CD4+ Regulatory T Cells Are Spared from Deletion by Antilymphocyte Serum, a Polyclonal Anti-T Cell Antibody

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CD4<sup>+</sup> Regulatory T Cells Are Spared from Deletion by Antilymphocyte Serum, a Polyclonal Anti-T Cell Antibody

Keisuke Minamimura,* Wenda Gao,† and Takashi Maki<sup>2*</sup>

Broad T cell depletion has been used as an integral part of treatment in transplantation and autoimmune diseases. Following depletion, residual T cells undergo homeostatic proliferation and convert to memory-like T cells. In this study, we investigated the effect of T cell depletion by antilymphocyte serum (ALS), a polyclonal anti-T cell Ab, on CD4<sup>+</sup> regulatory T cells. After ALS treatment, CD4<sup>+</sup>CD25<sup>+</sup> T cells underwent proliferation and expressed a memory T cell marker, CD44. One week after ALS treatment, both CD25<sup>+</sup> and CD25<sup>-</sup> T cells exhibited increased suppression of alloresponses in vitro, which waned thereafter to the levels mediated by naive CD25<sup>+</sup> and CD25<sup>-</sup> T cells. By real-time PCR analyses, ALS treatment of CD4-deficient mice adoptively transferred with Thy1.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and Thy1.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> T cells resulted in the appearance of Thy1.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> and Thy1.1<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> T cells, suggesting the conversion between CD25<sup>+</sup> and CD25<sup>-</sup> T cells. Naive CD25<sup>+</sup> T cells expressed a higher level of intracellular Bcl-x<sub>L</sub> than CD25<sup>-</sup> T cells. Up-regulation of the Bcl-x<sub>L</sub> molecule during ALS-induced homeostatic expansion further promoted survival of CD25<sup>+</sup> and, to a lesser degree, CD25<sup>-</sup> T cells. These results indicate that CD25<sup>+</sup> T cells are spared from ALS-mediated deletion, with some CD25<sup>-</sup> T cells converting to CD25<sup>+</sup> T cells, and continue to exhibit regulatory activity. The concomitant presence of T cell deletion and continuous regulatory T cell activity may underlie the therapeutic effect of ALS, particularly in treatment of autoimmune diseases.

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Broad T cell depletion by polyclonal anti-T cell Abs such as antilymphocyte serum (ALS) and antithymocyte globulin has been used as an integral part of tolerance induction in experimental transplantation (1–3) and clinical trials (4–6). These polyclonal Abs are a complex mixture of Abs with multiple specificities directed to both T cells and non-T cells (7, 8). They produce profound T cell depletion via complement-dependent lymphocyte lysis and Fas/Fas ligand-mediated apoptosis (9, 10). It has been reported that T cell depletion leads to homeostatic proliferation of residual T cells and their conversion into alloantigen-reactive memory-like T cells, potentially posing a barrier to the establishment of transplantation tolerance (11, 12). However, in addition to the T cell-depleting effect, use of ALS has been associated with subsequent emergence of regulatory (suppressor) T (Treg) cells in the experimental transplantation tolerance (13, 14) and autoimmune diabetes models (15). Association with Treg cell function was also seen with the use of monoclonal anti-CD3 Ab in clinical transplantation (16) as well as treatment of humans and NOD mice with early onset type 1 diabetes (17, 18).

There has been accumulating evidence indicating that normal individuals and rodents harbor naturally occurring Treg cells that express the IL-2R α-chain (CD25), develop during the normal process of T cell maturation in the thymus, persist in the periphery, and maintain self-tolerance (reviewed in Ref. 19). Another marker of natural Treg cells is forkhead box P3 (Foxp3) transcriptional factor that seems to control Treg cell development and expression of the suppressive phenotype (20–22). Because Treg cell activity was detectable in vitro coculture experiments immediately after ALS treatment (13, 14), we hypothesized that Treg cells may be spared by the depleting effect of anti-T cell Abs. In the present study, we tested our hypothesis by administering ALS to mice and determining the subsequent functional and phenotypic changes of CD4<sup>+</sup>CD25<sup>+</sup> natural Treg cells.

