow cytometry (CFC; BD Biosciences). We did not study IL-4 production, because available reagents are not suitable for this assay.

### *Suppressor assays*

To wash out confounding effects of extrinsic factors influencing a lupus patient's T cells (5, 9, 39); short-term T cell lines were derived from PBMCs by one-time stimulation by anti-CD3, anti-CD28, and IL-2, and then rested for 10 days before starting the suppression assays. We used these short-term $CD4^+$ and $CD8^+$ T cell lines, derived under identical conditions, from lupus patients or healthy donors to measure $CD8^+$ Treg suppressor functions. $CD4^+CD25^-$ or total $CD4^+$ T cells from the lines were used as target (responder) cells that were stimulated with anti-CD3/anti-CD28 for 3 days in the presence of 20 U/ml IL-2 and then rested for 10 days with IL-2 before starting the suppression assay. CFSE (2 $\mu$M; Molecular Probes) labeled target (responder) cells ($5 \times 10^5$ cells/well) were stimulated with 50 U/ml IL-2, or an equal number of irradiated allogeneic APCs (3000 rad), and cocultured with different numbers of autologous CD8 line T cells (the ratios of target T cells to CD8 T cells being 1:1, 1:0.5, 1:0.25, 1:0.125, 1:0.0625, and 1:0.03125), either in contact or separated by cell-impermeable membranes of transwell plates (Corning Costar; 0.4 $\mu$m pore size), and proliferation (CFSE dilution) of gated CD4 T cells was assessed by flow cytometry after culturing for 4 days. The percent of inhibition was calculated as [(% of proliferated target T cells cultured alone − % of proliferated target T cells in coculture with CD8 T cells)/(% of proliferated target T cells cultured alone)] × 100. In experiments to block suppressive effect of the post-CD8 T cells, XViVo-20 serum-free medium (Lonza) supplemented with penicillin-streptomycin was used and different concentrations of anti-TGF-$\beta$, anti-IL-10, or anti-IL-13 neutralization Ab, or isotype control were added at the day 0 and maintained throughout the experiment. The percent of blocking of suppression was calculated as [(% of proliferated target T cells in cultures with blocking Ab − % of proliferated target T cells cultured with respective isotype control)/(% of proliferated target T cells cultured in medium − % of proliferation target T cells cultured with isotype control)] × 100.

To measure the autoantigen-specific helper or suppression function of CD8 T cells, posttransplant PBMC were cocultured with autologous pretransplant or posttransplant CD8 T cells at ratios of 1:0.5 and stimulated with nucleosomal histone peptide epitopes or whole nucleosomes. In addition, PBMCs from drug-treated lupus patients were cocultured with allogeneic pre- or posttransplant CD8 T cells and stimulated with autoantigens. Intracellular cytokine response of target CD4 T cells in the PBMC was measured by CFC, as mentioned above.

### *Statistical analysis*

Data analyses were performed using Prism 4.0 software (GraphPad Softwear). Comparisons were performed by Student *t* tests. Results are expressed as mean ± SD; *p* values <0.05 were considered significant.

## Results

### *Autologous HSCT reduced IFN-γ response and increased immunoregulatory IL-13 response of CD4 T cells in fresh PBMCs to major peptide autoepitopes from nucleosomes*

Most pathogenic autoantibodies in lupus that fix complement and bind to activating FcγR on inflammatory cells belong to the IgG subclasses that are Th1 or IFN-γ dependent (7). Although T cells in freshly obtained lupus PBMCs proliferate poorly when tested immediately ex vivo for reasons mentioned above (4, 5, 9, 37, 39), the CD4 T cells in lupus patients respond strongly to the critical histone peptides in nucleosomes by producing IFN-γ, and those peptide epitopes also accelerate lupus in mouse models upon immunization (7–9). Herein, we wanted to know whether the CD4 T cells in fresh PBMCs from posttransplant lupus patients can still respond to those autoantigens. The results (Fig. 1) show that Th1 type IFN-γ response of CD4 T cells to critical peptide epitopes ($H1'_{22-42}$, $H3_{82-105}$, $H3_{115-135}$, $H4_{16-39}$, $H4_{71-94}$) and nucleosomes drops to background (normal) levels in the posttransplant lupus patients who were in remission. Alternatively, the counter-inflammatory IL-13 response of those T cells rises (posttransplant vs normal, $p < 0.008$ to $p < 0.01$; posttransplant vs pretransplant, $p < 0.05$ to $p < 0.01$). By contrast, the IFN-γ response of CD4 T cells in pretransplant lupus patients' PBMCs to the peptide autoepitopes was elevated (pretransplant vs normal, $p < 0.001$ to $p < 0.01$; pretransplant vs posttransplant, $p < 0.05$ to $p < 0.002$), similar to patients with active lupus that we had previously reported (9), but they had low IL-13 response (Fig. 1*A*). In this regard, we had previously found that $CD4^+$ T cells in lupus patients, who were in clinical remission after conventional drug therapy, still retained IFN-γ response to the peptide epitopes (9). Therefore, the posttransplant remission patients are different with regard to mechanisms down-regulating the autoimmune response.

### *CD8 T cells are the major contributors to the suppression of autoreactive response in posttransplant patients*

To address the question whether the reduced IFN-γ response of $CD4^+$ T cells to the peptide epitopes in the posttransplant lupus