Materials and Methods

Mice

C57BL/6 (B6, Thy1.2<sup>+</sup>) mice were purchased from Taconic Farms. Thy1.1<sup>+</sup>B6, CD4 Ag-deficient B6 (CD4-deficient), RAG-1-deficient B6 (RAG<sup>−/−</sup>), GFP-transgenic mice (GFP mice, B6 background), DBA/2, and CBA mice were purchased from The Jackson Laboratory. All care and handling of animals was conducted in accordance with guidelines provided in the Guide for the Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services.

Antilymphocyte serum

ALS was prepared by immunizing rabbits with lymph node cells harvested from C3H/He, DBA/2, (C57BL/6 × AJ)F<sub>1</sub>, and NOD mice as previously described (23). Two doses (0.5 ml/dose) of ALS were given 3 days apart (designated arbitrarily as days −1 and 2). Four different batches of ALS were used in the present study without significant difference in Treg cell activity described herein.

BrdU labeling

Mice were fed with BrdU in drinking water (0.8 mg/ml) for 7 days. BrdU-incorporated cells were detected by flow cytometry following fixation and permeabilization using a Fix and Perm reagent (Caltag Laboratories).

Flow cytometry

The cells were first incubated with an anti-CD16/32 mAb for 10 min to block nonspecific binding of the labeled Abs and stained with FITC<sup>-</sup>, PE<sup>-</sup>, or CyChrome-conjugated mAbs directed to CD4 (clone H129.19), CD8...
(53-6,7), CD19 (1D3), CD25 (PC61.5), and CD44 (IM7), all of which were purchased from BD Pharmingen. FITC-, PE-, or CyChrome-conjugated isotype Abs were used as controls. Stained cells were analyzed on a FACSscan (BD Biosciences).  

**Purification of CD4\(^+\)CD25\(^+\) T cells**

CD4\(^+\) T cells were incubated with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs for 30 min followed by isolation of CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells by a high-speed MoFlo cell sorter (DakoCytomation). The purity and viability of each subset was typically >95%. For in vitro assays of regulatory activity and in vivo reconstruction studies, T cells purified using a CD4 T cell isolation kit (Miltenyi Biotec) were incubated with PE-conjugated anti-CD25 mAb and separated into CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) cells using anti-PE MicroBeads and MACS separator (Miltenyi Biotec) according to the manufacturer’s instructions. After repeated separation, the purity of CD25\(^-\) and CD25\(^+\) cells was typically 80–90% and >90%, respectively, and their viability was >90%.

**Mixed lymphocyte response**

A single-cell suspension was prepared from naive wild-type B6 (Thy1.2\(^+\)) spleens and peripheral lymph nodes followed by purification of T cells using mouse T cell enrichment columns (R&D Systems). T cells were labeled with CFSE as described previously (24). CFSE-labeled B6 T cells using mouse T cell enrichment columns (R&D Systems). T cells were depleted of CD3\(^+\)CD4\(^+\)/CD8\(^+\) T cells (each 0.5–1.0 \(10^6\) cells tested) with 0.1–0.5 ml/dose of anti-CD3, anti-CD25, and anti-CD8 mAb (clone H-5; Santa Cruz Biotechnology). For intracellular staining, eosin-4, and CD25\(^+\) T cells (Fig. 1c). Adoptive transfer of CFSE-labeled B6 T cells into ALS-treated B6 mice also induced proliferation of the CD4, CD8, and CD25\(^+\) T cell subsets (Fig. 1c).

**Inhibition of in vivo alloresponses by post-ALS CD4\(^+\) T cells**

To test regulatory activity of post-ALS residual T cells, splenic and lymph node cells were harvested from Thy1.1\(^+\) B6 mice 1–4 wk after ALS treatment and separated into CD25\(^-\) and CD25\(^+\) T cells by a magnetic bead method. Each subset was added to 24-well plates containing CFSE-labeled wild-type B6 (Thy1.2\(^+\)) responder T cells and irradiated DBA/2 splenocytes. After a 4-day incubation, proliferation of each CD4\(^+\) and CD8\(^+\) T cell subset was determined by the CFSE profile after gating of cells by Thy1.2 and CD4 or CD8. The percentage of CFSE-labeled CD4\(^+\) or CD8\(^+\) cells that have divided one or more times was analyzed as reported previously by Wells et al. (24).

**Skin grafting**

Full-thickness DBA/2 skin was transplanted onto the lateral thorax of RAG\(^-/-\) mice using standard techniques. Following injection of naive B6 T cells with or without putative Treg cells, skin grafts were scored as described byBillingham et al. (25).