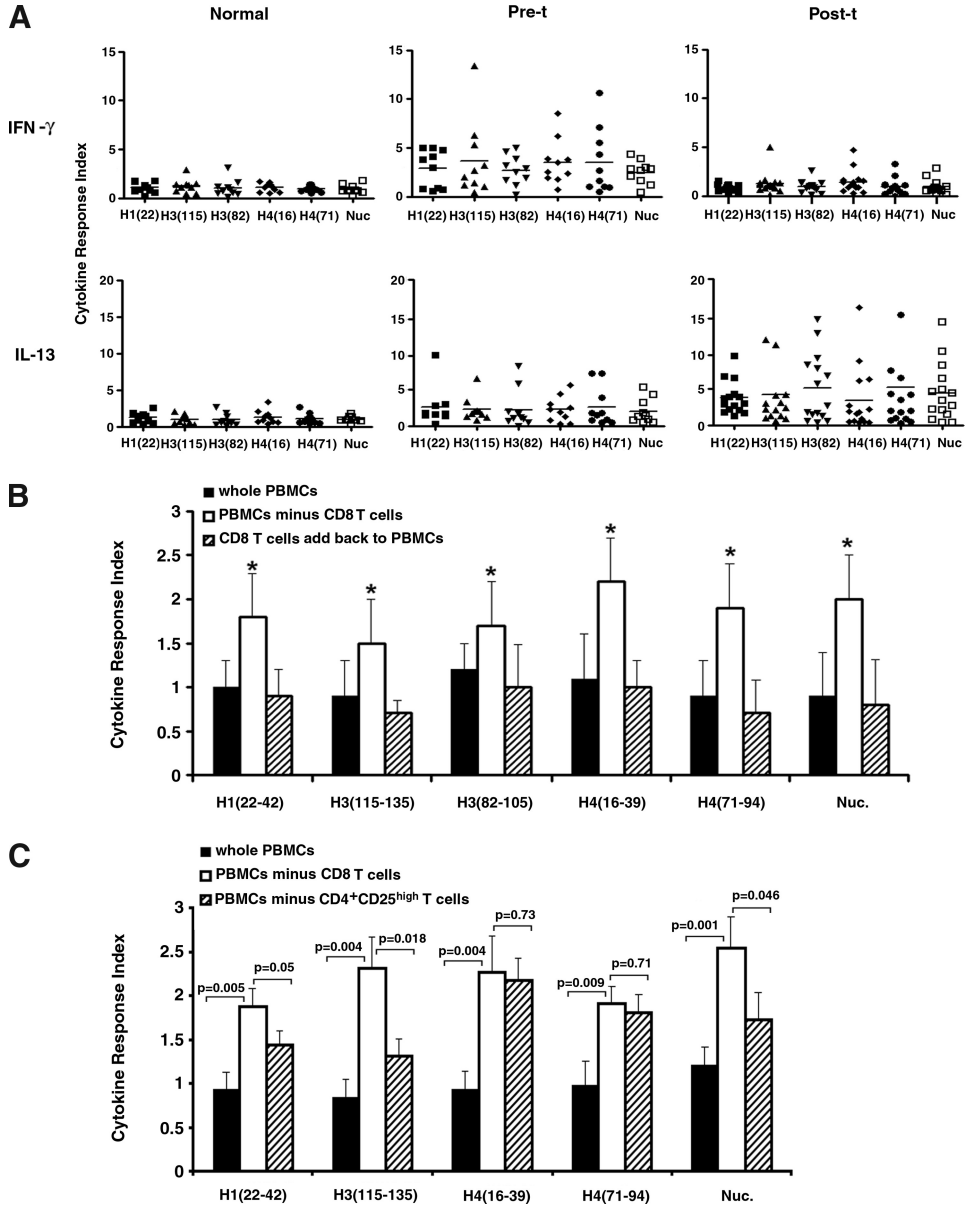


**FIGURE 1.** Autologous HSCT (posttransplant; Postt) reduced IFN-γ but increased IL-13 responses of $CD4^+$ T cell in fresh PBMCs to nucleosomal autoepitopes. *A*, Fresh PBMC samples were cultured with nucleosomal histone peptide epitopes or whole nuclesomes (Nuc.) in the presence of IL-2, IL-7, IL-15, and anti-CD28/CD49d for 3 days and then stained for surface CD4 and intracellular IFN-γ or IL-13 for flow cytometry analysis of gated $CD4^+$ T cells. Peptide epitope designations are abbreviated to fit the *x*-axis, for example $H1'_{22-42}$ is H1, $H3_{115-135}$ is H3(115), and so on. The baseline values of the IFN-γ response of CD4 T in PBMCs (cultured in medium only) were 0.06–0.27, and the baseline values of IL-13 response of CD4 T (medium only) were 0.07–0.34. The CRI ratios were calculated by dividing experimental values by the corresponding values of resting control CD4 cells (without autoantigen stimulation). CRI <2 is considered to be at background. Bar, mean. We used a BRN-3 transcription factor-related peptide, called eluted peptide-2 (sequence DWMEEEEGAQREKE), as a negative control peptide for the histone peptide epitopes (8) and $OVA_{323-339}$ as an irrelevant control Ag for nucleosomes; the positive control was stimulated with anti-CD3. The CRIs for IFN-γ response of CD4 T cells were 0.5–1.6 to eluted peptide-2, 0.4–1.2 to OVA, and 12.7–51 to anti-CD3. The CRIs for IL-13 response of CD4 T cells were 0.4–1.8 to eluted peptide-2, 0.6–1.5 to OVA, and 9.3–46 to anti-CD3. *B*, Posttransplant PBMCs were depleted of CD8 T cells and then cultured with nucleosomal autoepitopes to measure IFN-γ response of CD4 T cells as in *A*, along with two other groups: whole PBMC group; and a group with CD8 T cells added back to PBMC which were depleted of CD8 T cells. Experiments were repeated five times; bars, mean ± SD. ∗, $p < 0.01$. *C*, $CD4^+CD25^{high}$ ($CD25^{hi}$)T cells or CD8 T cells were removed from posttransplant PBMCs by sorting; then the whole PBMCs, or PBMCs without $CD4^+CD25^{high}$ or without CD8 T cells were cultured with nucleosomal autoepitopes to measure the IFN-γ response of CD4 T cells as in *A*. Experiments were repeated three times; bars, mean ± SD.

patients is caused by the suppressive function of CD8 Treg cells or because HSCT caused lower responsiveness of the $CD4^+$ T cells, or both, $CD8^+$ T cells were deleted from the posttransplant PBMCs, and then the intracellular IFN-γs of $CD4^+$ T cell response to peptide epitopes were measured in three groups: group A, total PBMC; group B, PBMCs deleted of $CD8^+$ T cells; and group C, $CD8^+$ T cells added back to group B. The IFN- γ response of $CD4^+$ T cells in the group of PBMCs deleted of $CD8^+$ T cells (group B) to peptide epitopes was significantly higher than that of $CD4^+$ T cells of other two groups (Fig. 1*B*), indicating that CD8 Treg cells play critical role in suppressing the autoreactive CD4 T cells response. $CD4^+CD25^{high}$ Treg cells in the PBMC might also have contributed to suppression because the response was not restored to pretransplant levels after CD8 T cell removal, which we

address in the next section using short-term line T cells. Indeed, when the $CD4^+CD25^{high}$ cell subset (range, 0.9–1.25%) was deleted from posttransplant PBMCs, the IFN-$\gamma$ response of CD4 T cells to some histone epitopes was also significantly restored, but not as strongly as deletion of total CD8 T cells did in the case of some of the peptides and whole nucleosomes (Fig. 1*C*).

### *Increase in percentage of $CD4^+CD25^{high}FoxP3^+$ and $CD8^+FoxP3^+$ and $CD8^+CD103^+$ T cells in short-term T cell lines from posttransplant lupus patients*

Human peripheral blood contains Treg cells, and the transcription factor FoxP3 is critical for Treg cells. To compare the numbers of Treg cells in pre- and posttransplant, as well as conventional drug-treated lupus patients' peripheral blood, we used three-color flow cytometry to distinguish two types of CD8 Treg cell subsets based on the expression of CD28, namely $CD8^+CD28^-FoxP3^+$ and $CD8^+CD28^+FoxP3^+$ cells; and we also examined the $CD4^+CD25^{high}FoxP3^+$ Treg subset. We stained fresh PBMC directly ex vivo, as well as short-term CD4 and CD8 line T cells. To wash out confounding effects of extrinsic factors influencing lupus patients' T cells (5, 9, 39); short-term T cell lines were derived from PBMCs by one-time stimulation by anti-CD3, anti-CD28, and IL-2 and then rested for 10 days. Such short-term line T cells retain the autoimmune characteristics of lupus T cells (5, 9, 39). Fig. 2*A*, shows that the percent of $FoxP3^+$ cells in short-term line $CD8^+$ ($CD28^-$ or $CD28^+$) T cells from posttransplant patients were increased considerably, as compared with pretransplant samples; however, this difference cannot be seen in $CD8^+CD28^-$ cells in fresh PBMCs (Fig. 2*C*), probably because the differences in FoxP3 expression become evident after T cell activation. Nevertheless, the short-term culture T cells were rested following stimulation for 10 days, demonstrating a stable and sustained elevation of FoxP3 expression only in the posttransplant and normal T cells. Although it was believed that the $CD28^-$ phenotype can be used as a T suppressor cell marker (60), our result shows that the percentages of $FoxP3^+$ cells in the $CD8^+$ T cells from posttransplant patients were significantly increased not only in $CD28^-$ subpopulations but also in $CD28^+$; and in contrast to pretransplant patient samples and drug-treated patient samples, $FoxP3^+$ cells were also significantly increased in the $CD4^+$ $CD25^{high}$ T cell lines from posttransplant patients.

We wanted to define the surface marker/s which is/are coexpressed in $FoxP3^+CD8^+$ T cells by flow cytometry. We detected that CD103, PD1, PDL-1, and CTLA-4 (61–63) were all significantly increased in posttransplant CD8 line T cells compared with pretransplant samples (Fig. 2*A*). Although PD-1, PD-L1, and CTLA-4 expression in CD8 line T cells from conventional drug-induced remission patients were not significantly different from those in posttransplant CD8 line T cells, expression of CD103 and intracellular Foxp3 was significantly lower in CD4 and CD8 line T cells from drug-induced remission lupus patients. However, none of the PD-1, PD-L1, CTLA-4, or CD103 markers showed correlated expression with FoxP3 in CD8 T cells at the same time point (data not shown). When CD103 expression was compared in pre- and posttransplant patients' CD8 line T cells after rest periods following TCR activation, different kinetics were observed in both the $CD28^+$ and $CD28^-$ subsets. Results showed that although the CD103 expression were high in CD8 line T cells from posttransplant patients and low in CD8 line T cells from pretransplant patients before anti-CD3/anti-CD28 stimulation, the CD103 level in both pre- and posttransplant CD8 line T cells increased markedly after TCR activation. However, posttransplant CD8 T cells retained high level of CD103 even after cells were rested for 9 days, but the CD103 level of pretransplant CD8 T cells decreased rapidly after cells were rested for only 6 days. These temporal changes in CD103 level showed the same trend in both $CD28^+$ and $CD28^-$ subsets (Fig. 2*B*).

### *HSCT, but not conventional drug therapy, restored suppressive activity in $CD8^+$ T cells of lupus patients*

To determine whether the CD8 T cells had suppressor activity, CD4 line T cells were used as target (responding) cells. In most experiments, $CD25^+$ cells were removed from the $CD4^+$ target (responder) T cell population, in case the $CD4^+CD25^{high}$ cells confound the results of $CD8^+$ Treg activity. In our suppression assay system, we used IL-2 to induce proliferation of the rested CD4 line T cells (target cells), as well as to activate any CD8 line Treg cells, because that is a more physiological stimulus than overwhelming and artificial anti-CD3 stimulation. Allogeneic irradiated APCs were also potent stimulators for T cells, but not consistently, because in some lines they could not stimulate CD4 or CD8 T cells very well. This might have been due to some degrees of MHC match between the allogeneic APCs and the T cells by coincidence. Because it is well known that high dose of IL-2 alone can induce proliferation of TCR-activated and subsequently rested T cells (64, 65), we searched for the optimal IL-2 dose and found that 20, 50, and 100 U/ml IL-2 all can activate CD4 and CD8 line T cells and the suppression results were consistent even at the highest level of IL-2 (dose data not shown), indicating that IL-2 hogging by CD8 T cells was not the cause of suppression. Therefore, 50 U/ml IL-2 was chosen in our suppression assays. Because there is no unique cell surface marker to isolate CD8 Treg cells, total CD8 line T cells were used in coculture with autologous target CD4 T cells at different ratios (ratio of target CD4T to Treg at 1:1, 1:0.5, 1:0.25, 1:0.125, 1:0.0625, and 1:0.03125). We found that ratios of 1:1, 1:0.5, and even 1:0.25 could show a strong suppressive effect (Fig. 3*C*), and the 1:0.5 ratio was used in most of our experiments, which showed that the CD8 T cells from posttransplant patients significantly decreased the proliferative responses of autologous CD4 T cells to a high dose of IL-2 (50 U/ml), or to allogeneic APCs (Fig. 3*D*). Collectively, in the presence of posttransplant CD8 T cells, the percent suppression of proliferation of CD4 target T cells was 35–48%, but this suppressive effect could not be found with conventional drug-induced remission patients' $CD8^+$ T cells. $CD8^+$ T cells from pretransplant lupus patients could not suppress their autologous CD4 T cells proliferation. Instead, those patients' CD8 T cells actually enhanced autologous CD4 T cell proliferation that was induced by IL-2.

### *Posttransplant CD8 T cells even without Treg enrichment, have stronger suppressive function than autologous $CD4^+CD25^{high}$ Treg subset*

We compared the suppressive effects of posttransplant, short-term line, unfractionated $CD8^+$ T cells with $CD4^+CD25^+$ T cells purified from $CD4^+$ line T cells on autologous target cells (short-term line $CD4^+$ T cells as a whole or $CD4^+CD25^-$ T cells purified from it) side by side. $CD4^+CD25^+$ T cells or CD8 T cells were cocultured with the target T cells in cell contact or separated by transwell membrane, and results showed that the posttransplant $CD4^+CD25^+$ T cells had a minor suppressive effect on target T cells proliferation in both coculture conditions (the percentages of suppression were 16% on $CD4^+CD25^-$ target T cells and 18% on total CD4 target T cells), whereas the autologous CD8 Treg cells showed significantly higher inhibitory function (the percentages of suppression were 40% on $CD4^+CD25^-$ target T cells and 36% on total CD4 target T cells; Fig. 4*A*). We found that posttransplant CD4 line T cells were 90% $CD4^+CD127^-$ following the one-time

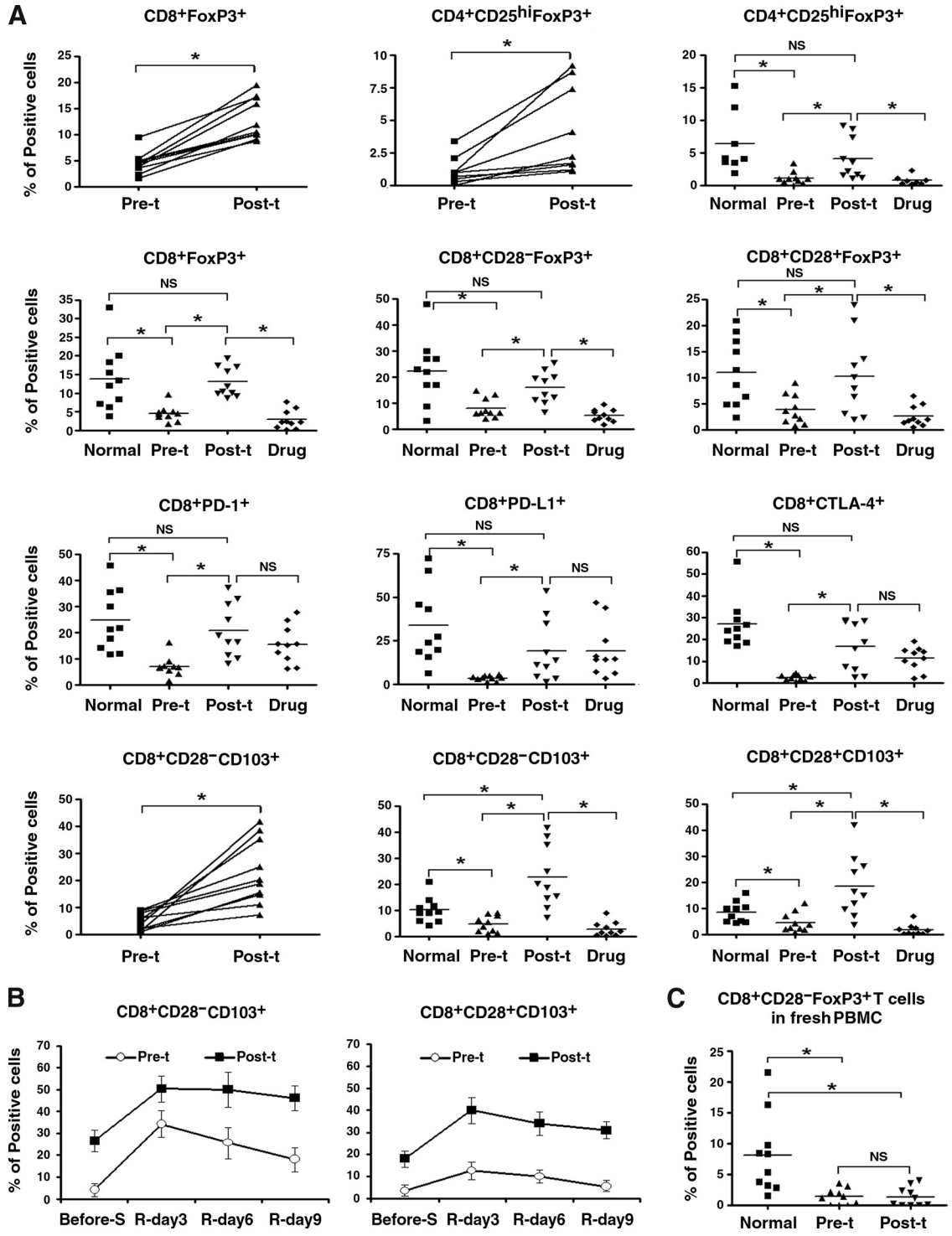


**FIGURE 2.** Increase in $CD8^+FoxP3^+$ and $CD4^+CD25^{high}$ ($CD25^{hi}$)$FoxP3^+$ Treg cells after stem cell transplant. *A*, Short-term CD4 and $CD8^+$ T cell line cells from normal subjects and pre- and posttransplant (pre-t and post-t), as well as conventional drug-treated (drug) lupus patients were stained for CD4, CD25 or CD8, CD28, CD103, PD-1, PD-L1, and intracellular FoxP3, CTLA-4 (to stain for PD-1, PD-L1, and CTLA-4, the CD8 T cells were stimulated with anti-CD3 for 24 h). Where samples were available from the same patient, connected plots are shown first for pre- and posttransplant samples, in addition to the collective data. *y*-axes show percent of positive cells among viable T cells gated for being $CD4^+$ or $CD8^+$. $n = 10$; horizontal line, mean; ∗, $p < 0.01$. *B*, Pre- and posttransplant CD8 line T cells were stained with CD8, CD28, and CD103 before cells were stimulated with anti-CD3/CD28 for 24 h in the presence of 20 U/ml IL-2, and then cells were rested for 9 days, and levels of CD8, CD28, and CD103 expression were measured at days 3, 6, and 9 of resting. Results are from three experiments; bars, mean ± SD, ∗, $p \leq 0.05$. *C*, percent of $CD8^+CD28^-FoxP3^+$ cell subset was measured in fresh PBMC from pre- and posttransplant lupus patients, as well as normal subjects. $n = 10$, horizontal line, mean; ∗, $p < 0.01$.

activation and rest; therefore, most of the $CD4^+CD25^+$ T cells used there were $CD127^-$. We next compared the suppressive effects of posttransplant, short-term line $CD8^+$ T cells as a whole with $CD4^+CD127^-CD25^{high}$ Treg cells sorted from the $CD4^+$ line T cells on autologous $CD4^+CD25^-$ target cells in cell contact and found that the suppressive effect of those posttransplant