**Intracellular staining**

The lymph node cells were separated into CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells using FITC-conjugated anti-CD25 and CyChrome-conjugated anti-CD4 mAbs. Each subset of cells was fixed and permeabilized with Cytofix (BD Pharmingen) followed by intracellular staining with PE-conjugated mAbs directed to Bcl-x\(_L\) (clone H-5; Santa Cruz Biotechnology). For intracellular double staining, FITC-conjugated anti-CD4 (RM4-5; eBioscience), allophycocyanin-conjugated anti-CD25 (PC61.5; eBioscience), PE-conjugated anti-Bcl-x\(_L\), and biotin-conjugated anti-Foxp3 (FKH-166; eBioscience) mAbs were used. Stained cells were analyzed on a FACSscan.

**Real-time PCR**

For quantitative PCR analysis, RNA was extracted using an RNasy Mini kit (Qiagen) according to the manufacturer’s instructions, and cDNA was prepared with a TaqMan Reverse Transcription kit (Applied Biosystems) using random hexamers. Message levels were quantified by real-time PCR using the ABI 7700 Sequence Detection system (Applied Biosystems). Amplification was performed in a total volume of 25 \(\mu\)l for 40 cycles of 15 s at 95°C and 1 min at 60°C, and products were detected using probes labeled with VIC or FAM (Applied Biosystems), or primers labeled with SYBR Green I dye (Molecular Probes). The relative expression of each target gene was determined by normalizing the expression to GAPDH. Primers for CD25, Foxp3, Bcl-2, Bcl-x\(_L\), CTLA-4, IL-10, and GADPH were purchased from Applied Biosystems (sequence information of each primer was not provided).

**Statistical analysis**

Statistical significance was evaluated by the Kaplan-Meier life table method as well as Student’s t test. Differences with \(p < 0.05\) are deemed significant.

**Results**

**Phenotypic changes after T cell depletion by ALS**

We treated C57BL/6 (B6) mice with two doses (0.5 ml/dose) of ALS on days -1 and 2. This caused a 90–95% reduction of CD3\(^+\), CD4\(^+\) and CD8\(^+\) T cell numbers in the peripheral lymph nodes on day 7 (Fig. 1a). The majority of residual T cells (75–90% of CD4 and ~70% of CD8 T cells) expressed CD44\(^\text{high}\) but was negative for CD62L (data not shown). B cell counts remained relatively stable after ALS treatment. T cell numbers gradually recovered to the pre-ALS levels in 5 wk. The number of CD4\(^+\)CD25\(^-\) (CD25\(^+\)) and CD4\(^+\)CD25\(^+\) (CD25\(^+\)) T cells in the lymph nodes decreased to 10 and 25% of the pre-ALS levels, respectively, at 1 wk after ALS treatment. Unlike naive CD25\(^+\) T cells, the majority of post-ALS 1-wk CD25\(^+\) T cells were CD44\(^\text{high}\) (Fig. 1b). Similar phenotypic changes were observed in splenic T cells as well as other strains of inbred mice (data not shown). When ALS-treated, T cell-depleted mice were fed BrdU from day 7 until sacrifice at day 14, increased BrdU incorporation was observed in residual CD4\(^+\) T cells and CD8\(^+\) T cells as well as in CD4\(^+\)CD25\(^+\) T cells (Fig. 1c).

**In vivo regulatory activity of CD4\(^+\) T cells**

We tested the in vivo suppressive activity of post-ALS CD25\(^+\) T cells using a skin allograft model, a stringent model of alloresponsiveness. We transplanted groups of RAG\(^-/-\) mice with DBA/2 skin grafts and injected 0.1 \(\times\) 10\(^6\) naive B6 T cells with or without naive or post-ALS B6 CD25\(^+\) T cells (0.5 \(\times\) 10\(^6\) or 1.0 \(\times\) 10\(^6\)). We had predetermined that 0.1 \(\times\) 10\(^6\) was the minimum number of naive T cells capable of causing acute rejection. Addition of post-ALS CD25\(^+\) T cells induced statistically significant, but small, prolongation of graft survival over naive B6 T cell controls (\(p < 0.05\) for all three groups) (Table I). Although naive CD25\(^+\) T cells failed to induce significant graft prolongation, the differences in graft survival between recipients of naive vs post-ALS CD25\(^+\) T cells were significant only with 0.5 \(\times\) 10\(^6\) cells tested (\(p = 0.030\),
but not with $1.0 \times 10^6$ cells ($p = 0.462$ or 0.667 for 3 or 1 wk post-ALS cells).

**Post-ALS switch of the CD25 marker**

A characteristic of Treg cells is their high expression of the Foxp3 transcriptional factor. Therefore, we investigated the expression of the Foxp3 and CD25 transcripts by real-time PCR in naive and post-ALS CD25$^+$ and CD25$^-$ T cells that were purified by MoFlo sorting from spleen and lymph node T cells (Fig. 3a). CD25 expression remained high in CD25$^+$ and low in CD25$^-$ T cells after ALS treatment. Foxp3 was highly expressed in naive CD25$^+$ cells but not in naive CD25$^-$ cells. Foxp3 expression in CD25$^+$ T cells was slightly reduced at 1 wk post-ALS but returned to high levels thereafter. CD25$^-$ T cells transiently up-regulated Foxp3 expression 10-fold at 1 wk post-ALS, but returned to low expression thereafter.

To correlate the expression of CD25 and Foxp3 in post-ALS CD25$^+$ and CD25$^-$ T cells, we injected a mixture of magnetic beads-separated Thy1.2$^+$CD4$^+$CD25$^+$ cells ($2 \times 10^6$) and Thy1.1$^+$CD4$^+$CD25$^-$ cells ($20 \times 10^6$) into nonlymphopenic CD4-deficient mice and treated them 1 wk later with two doses of ALS. CD4$^+$ T cells injected into CD4-deficient mice do not undergo homeostatic expansion (data not shown). In addition, there is no new thymic emigration of CD4$^+$ T cells in CD4-deficient mice.

Three weeks after ALS injection, we determined the expression of the regulatory cell marker Foxp3 by real-time PCR in MoFlo-sorted Thy1.2$^+$CD25$^+$ cells and Thy1.1$^+$CD25$^-$ cells as well as in Thy1.2$^+$CD25$^+$ cells and Thy1.1$^+$CD25$^-$ cells (Fig. 3b) in the lymph nodes and spleens. After ALS treatment, Thy1.2$^+$ cells included CD25$^+$Foxp3$^+$ cells as well as CD25$^-$Foxp3$^+$ T cells while post-ALS Thy1.1$^+$ cells included CD25$^+$Foxp3$^+$ cells as well as CD25$^-$Foxp3$^+$ T cells.

In similar reconstitution experiments in which Thy1.2$^+$CD4$^+$CD25$^+$ cells and Thy1.1$^+$CD4$^+$CD25$^-$ cells were injected into CD4-deficient mice, we analyzed expression of several genes before and after reconstitution and ALS treatment by real-time
MoFlo-sorted T cells were used for analyses. Representative results of three separate experiments are shown in Table II. Although preinjection CD4+CD25+ Foxp3, CTLA-4, glucocorticoid-induced TNFR (GITR), and IL-10 expression than preinjection CD4+CD25− cells, regardless of their Thy1 phenotypes, so did post-ALS CD4+CD25+ cells although CD25 and Foxp3 expression was lower than preinjection CD4+CD25+ cells. Post-ALS Thy1.2+CD4+CD25+ cells showed a similar profile as preinjection Thy1.2+CD4+CD25− cells, except absence of CD25 expression and somewhat lower Foxp3 expression. Post-ALS Thy1.1+CD4+CD25+ cells remained negative for CD25 and Foxp3 expression but showed moderate up-regulation of CTLA-4 and GITR expression.

To further analyze the changes of CD25 markers post-ALS, we injected a mixture of magnetic beads-sorted GFP−CD25+ (2 × 10^6) and wild-type (GFP+) CD25+ (2 × 10^6) T cells into CD4-deficient mice. One week later, mice were given two doses of ALS. At various time points (1–4 wk) after ALS, we determined the proportion of CD25+ and CD25− T cells in the lymph nodes and spleens by flow cytometry (Fig. 3c). Flow cytometric analysis of the cell mixture before injection showed that the GFP+CD25− cell preparation contained small numbers of CD25+ cells (4–9%). The GFP−CD25+ cell preparation also contained CD25+ cells (13–23%). After ALS treatment, the proportion of GFP−CD25+, GFP+CD25+, and GFP−CD25− markedly increased to ~34%.