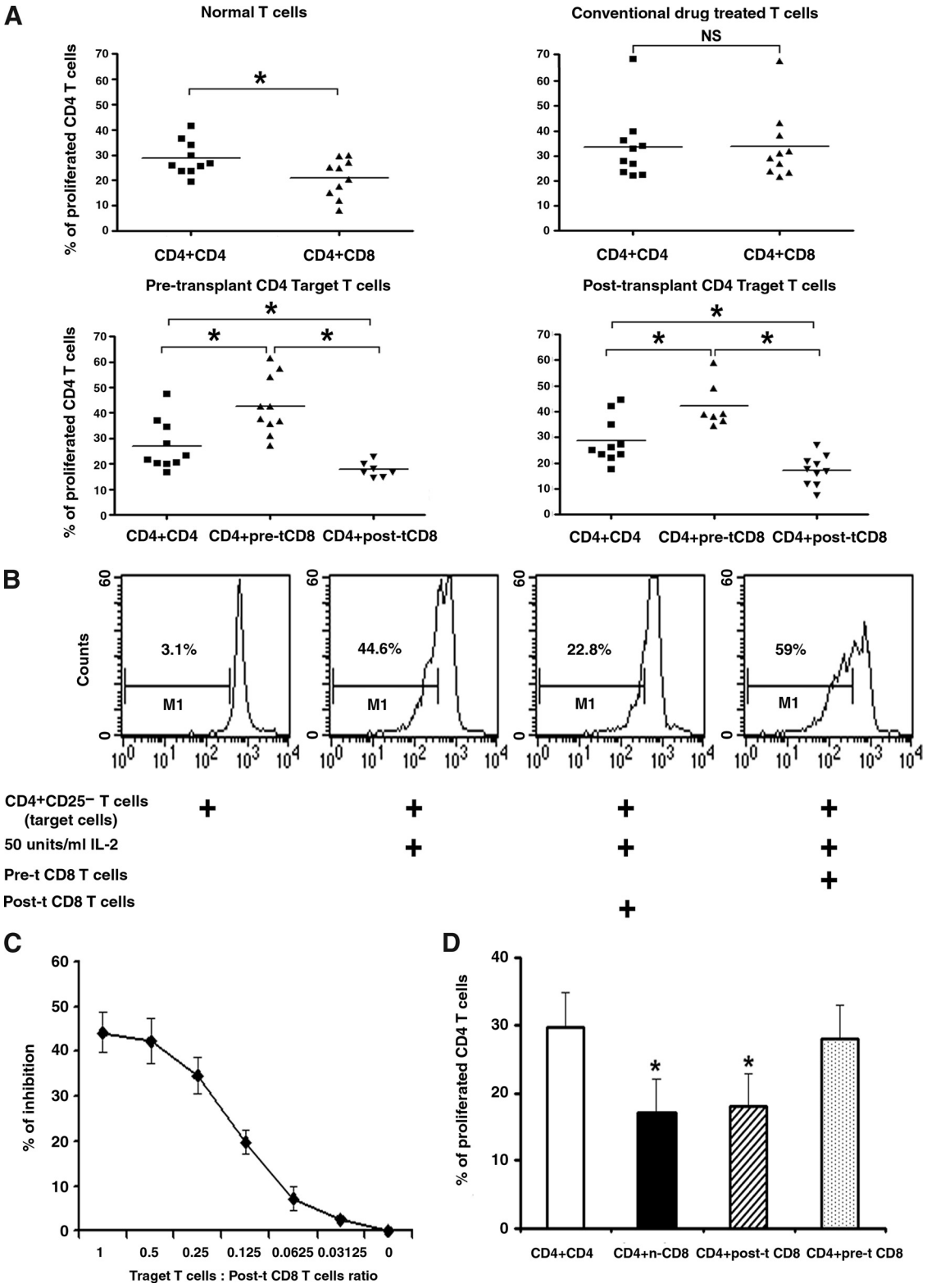


**FIGURE 3.** Restoration of suppressive activity in $CD8^+$ T cells of lupus patients after stem cell transplantation. CFSE-labeled target cells (short-term line $CD4^+$T cells or $CD4^+CD25^-$ T cells) at $5 \times 10^5$ cell/well were cocultured with autologous pre- (pre-t) or posttransplant (post-t) CD8 line T cells at a ratio of 1:0.5 for 4 days either in contact or separated by transwell membrane, and 50 U/ml IL-2 or $7 \times 10^5$/well-irradiated allogeneic APCs were used as stimulators in the culture system. *A*, Target CD4 T cells and autologous CD8 line T cells from normal donors, or pre- and posttransplant, as well as conventional drug-treated lupus patients were cocultured in contact in the presence of 50 U/ml IL-2, $n = 7$–10; horizontal line, mean. *B*, Representative histograms, showing proliferation of CFSE-labeled posttransplant target $CD4^+CD25^-$ T cells with IL-2 by themselves or cocultured with autologous pre- or posttransplant CD8 T cells. *C*, Titration of posttransplant CD8 Treg cells in suppression assay; results from three experiments are shown. The percent of inhibition was determined by comparing proliferation of target T cells alone to the percent of proliferation of target T cells in coculture with CD8 T cells. *D*, Posttransplant target CD4 cells were cocultured with autologous pre- and posttransplant CD8 line T cells, or with allogeneic normal CD8 T cells in transwell membrane system, and both stimulated by irradiated allogeneic APCs; n-CD8, normal CD8. *D*, Results from five experiments; bars, mean ± SD. *, $p < 0.01$.

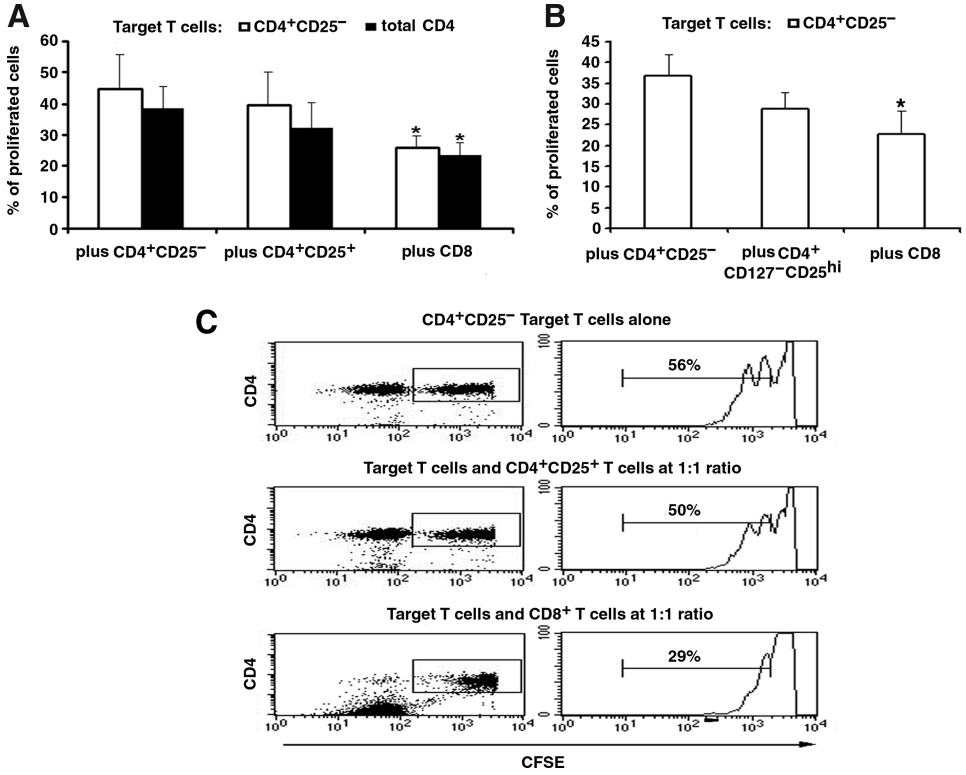


**FIGURE 4.** Posttransplant CD8 Treg cells have stronger suppressive function than their autologous $CD4^+CD25^+$ Treg cells. *A*, As in Fig. 3, posttransplant CD8 line T cells or $CD4^+CD25^+$ T cells sorted from autologous posttransplant CD4 line T cells were cocultured in contact, at the ratio of 1:1, with IL-2-stimulated autologous CD4 line T cells as a whole or $CD4^+CD25^-$ T cells purified from it, as target. Results are from three experiments; bar, mean ± SD; *, $p < 0.05$. *B*, As in *A*, posttransplant CD8 line T cells as a whole, or $CD4^+CD127^-CD25^{high}$ ($CD25^{hi}$) T cells that were sorted from autologous posttransplant CD4 line T cells were cocultured in contact with IL-2-stimulated target cells (sorted autologous $CD4^+CD25^-$ T cells), at the ratio of 1:1. *, $p < 0.05$. *C*, Representative histograms showing CFSE-labeled posttransplant, target $CD4^+CD25^-$ T cells proliferation when cocultured with autologous $CD4^+CD25^+$ T cells or CD8 T cells. Proliferation (CFSE dilution) of gated CD4 T cells was assessed by flow cytometry after coculturing for 4 days (see *Materials and Methods*).

$CD4^+CD127^-CD25^{high}$ T cells were not as strong as the posttransplant CD8 T cells, even though the latter could not be fractionated for further enrichment of putative CD8 Treg (Fig. 4*B*). These results suggest that CD8 Treg subset generated by HSCT play the major role in restoring immunoregulation.

### *CD8 Treg cell-mediated suppression of CD4 T cells is contact independent*

A transwell system was used to explore whether posttransplant CD8 Treg cells down-regulate CD4 T cell response via cytokine production. CD8 T cells and CD4 T cells were separated by a membrane with 0.4-$\mu$m pores and cultured with irradiated allogeneic APCs or 50 U/ml IL-2. The proliferative response of CD4 T cells was inhibited to the same degree when a membrane was placed between the target CD4 and normal or posttransplant CD8 T cells (Fig. 5). These experiments indicated soluble suppressive factors are the main mechanism of suppression excluding any cytotoxic contact effect. Alternatively, the helper activity of pretransplant lupus CD8 T cells enhancing CD4 T cell proliferation disappeared when the CD4 T cells were separated from pretransplant CD8 T cells by a transwell membrane (Fig. 5), suggesting that the helper function of pretransplant CD8 T cells needed cell to cell contact.

### *$CD8^+$ Treg cells from posttransplant lupus patients also have autoantigen-specific suppressive activity*

As mentioned in *Materials and Methods*, we derived CD4 and CD8 short-term T cell lines from PBMCs. Such short-term T cells from patients with lupus retain specificity for nuclear autoantigens (9, 37). To explore whether post- or pretransplant $CD8^+$ T cells also have lupus autoantigen-specific suppressive or helper activity, freshly obtained whole PBMCs must to be used as target, because live APCs are needed for presentation of lupus autoantigen epitopes. However, posttransplant lupus PBMCs do not respond significantly to autoepitopes (Fig. 1*A*). Therefore, in an autologous combination, where matched pretransplant CD8 line T cells and posttransplant PBMCs (fresh) from the same patient were available, they could be cocultured to determine the activity of pretransplant CD8 T cells. The converse experiment could not be done because pretransplant PBMCs lose APC activity in storage, by the time posttransplant CD8 T cells could be derived from the

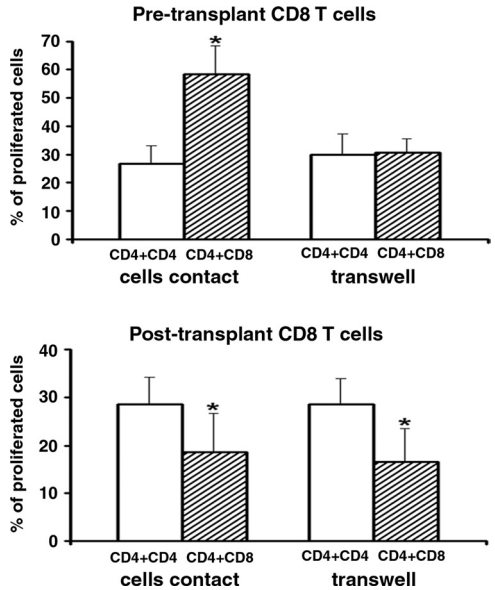


**FIGURE 5.** Posttransplant CD8 Treg cells mediate contact-independent suppressive activity, whereas pretransplant CD8 T cells show contact-dependent helper function. Pretransplant or posttransplant CD8 cells were cocultured with autologous IL-2-stimulated CD4 line T cells in contact or separated by transwell membrane by a target T cells-CD8 T cells ratio of 1:0.5. Culture conditions were similar to that in Fig. 3; results are from five experiments; bars, mean ± SD; *, $p < 0.01$.