Expression of Bcl-xL by CD25+ and CD25− T cells

The degree of CD25+ T cell deletion after ALS treatment was always less than that of CD25− T cell deletion (Fig. 1a). This appeared to support our previous postulation that suppressor T cells might escape deletion by ALS (13, 26). To investigate this possibility, we analyzed the expression of anti-apoptotic genes, Bcl-2 and Bcl-xL, in naive CD25+ and CD25− T cells by real-time PCR. Although Bcl-xL expression was always slightly higher in

**FIGURE 2.** Regulat ory activity of CD25+ and CD25− T cells. CD25+ and CD25− cells were prepared from naive or ALS-treated Thy1.1+B6 mice and added to 24-well plates containing CFSE-labeled naive Thy1.2+B6 responder T cells and irradiated DBA/2 splenocytes. After a 4-day incubation, proliferation of each CD4+ or CD8+ T cell subset was determined by the CFSE profile and the percentage of cells that underwent more than one cell division.

a, Representative results of an experiment in which CD25+ and CD25− cells harvested 2 wk after ALS treatment were used. b, Inhibition of CD4+ and CD8-proliferative responses by naive or post-ALS CD25+ and CD25− cells. Mean ± SD of four separate experiments is shown. *, **, and ***p < 0.05, p < 0.025, and p < 0.001, respectively, against regulatory activity of naive CD25+ or CD25− T cells. c, Dose response of regulatory activity (representative results). Indicated numbers of post-ALS Thy1.1+B6 CD25+ cells were added to the 24-well plates containing CFSE-labeled naive Thy1.2+B6 responder T cells and irradiated DBA/2 splenocytes. After a 4-day incubation, proliferation of the CD4+ T cell subset was determined.
CD25+ T cells than in CD25− T cells, there was no significant difference in Bcl-2 expression between the two cell populations (Fig. 4a). After ALS treatment, there was no up-regulation of Bcl-xL expression in CD25+ T cells while slight up-regulation was always seen in CD25− T cells (Fig. 4b). We also determined expression of Bcl-xL protein in both cell populations by intracellular staining before and after ALS treatment. As shown in Fig. 4c, a subset of naive CD25+ T cells (range, 22–51%, n = 3) stained strongly for intracellular Bcl-xL protein with a distinct peak (Bcl-xLhigh cells), whereas no such distinct peak was seen for CD25− T cells (0.2–3%). At 1 wk after ALS treatment, the majority of CD25+ T cells (81–94%, n = 3) was Bcl-xLhigh. The proportion of Bcl-xLhighCD25+ T cells gradually returned to the pre-ALS levels in 4 wk. There was a slight increase in the proportion of Bcl-xLhighCD25− T cells 1 wk post-ALS (6–20%, n = 3).

To further define the Bcl-xLhigh cells, we determined the expression of both Foxp3 and Bcl-xL proteins in naive and post-ALS CD4+CD25+ and CD4+CD25− T cells by double intracellular staining. In one experiment shown in Fig. 5, Bcl-xLhigh cells comprised 20% of naive CD25+ cells and 4% of naive CD25− cells. Although ~70% of naive CD25+ cells expressed intracellular Foxp3, 14% of Foxp3−CD25+ cells or 50% of Bcl-xLhighCD25+ cells were Bcl-xLhighFoxp3−. Most of the naive CD25− cells (90%) were Foxp3+. At 1 wk after ALS treatment, the proportion of Bcl-xLhigh cells increased to 52% in CD25+ cells and to 28% in CD25− cells. The majority of Bcl-xLhigh cells also expressed Foxp3 in both CD25+ (80%) and CD25− (70%) cells. The overall proportion of Foxp3+ cells also increased in both CD25+ (82%) and CD25− cells (40%) while 50% of Foxp3+ T cells were Bcl-xLhighFoxp3+ in both populations.

**Discussion**

A recent study by Wu et al. (11) demonstrated that administration of depleting anti-CD4 plus anti-CD8 mAb caused homeostatic expansion of residual T cells and their conversion to alloantigen-reactive memory T cells. Lymphopenic acid mice that were adoptively transferred with postexpansion memory T cells showed dominant resistance to tolerance induction by a combination of donor-specific Ag administration and CTLA-4Ig, suggesting that incomplete T cell depletion may pose a barrier to the establishment of transplantation tolerance. The proportion and suppressor activity of CD4+CD25+ T cells in their lymph nodes and spleens after cessation of homeostatic proliferation (at 12 wk) remained the same as naive control mice. More recently, Pearl et al. (12) reported that residual T cells present following T cell depletion by Campath-1H or ALS in transplant patients were predominantly activated memory-like T cells. These memory-like T cells were found to be sensitive to calcineurin-inhibitors. CD4+CD25+ T cells were relatively spared from deletion and their proportion was not significantly different from that in unmanipulated individuals. Regulatory function of residual CD4+CD25+ T cells was not determined in this study.