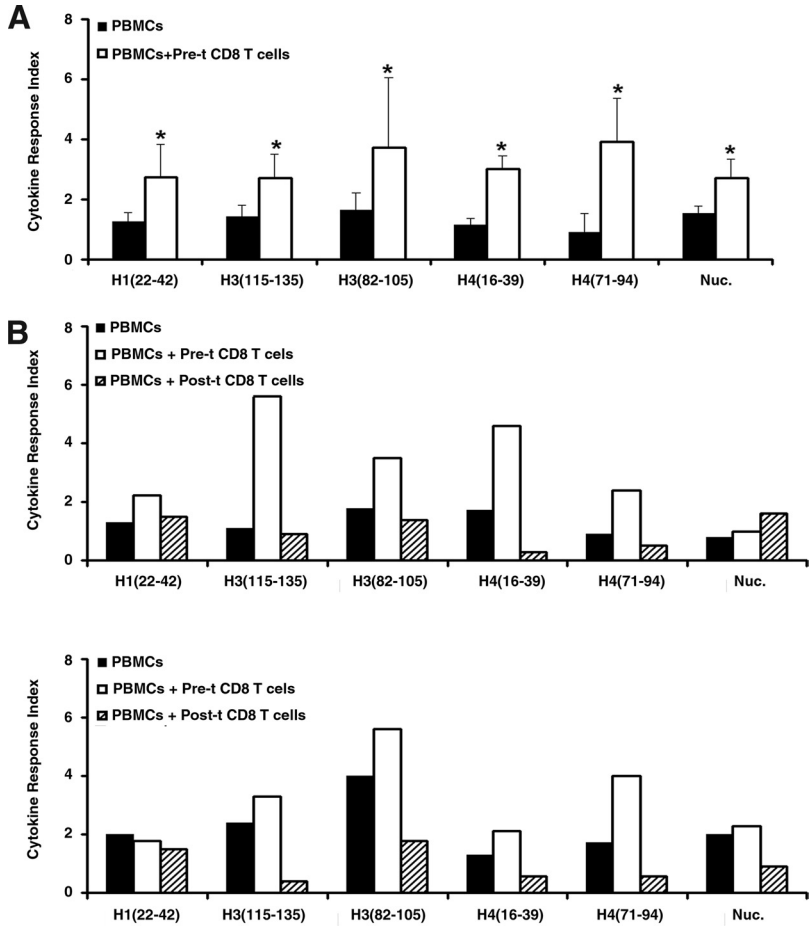


**FIGURE 6.** Pre- (Pre-t) and posttransplant (Post-t) CD8 T cells show autoantigen-specific help or suppression function, respectively. *A*, Posttransplant PBMCs were cocultured with autologous pretransplant CD8 T cells. *B*, Drug-treated lupus patients' PBMCs were cocultured with allogeneic pre- or posttransplant lupus CD8 T cells, at a ratio of 1:0.5 for 3 days. Gated viable $CD4^+$ T cells were then analyzed for IFN-$\gamma$ production by CFC, as in Fig. 1. Experiments were repeated five times; bars, mean $\pm$ SD. $*$, $p < 0.01$, in *A*. Because of the complexity of using allogeneic combinations, the experiments in *B* were done only with two separate sets of samples. Nuc., Nucleosome.

same patients months later after stem cell transplantation. Therefore, we did attempt to study any suppressive effect of posttransplant CD8 T cells in allogeneic combinations using PBMCs from a nonautologous lupus patient.

First, pretransplant CD8 line T cells were cocultured with their autologous posttransplant whole PBMCs in the presence of nucleosomes or one of the histone peptide epitopes, and responses of CD4 T cells in the target PBMC were measured by CFC (Fig. 6*A*), using procedures described in Fig. 1*A*. Then, in the same way, pre- or posttransplant CD8 line T cells were cocultured with the allogeneic, drug-treated lupus patients' PBMCs that retain autoimmune responsiveness to nucleosome autoepitopes. The peptide epitopes have both class II and class I motifs for stimulation of $CD4^+$ target responder and $CD8^+$ Treg cells (30). The low level of IFN-$\gamma$ response to the autoepitopes in posttransplant CD4 T cells was reversed to pathogenic high levels when cultured with autologous pretransplant CD8 T cells (Fig. 6*A*). On the other hand, response to the autoepitopes in drug-treated patients' CD4 T cells was reduced when cultured with allogeneic posttransplant lupus CD8 T cells, but it increased when cultured with allogeneic pretransplant CD8 T cells (Fig. 6*B*). In the latter case, the increased response in this short 3-day assay was not related to allogeneic MHC stimulation, because the control wells of coculturing the target PBMCs with the allogeneic CD8 T cells, without peptide or nucleosome, did not show any disproportionate increase in IFN-$\gamma$ response as compared with controls in Fig. 1*A* containing only autologous cells. Moreover, the same allogeneic posttransplant CD8 T cells showed a suppressive effect on IFN-$\gamma$ response (Fig. 6*B*). These results along with those in Fig. 1*B* indicate that pre- and posttransplant CD8 T cells can exhibit autoantigen-specific help or suppressive activity, respectively.

### *Blockade of TGF-$\beta$ predominantly abrogates the suppressive function of posttransplant CD8 T cells*

Having shown that the suppression effect of posttransplant CD8 T cells on CD4 T cells is mediated by soluble suppressive factor (Fig. 5) and that posttransplant CD4 T cells produce higher levels of IL-13 in response to nucleosomal autoepitopes (Fig. 1*A*), we asked whether CD8 line T cells from a posttransplant lupus patient expressed higher levels of the TGF-$\beta$ LAP, or IL-10, and whether blocking TGF-$\beta$, IL-10, or IL-13 abrogates the suppression function. As shown in Fig. 7, *A* and *B*, IL-2 treated posttransplant CD8 T cells expressed markedly higher levels of LAP than did IL-2-treated CD8 T cells from pretransplant and conventional drug-treated lupus patients. Although the levels of IL-10 in posttransplant CD8 T cells were much lower than those of LAP, it was still significantly higher than that in pretransplant and conventional drug-treated CD8 T cells. Anti-TGF-$\beta$ neutralization Ab significantly blocked the ability of the suppressive effect of posttransplant CD8 T cells, the lowest effective concentration of anti-TGF-$\beta$ being 10 ng/ml. Blocking IL-10 could also reduce the suppressive function of posttransplant CD8 Treg cells, however, the blocking effect was not as strong as with blocking of TGF-$\beta$ (Fig. 7, *C* and *D*), indicating that TGF-$\beta$ plays a more important role in posttransplant CD8 Treg-suppressive function. On the other hand, anti-IL-13 neutralization Ab could not abrogate the suppressive effect of the CD8 T cells, suggesting IL-13 may not play a role in CD8 Treg function here.