We demonstrated in the present study that residual T cells remaining after T cell deletion by ALS, a polyclonal anti-T cell Ab, not only underwent homeostatic proliferation and conversion to phenotypically memory-like T cells but also continuously exhibited regulatory activity in vitro. Regulatory activity peaked 1 wk after ALS treatment when both CD4+CD25+ and CD4+CD25− T cells mediated suppression of alloresponses. By 3 wk after ALS treatment, the regulatory activity of CD25+ or CD25− T cells returned to the level of suppression mediated by naive CD25+ T cells (~30% inhibition) or naive CD25− T cells (0–5%), respectively. These results confirmed our earlier report (26) in which we showed that Lyt-1−(CD4+)Lyt-2− (CD8−) cells prepared from ALS-treated mice inhibited the proliferative response as well as generation of cytotoxicity in vitro in an Ag-nonspecific and dose-dependent manner. The suppression was maximum 4 days after ALS treatment (96%) and gradually decreased thereafter (26).

**Table I. In vivo suppressive effect of naive and post-ALS Tregsa**

<table>
<thead>
<tr>
<th>Naive T Cells</th>
<th>CD25+ T Cells</th>
<th>n</th>
<th>Graft Survival (days)</th>
<th>Median Survival Time</th>
<th>p b</th>
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</thead>
<tbody>
<tr>
<td>0.1 × 10^6</td>
<td>None</td>
<td>7</td>
<td>13, 14, 14, 14, 14, 16</td>
<td>14</td>
<td>—</td>
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<tr>
<td>0.1 × 10^6</td>
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<td>7</td>
<td>13, 14, 14, 14, 15, 17, 22</td>
<td>14</td>
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<tr>
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<td>Naive, 1.0 × 10^6</td>
<td>6</td>
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<td>16</td>
<td>0.079</td>
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<td>Post-ALS, 0.5 × 10^6</td>
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<td>13, 14, 15, 15, 16, 17, 18, 24</td>
<td>15</td>
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<td>17</td>
<td>0.000</td>
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<tr>
<td>0.1 × 10^6</td>
<td>Post-ALS, 1.0 × 10^6</td>
<td>4</td>
<td>17, 20, 22, 23</td>
<td>21</td>
<td>0.002</td>
</tr>
</tbody>
</table>

a Recipients: RAG−/− mice; skin donor: DBA/2 mice.

b p against naive T cells alone.

—, Tregs were harvested at 1 wk after ALS treatment whereas all other Tregs were harvested at 3 wk after ALS treatment.

**Table II. Changes in gene expression following reconstitution and ALS treatmenta**

<table>
<thead>
<tr>
<th>Cell Subsets</th>
<th>Preinjection/Post-ALS</th>
<th>CD25</th>
<th>Foxp3</th>
<th>CTLA-4</th>
<th>GITR</th>
<th>IL-10</th>
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<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Thy.1.2 CD4+CD25+</td>
<td>Preinjection</td>
<td>79</td>
<td>369</td>
<td>108</td>
<td>84</td>
<td>6</td>
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<tr>
<td>Thy.1.2 CD4+CD25−</td>
<td>Pre (not injected)</td>
<td>1</td>
<td>5</td>
<td>16</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Thy.1.2 CD4+CD25+</td>
<td>Post-ALS</td>
<td>23</td>
<td>237</td>
<td>67</td>
<td>82</td>
<td>6</td>
</tr>
<tr>
<td>Thy.1.1 CD4+CD25+</td>
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<td>2</td>
<td>9</td>
<td>16</td>
<td>22</td>
<td>1</td>
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<tr>
<td>Thy.1.1 CD4+CD25+</td>
<td>Pre (not injected)</td>
<td>73</td>
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<td>Thy.1.1 CD4+CD25+</td>
<td>Post-ALS</td>
<td>30</td>
<td>175</td>
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<td>Preinjection</td>
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<td>99</td>
<td>17</td>
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<td>1</td>
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<tr>
<td>Thy.1.2 CD4+CD25−</td>
<td>Post-ALS</td>
<td>&lt;1</td>
<td>40</td>
<td>47</td>
<td>25</td>
<td>3</td>
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<tr>
<td>Thy.1.1 CD4+CD25+</td>
<td>Preinjection</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Thy.1.1 CD4+CD25−</td>
<td>Post-ALS</td>
<td>&lt;1</td>
<td>3</td>
<td>17</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