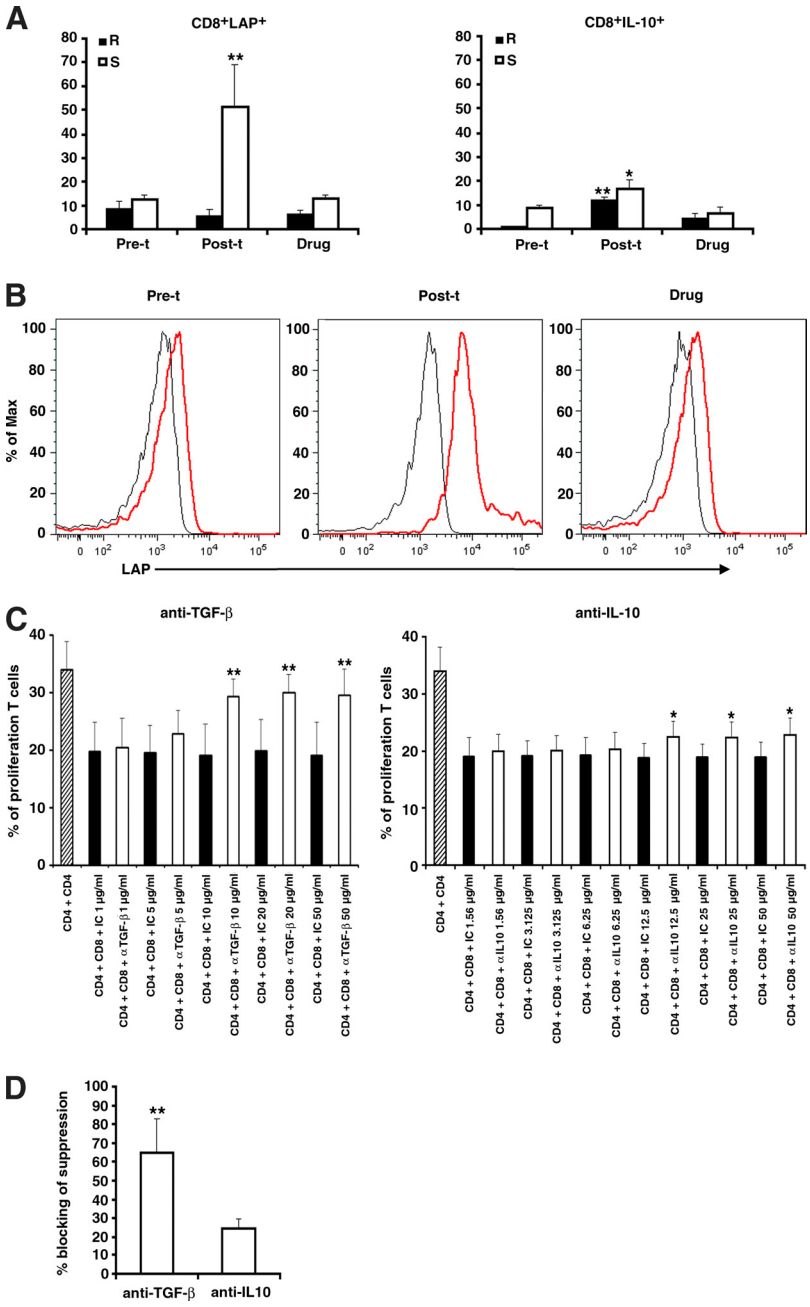


**FIGURE 7.** Posttransplant CD8 T cells suppress autologous CD4 T cell proliferation by secreting TGF-$\beta$ mainly and IL-10 to a lesser extent. *A*, Rested, short-term CD8$^+$ T cell line cells from pre- and posttransplant (Pre-t and Post-t), as well as conventional drug-treated (drug) lupus patients were stained for CD8 and LAP or intracellular IL-10 before (R) or after (S) culturing with 20 U/ml IL-2 for 48 h, *y*-axes show percent of LAP or IL-10$^+$ cells among viable T cells gated for being CD8$^+$. Results are from four experiments; bars, mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$. *B*, Examples of histograms of LAP expression (red line) in pre- and posttransplant, as well as conventional drug-treated CD8 T cells after culturing with IL-2 (20 U/ml) for 48 h (black line for isotype control). *C*, Different concentrations of anti-TGF-$\beta$ or anti-IL-10-neutralizing Ab or the relevant isotype control (IC) were added to the posttransplant CD4$^+$ target T cells cocultured with the autologous CD8 T cells in XViVo-20 serum-free medium. Results show that 10–50 $\mu$g/ml anti-TGF-$\beta$-neutralizing Ab could significantly block the suppression function of posttransplant CD8 on CD4 proliferation, and 12.5–50 $\mu$g/ml anti-IL-10-neutralizing Ab also showed blocking of the suppressive effect of the CD8 T cells. *D*, For comparison, data in *C* are shown as blocking of suppression by anti-TGF-$\beta$ or anti-IL-10-neutralizing Ab at the highest optimal concentration in the suppression assays. The percent blocking of suppression was calculated as stated in *Materials and Methods*. *C* and *D*, results from five experiments; bars, mean $\pm$ SD; *, $p < 0.05$ and **, $p < 0.01$.

## Discussion

Although aggressive immunosuppressive therapy can control disease activity and reduce organ damage, it is impossible to achieve the goal of drug-free remission or cure, given that patients with organ-threatening active SLE still have 20% 2-year and 35% 5-year disease-related mortalities with conventional treatment (66, 67). The approach of HSCT was first reported for patients with SLE in 1997 showing encouraging responses and inducing long-term remission and low (~1.5%) mortalities. HSCT thus became a great opportunity for patients with refractory SLE long-term remission. Even if the disease returned in some patients, it was mild and easy to treat (55, 66).

There are three hypotheses formulated for the mechanisms of stem cell transplantation therapy: 1) immune ablation eliminates autoreactive T cell clones; 2) autoreactive T cell clones are rendered tolerant; and 3) regulatory networks controlling the autoreactive T cells are restored. It was suggested that an immune reset occurs following autologous HSCT in patients with autoimmune disease (56); in fact, upon immune reconstitution, the default mechanism of the immune system is self-tolerance, but the exact mechanism by which self-tolerance returns following a HSCT remains undefined. In an attempt to address this issue, we studied both Treg function and nucleosomal autoantigen-specific T cell response before and after HSCT in lupus patients who were in remission after HSCT and compared them with those of lupus patients who received conventional drug treatment and were in clinical remission, and healthy controls as well.

Most studies on Treg cells in human SLE have focused on CD4$^+$ Treg cells, but results are very conflicted. Valencia et al. (28) described reduced numbers of suppressive CD4$^+$CD25$^{high}$ FoxP3 Treg cells in the peripheral blood of patients with active SLE (aSLE), compared with patients with inactive SLE (iSLE) and normal donors, and those aSLE CD4 Treg cells failed to suppress CD4$^+$CD25$^-$ T cell proliferation, whereas iSLE CD4 Treg

showed normal suppressive function. However, Yan et al. (68) found that the number of $CD4^+CD25^{high}$FoxP3 Treg cells in aSLE patients' fresh PBMCs was actually higher than that in iSLE and normal controls, but aSLE $CD4^+CD25^{high}$ Treg cells showed impaired suppressive function when in the presence of lupus APC. Venigalla et al. (35) also reported that the number of $CD4^+CD25^{high}$ FoxP3 Treg cells in aSLE fresh PBMC were higher than that in iSLE, but the suppressive function of both aSLE and iSLE CD4 Treg cells was impaired. Although those three groups used similar methods, they got different results, most likely because Treg studies were done with patient blood samples immediately ex vivo. We have shown previously that lupus T cells need to rest and recover in vitro, because many extrinsic factors may affect immune cell function in lupus in vivo, such as excessive IFN-$\alpha$, IL-6, and IL-10 production; recurrent autoantigenic stimulation; autoantibodies against immune cell surface molecules; and global immunosuppressant drugs, such as steroids, Cytoxan, etc. (4, 5, 9, 37, 39). In our study comparing the autologous pre- and posttransplant lupus patients' CD4 line T cells that had been rested, we found the number of pretransplant $CD4^+CD25^{high}FoxP3^+$ Treg cells to be significantly lower, and the frequency of this subset in posttransplant lupus patients was restored to levels seen in nonlymphopenic, normal subjects" short-term lines derived by an identical procedure. We also analyzed the suppressive function of $CD4^+CD25^{high}$ Treg cells which come from posttransplant lupus patients; but those cells had minor suppressive function, nowhere as strong as their autologous posttransplant CD8 Treg cells (Fig. 4).

Although very little is known about CD8 Treg cells in human SLE, observations indicated the important role of CD8 Treg in human organ transplantation, murine SLE (11, 50) and experimental autoimmune encephalomyelitis, but these CD8 Tregs are different in different situations (69). Our study found that the number of $CD8^+FoxP3^+$ Treg ($CD28^-$ or $CD28^+$) cells significantly increased in posttransplant CD8 line T cells as compared with pretransplant and drug-treated lupus patients; moreover, those cells showed a strong suppressive effect on CD4 T cells proliferation. However, our data did not show any significant difference in the numbers of $CD8^+CD28^-FoxP3^+$ Treg cells in fresh PBMC from pre- and posttransplant lupus patients, although those CD8 Treg cell numbers are much higher in normal PBMCs (Fig. 2*C*), indicating that unknown in vivo influences still exists between posttransplant lupus and normal control subjects. The one-time TCR stimulation with IL-2 in vitro followed by resting probably removes the extrinsic in vivo influences mentioned above and brings out these Treg cells in posttransplant lupus patients to the levels seen in normal subjects. Nevertheless, the short-term culture T cells were rested following stimulation for 10 days, demonstrating a stable and sustained elevation of FoxP3 expression only in the posttransplant and normal T cells. The CD8 Treg activity in the posttransplant patients and normal subjects reported here is mediated by soluble factors, such as TGF-$\beta$, and not through cytotoxic activity. Filaci et al. (43), used different conditions to generate CD8 Treg cells in vitro by first culturing CD8 T cells with irradiated autologous monocytes in the presence of IL-2 and GM-CSF for 7 days and then purified CD8 T cells to study Treg activity. They reported that these cytokine-induced CD8 Treg cells from conventional drug-treated, inactive lupus patients mediated suppressive function by secreting IL-6 and IFN-$\gamma$, which cytokines paradoxically may aggravate lupus. By contrast, our results show that the CD8 cells from lupus patients with conventional drug-induced remission, actually have no suppressive function on CD4 T cell proliferation. The results indicate different mechanisms in HSCT and conventional drug treatment, and understanding those mechanisms may be helpful to search for better therapy for lupus.

As Table I shows, most posttransplant lupus patients in our study were in remission (SLEDAI $< 3$), and the CD8 Treg cells from these patients showed strong suppressive function. We have one posttransplant lupus patient in our study (patient 5-B) with a SLEDAI of 4, and the CD8 Treg from this patient did not show strong suppressive function (the percent of inhibition was only 8%). In the future, we will compare autoimmune responses and CD8 Treg suppressive function in rare posttransplant lupus patients who have relapsed years after transplantation. It was also reported that target $CD4^+CD25^-$ T cells from active SLE patients show low sensitivity to the suppressive function of their autologous $CD4^+CD25^{high}$ $CD127^{-/low}$ Treg cells (35). We could not confirm this result (Fig. 4). Indeed, the posttransplant CD8 Treg cells could suppress to a similar degree both pre- and posttransplant CD4 T target cells proliferation, just like normal CD8 Treg cells. The mechanisms by which transplanted stem cells can induce more functional CD8 Treg cells to grow up in SLE is unclear; it may be related to the fact that immune ablation in HSCT eliminates autoreactive T and B cells giving more space for homeostatic proliferation of Treg cells (70), but this is not the case in nonlymphopenic normal subjects where these Treg cells are also prevalent. In contrast to posttransplant patients' CD8 Treg cells, our results show that pretransplant CD8 line T cells markedly augmented proliferation of autologous CD4 target T cells in contact cultures and helped in CD4 T cells response to histone peptide or nucleosomes (Figs. 5 and 6). Horwitz et al. (40) also reported that deleting CD8 T cells from active lupus PBMCs can reduce the polyclonal IgG production and that adding autologous CD8 T cells back to PBMCs can reconstitute this Ab production, but the underlying mechanisms of this helper activity are still not clear.

Nucleosomes and their histone peptides have been identified to be the major immunogens that initiate cognate interactions between autoimmune Th and B cells for the production of pathogenic antinuclear autoantibodies in lupus. Previous studies have shown that in contrast to normal T cells, conventional drug-treated lupus patients' T cells responded strongly to the nucleosomal histone peptide epitopes by producing intracellular IFN-$\gamma$, irrespective of the patient's disease status (9). These peptides can also induce anti-DNA autoantibodies and nephritis in lupus-prone mice on immunization (7, 8). Our study established here that after HSCT, IFN-$\gamma$ production response of $CD4^+$ T cell in fresh PBMC to nucleosomal autoepitopes was reduced to background levels in the posttransplant lupus patients, whereas immunoregulatory IL-13 response was increased, and this reduced IFN-$\gamma$ response rose when CD8 T cells were deleted from the PBMCs, considerably more than when $CD4^+CD25^{high}$ subset was deleted. Moreover, IFN-$\gamma$ response to the autoepitopes in posttransplant PBMC-CD4 T cells was reversed to pathogenic high levels when cultured with autologous pretransplant CD8 T cells. Similarly, in an allogeneic culture combination, the IFN-$\gamma$ response of PBMC-CD4 T cells from conventional drug-treated patients to nucleosomes and its peptide epitopes was suppressed when cultured with posttransplant CD8 T cells, but this response was increased when cultured with pretransplant CD8 T cells. Overall, the observations indicate that HSCT-induced CD8 Treg cells play very important role in restoring self-tolerance in posttransplant lupus patients, and the mechanisms of HSCT induced remission are different from conventional drug induced remission.

T suppressor or $CD8^+$ Treg cells have been studied in other systems, but no unique surface marker has been identified thus far which can be used to distinguish them. Although the transcription factor FoxP3 is critical for Treg cells, it is expressed intracellularly. CD28 Ag was believed to be poorly expressed on CD8 Treg cells; however, our results show that the percentages of $CD8^+$

$CD28^-$ cells are not markedly different in pre- and posttransplant lupus patients" fresh PBMCs and CD8 line T cells. In fact, the percent of $CD8^+CD28^-$ was >90% in both pre- and posttransplant CD8 line T cells, but the former showed a helper function and the latter showed a suppressive function. In addition, the percent of $FoxP3^+$ cells in the $CD8^+$ T cell lines from posttransplant patients as compared with pretransplant samples were significantly increased not only in $CD28^-$ subpopulations, but also in the $CD28^+$ subset. Therefore, CD28 may not be a suitable marker for CD8 Treg cells of lupus. In our study, the short-term posttransplant CD8 line T cells highly express LAP, CD103, PD-1, PD-L1, and intracellular CTLA-4, as compared with pretransplant CD8 line T cells. Moreover, CD103 expression remained at high levels in posttransplant CD8 line T cells even after the cells had been rested for 9 days after TCR stimulation, whereas CD103 expression decreased rapidly in pretransplant CD8 line T cells under the same conditions, but again, those molecular markers are not always coexpressed with FoxP3 at the same time point. We also measured CD27, CD56, and CD62L expression level in CD8 line T cells, but we could not find significant differences for those in pre- and posttransplant CD8 T cells (data not shown). To explore unique marker(s) for CD8 Treg cells, further work is being done.

In conclusion, HSCT can induce immunological self-tolerance in refractory SLE by restoring the $CD8^{TGF\text{-}\beta}FoxP3^+$ regulatory network in particular, accompanied by almost complete inhibition of pathogenic T cell response to autoepitopes from nucleosomes. The CD8 Treg cells generated in posttransplant lupus patients are much more potent than $CD4^+CD25^{high}$ Treg in suppressing lupus autoimmunity. These $CD8^+FoxP3^+$ TGF-$\beta$-producing Treg are more relevant for controlling lupus, as shown in stem cell transplant patients here and in peptide-treated murine models (11, 19, 30, 50), and they are different from cytotoxic, contact-dependent $CD8^+$ Treg cells described in other situations, such as transplantation and organ-specific autoimmune disease (20–24, 69, 71).

## Disclosures

The authors have no financial conflict of interest.

## References


1. Kotzin, B. L. 1996. Systemic lupus erythematosus. *Cell* 85: 303–306.
2. Rahman, A., and D. A. Isenberg. 2008. Systemic lupus erythematosus. *N. Engl. J. Med.* 358: 929–939.
3. Datta, S. K. 2003. Major peptide autoepitopes for nucleosome-centered T and B cell interaction in human and murine lupus. *Ann. NY Acad. Sci.* 987: 79–90.
4. Krishnan, S., D. L. Farber, and G. C. Tsokos. 2003. T cell rewiring in differentiation and disease. *J. Immunol.* 171: 3325–3331.
5. Datta. S. K., L. Zhang, and L. Xu. 2005. T-helper cell intrinsic defects in lupus that break peripheral tolerance to nuclear autoantigens. *J. Mol. Med.* 83: 267–278.
6. Mohan, C., S. Adams, V. Stanik, and S. K. Datta. 1993. Nucleosome: a major immunogen for the pathogenic autoantibody-inducing T cells of lupus. *J. Exp. Med.* 177: 1367–1381.
7. Kaliyaperumal, A., C. Mohan, W. Wu, and S. K. Datta. 1996. Nucleosomal peptide epitopes for nephritis-inducing T helper cells of murine lupus. *J. Exp. Med.* 183: 2459–2469.
8. Kaliyaperumal, A., M. A. Michaels, and S. K. Datta. 2002. Naturally processed chromatin peptides reveal a major autoepitope that primes pathogenic T and B cells of lupus. *J. Immunol.* 168: 2530–2537.
9. Lu, L., A. Kaliyaperumal, D. T. Boumpas, and S. K. Datta. 1999. Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J. Clin. Invest.* 104: 345–355.
10. Kaliyaperumal, A., M. A. Michaels, and S. K. Datta. 1999. Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: tolerance spreading impair pathogenic function of autoimmune T and B cells. *J. Immunol.* 162: 5775–5783.
11. Kang, H.-K., M. A. Michaels, B. R. Berner, and S. K. Datta. 2005. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J. Immunol.* 174: 3247–3255.
12. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101: 455–458.
13. Shevach, E. M. 2000. Regulatory T cells in autoimmunity. *Annu. Rev. Immunol.* 18: 423–449.
14. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T cell subset inhibits antigen-specific T cell responses and prevents colitis. *Nature* 389: 737–742.
15. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265: 1237–1240.
16. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
17. 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.
18. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-$\beta$ induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
19. Zheng, S. G., J. H. Wang, M. N. Koss, F. Quismorio, Jr., J. D. Gray, and D. A. Horwitz. 2004. $CD4^+$ and $CD8^+$ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J. Immunol.* 172: 1531–1539.
20. Gershon, R. K., and K. Kondo. 1971. Infectious immunological tolerance. *Immunology* 21: 903–914.
21. Filaci, G., and N. Suciu-Foca. 2002. $CD8^+$ T suppressor cells are back to the game: are they players in autoimmunity? *Autoimmunity Rev.* 1: 279–283.
22. Balashov, K. E., S. J. Khoury, D. A. Hafler, and H. L. Weiner. 1995. Inhibition of T cell responses by activated human $CD8^+$ T cells is mediated by interferon-$\gamma$ and is defective in chronic progressive multiple sclerosis. *J. Clin. Invest.* 95: 2711–2719.
23. Gilliet, M., and Y. J. Liu. 2002. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.* 195: 695–704.
24. Jiang, H., N. S. Braunstein, B. Yu, R. Winchester, and L. Chess. 2001. $CD8^+$ T cells control the TH phenotype of MBP-reactive $CD4^+$ T cells in EAE mice. Proc. *Natl. Acad. Sci. USA* 98: 6301–6306.
25. Kosiewicz, M. M., P. Alard, S. Liang, and S. L. Clark. 2004. Mechanisms of tolerance induced by transforming growth factor-$\beta$-treated antigen-presenting cells: CD8 regulatory T cells inhibit the effector phase of the immune response in primed mice through a mechanism involving Fas ligand. *Int. Immunol.* 16: 697–706.
26. Mellor-Pita, S., M. J. Citores, R. Castejon, P. Tutor-Ureta, M. Yebra-Bango, J. L. Andreu, and J. A. Vargas. 2006. Decrease of regulatory T cells in patients with systemic lupus erythematosus. *Ann. Rheum. Dis.* 65: 553–554.
27. 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.
28. Valencia, X., C. Yarboro, G. Illei, and P. E. Lipsky. 2007. Deficient $CD4^+CD25^{high}$ T regulatory cell function in patients with active systemic lupus erythematosus. *J. Immunol.* 178: 2579–2588.
29. Wu, H. Y., and N. A. Staines. 2004. A deficiency of $CD4^+CD25^+$ T cells permits the development of spontaneous lupus-like disease in mice, and can be reversed by induction of mucosal tolerance to histone peptide autoantigen. *Lupus* 13: 192–200.
30. Kang, H.-K., M. Liu, and S. K. Datta. 2007. Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific Treg cells and contraction of inflammatory TH17 cells (cover page figure). *J. Immunol.* 178: 7849–7858.
31. La Cava, A., F. M. Ebling, and B. H. Hahn. 2004. Ig-reactive $CD4^+CD25^+$ T cells from tolerized (New Zealand Black × New Zealand White) $F_1$ mice suppress in vitro production of antibodies to DNA. *J. Immunol.* 173: 3542–3548.
32. Sharabi, A., H. Zinger, M. Zborowsky, Z. M. Sthoeger, and E. Mozes. 2006. A peptide based on the complementarity-determining region 1 of an autoantibody ameliorates lupus by up-regulating $CD4^+CD25^+$ cells and TGF-$\beta$. *Proc. Natl. Acad. Sci. USA* 103: 8810–8815.
33. Hahn, B. H., M. Anderson, E. Le, and A. La Cava. 2008. Anti-DNA Ig peptides promote Treg cell activity in systemic lupus erythematosus patients. *Arthritis Rheum.* 58: 2488–2497.
34. Monk, C. R., M. F. Spachidou, F. Rovis, E. Leung, M. Botto, R. I. Lechler, and O. A. Garden. 2005. MRL/Mp $CD4^+CD25^-$ T cells show reduced sensitivity to suppression by $CD4^+CD25^+$ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. *Arthritis Rheum.* 52: 1180–1184.
35. Venigalla, R. K., T. Tretter, S. Krienke, R. Max, V. Eckstein, N. Blank, C. Fiehn, A. D. Ho, and H. M. Lorenz. 2008. Reduced $CD4^+$,$CD25^-$ T cell sensitivity to the suppressive function of $CD4^+$,$CD25^{high}CD127^{-/low}$ regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum.* 58: 2120–2130.
36. Wohlfert, E. A., M. K. Callahan, and R. B. Clark. 2004. Resistance to $CD4^+CD25^+$ regulatory T cells to TGF-$\beta$ in Cbl-b−/− mice. *J. Immunol.* 173: 1059–1065.
37. Yi, Y., M. McNerney, and S. K. Datta. 2000. Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J. Immunol.* 165: 6627–6634.
38. Jury, E. C., P. S. Kabouridis, F. Flores-Borja, R. A. Mageed, and D. A. Isenberg,. 2004. Altered lipid raft-associated signaling and ganglioside expression in T lymphocytes from patients with systemic lupus erythematosus. *J. Clin. Invest.* 113: 1176–1187.
39. Xu, L., L. Zhang, Y. Yi, H.-K. Kang, and S. K. Datta. 2004. Human lupus T cells resist inactivation and escape death by upregulating COX-2. *Nat. Med.* 10: 411–415.

40. Linker-Israeli, M., F. P. Quisimoro, and D. A. Horwitz. 1990. $CD8^+$ lymphocytes from patients with systemic lupus erythematosus sustain, rather than suppress, spontaneous polyclonal IgG production and synergize with $CD4^+$ cells to support autoantibody synthesis. *Arthritis Rheum.* 33: 1216–1225.
41. Desai-Mehta, A., L. Lu, R. Ramsey-Goldman, and S. K. Datta. 1996. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J. Clin. Invest.* 97: 2063–2073.
42. Stohl, W., J. E. Elliott, L. Li, E. R. Podack, D. H. Lynch, and C. O. Jacob. 1997. Impaired nonrestricted cytolytic activity in systemic lupus erythematosus: involvement of a pathway independent of Fas, tumor necrosis factor, and extracellular ATP that is associated with little detectable perforin. *Arthritis Rheum.* 40: 1130–1137.
43. Filaci, G., S. Bacilieri, M. Fravega, M. Monetti, P. Contini, M. Ghio, M. Setti, F. Puppo, and F. Indiveri. 2001. Impairment of $CD8^+$ T suppressor cell function in patients with active systemic lupus erythematosus. *J. Immunol.* 166: 6452–6457.
44. Crispín, J. C., M. Oukka, G. Bayliss, R. A. Cohen, C. A. Van Beek, I. E. Stillman, V. C. Kyttaris, V. T. Juang, and G. C. Tsokos. 2008. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J. Immunol.* 181: 8761–8766.
45. Hsu, H. C., P. Yang, J. Wang, Q. Wu, R. Myers, J. Chen, J. Yi, T. Guentert, A. Tousson, A. L. Stanus, et al. 2008. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat. Immunol.* 9: 166–175.
46. Bauquet, A. T., H. Jin, A. M. Paterson, M. I. Mitsdeorffer, I. C. Ho, A. H. Sharpe, and V. K. Kuchroo. 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and Th-17 cells. *Nat. Immunol.* 10: 167–175.
47. Wu, H. Y., F. J. Quintana, and H. L. Weiner. 2008. Nasal anti-CD3 antibody ameliorates lupus by inducing an IL-10-secreting $CD4^+CD25^-LAP^+$ regulatory T cell and is associated with down-regulation of $IL\text{-}17^+CD4^+ICOS^+CXCR5^+$ follicular helper T cells. *J. Immunol.* 181: 6038–6050.
48. Malissen, B. 2009. Revisiting the follicular helper T cell paradigm. *Nat. Immunol.* 10: 371–372.
49. Mohan, C., Y. Shi, J. D. Laman, and S. K. Datta. 1995. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J. Immunol.* 154: 1470–1480.
50. Singh, R. P., A. La Cava, M. Wong, F. Ebling, and B. H. Hahn. 2007. $CD8^+$ T cell-mediated suppression of autoimmunity in a murine lupus model of peptide-induced immune tolerance depends on Foxp3 expression. *J. Immunol.* 178: 7649–7657.
51. de Kleer, I., B. Vastert, M. Klein, G. Teklenburg, G. Arkesteijn, G. P. Yung, S. Albani, W. Kuis, N. Wulffraat, and B. Prakken. 2006. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the $CD4^+CD25^+$ immune regulatory network. *Blood* 107: 1696–1702.
52. Burt, R. K., A. Traynor, and R. Ramsey-Goldman. 1997. Hematopoietic stem-cell transplantation for systemic lupus erythematosus. *N. Engl. J. Med.* 337: 1777–1778.
53. Traynor, A. E., J. Schroeder, R. M. Rosa, D. Cheng, J. Stefka, S. Mujais, S. Baker, and R. K. Burt. 2000. Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. *Lancet* 356: 701–707.
54. Burt, R. K., A. Traynor, L. Statkute, W. G. Barr, R. Rosa, J. Schroeder, L. Verda, and N. Krosnjar. 2006. Nonmyeloablative hematopoietic stem cell transplantation for systemic lupus erythematosus. *JAMA* 295: 527–535.
55. Burt, R. K., A. Marmont, Y. Oyama, S. Slavin, R. Arnold, F. Hiepe, A. Fassas, and J. Snowden. 2006. Randomized controlled trials of autologous hematopoietic stem cell transplantation for autoimmune diseases: the evolution from myeloablative to lymphoablative transplant regimens. *Arthritis Rheum.* 54: 3750–3760.
56. Muraro, P. A., D. C. Douek, A. Packer, K. Chung, F. J. Guenaga, R. Cassiani-Ingoni. C. Campbell, S. Memon, J. W. Nagle, F. T. Hakim, et al. 2005. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. *J. Exp. Med.* 201: 805–816.
57. 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.
58. 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.
59. Baecher-Allan, C. M., and D. A. Hafler. 2006. The purification and functional analysis of human $CD4^+CD25^{high}$ regulatory T cells. In *Current Protocols in Immunology.* J. E. Coligan, B. E. Bierer, D. H. Margulies, E. M. Shevach, and W. Strober, eds. John Wiley & Sons, New York, 7.4B. 1–7.4B.2.
60. Cortesini, R., J. LeMaoult, R. Ciubotariu, and N. S. Cortesini. 2001. $CD8^+CD28^-$ T suppressor cells and the induction of antigen-specific, antigen-presenting cell-mediated suppression of Th reactivity. *Immunol. Rev.* 182: 201–206.
61. Keino, H., S. Masli, S. Sasaki, J. W. Streilein, and J. Stein-Streilein. 2006. $CD8^+$ T regulatory cells use a novel genetic program that includes CD103 to suppress Th1 immunity in eye-derived tolerance. *Invest. Ophthalmol. Vis. Sci.* 47: 1533–1542.
62. Okazaki, T., Y. Iwai, and T. Honjo. 2002. New regulatory co-receptors: inducible co-stimulator and PD-1. *Curr. Opin. Immunol.* 14: 779–782.
63. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over $Foxp3^+$ regulatory T cell function. *Science* 322: 271–275.
64. Ma, A., R. Koka, and P. Burkett. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu. Rev. Immunol.* 24: 657–679.
65. Gaffen, A. L., and K. D. Lie. 2004. Overview of interleukin-2 function, production and clinical applications. *Cytokine* 28: 109–123.
66. Burt, R. K., and A. E. Traynor. 2003. SLE: hematopoietic stem cell transplantation for systemic lupus erythematosus. *Arthritis Res. Ther.* 5: 207–209.
67. Pavletic, S. Z., and G. G. Illei. 2005. The role of immune ablation and stem cell transplantation in severe SLE. *Best Pract. Res. Clin. Rheumatol.* 19: 839–858.
68. Yan, B., S. Ye, G. Chen, M. Kuang, N. Shen, and S. Chen. 2008. Dysfunctional $CD4^+$,$CD25^+$ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-$\alpha$-producing antigen-presenting cells. *Arthritis Rheum.* 58: 801–812.
69. Lu, L., H. J. Kim, M. B. Werneck, and H. Cantor. 2008. Regulation of $CD8^+$ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc. Natl. Acad. Sci. USA* 105: 19420–19425.
70. Barthlott, T., G. Kassiotis, and B. Stockinger. 2003. T cell regulation as a side effect of homeostasis and competition. *J. Exp. Med.* 197: 451–460.
71. Shustov, A., I. Luzina, P. Nguyen, J. C. Papadimitriou, B. Handwerger, K. B. Elkon, and C. S. Via. 2000. Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. *J. Clin. Invest.* 106: R39–R47.