a Numbers represent the relative expression of each target gene normalized to GAPDH expression.
Adoptive transfer of post-ALS CD25\(^+\) T cells to lymphopenic RAG\(^{-/-}\) mice along with effector T cells induced only a small prolongation of skin allograft survival.

Naive CD25\(^+\) cells expressed high levels of Foxp3, whereas Foxp3 expression of naive CD25\(^-\) cells was extremely low. By intracellular staining, the majority of naive CD25\(^-\) cells was Foxp3\(^-\) while most of the naive CD25\(^+\) cells were Foxp3\(^+\). A small percentage of naive CD25\(^-\) cells expressed the intracellular Foxp3 protein as previously reported (27). At 1 wk after ALS treatment, CD25\(^-\) cells showed a 10-fold up-regulation of Foxp3 gene expression and a 4-fold increase in intracellular Foxp3 expression. CD25\(^+\) cells also showed an increase in intracellular Foxp3 expression, although up-regulation of Foxp3 gene expression was not always observed. An increase in Foxp3 protein expression in the CD25\(^-\) and CD25\(^+\) subsets at 1 wk post-ALS corresponded to their marked regulatory activity.

Adoptive transfer of the purified Thy1.2\(^+\)CD4\(^+\)CD25\(^-\) and Thy1.1\(^+\)CD4\(^+\)CD25\(^+\) T cell subsets into CD25-deficient mice revealed that ALS treatment led to conversion between CD25\(^-\) and CD25\(^+\) cells. By real-time PCR analyses, Thy1.2\(^+\)CD4\(^+\)CD25\(^-\) cells remained Foxp3\(^-\) following adoptive transfer, although its expression was somewhat lower than naive CD25\(^+\) T cells. The reconstitution studies with similarly purified GFP\(^+\)CD25\(^-\) and GFP\(^+\)CD25\(^+\) T subsets in CD25-deficient mice showed that contaminating GFP\(^+\)CD25\(^+\) cells in the GFP\(^+\)CD25\(^-\) T preparation underwent substantial expansion following ALS treatment. Thus, post-ALS Thy1.2\(^+\)CD4\(^+\)CD25\(^-\)Foxp3\(^-\) cells might also include a small number of contaminating CD25\(^+\)Foxp3\(^+\) cells that were converted to CD25\(^-\) cells (28), and/or CD25\(^+\)Foxp3\(^-\) cells that became CD25\(^+\) and attained Foxp3 expression (29), after homeostatic expansion. The emergence of Thy1.2\(^+\)CD4\(^+\)CD25\(^-\)Foxp3\(^+\) cells is attributable to loss of the CD25 marker during ALS-induced homeostatic proliferation by the naive CD25\(^+\) T cells since their gene profile is similar to that of CD4\(^+\)CD25\(^-\) cells except for the absence of CD25 and somewhat lower Foxp3 expression. Gavin et al. (30) reported that CD4\(^+\)CD25\(^+\) Treg cells were capable of undergoing homeostatic proliferation in a lymphopenic environment and acquired memory-like CD44\(^{hi}\)CD45RB\(^{lo}\) expression with loss of CD25 and CD62L expression but exhibit substantially augmented suppressive function. The appearance of Thy1.1\(^+\)CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells after ALS treatment is probably due to the expansion of a small number of CD25\(^+\)Foxp3\(^+\) cells and contaminating CD25\(^+\)Foxp3\(^-\) cells in the Thy1.1\(^+\)CD4\(^+\)CD25\(^+\) preparation and/or the conversion of CD25\(^-\)Foxp3\(^-\) T cells to CD25\(^+\)Foxp3\(^+\) cells during ALS-induced expansion (29).

Following ALS treatment, reduction of CD4\(^+\)CD25\(^+\) T cell numbers (70–75% at 1 wk after ALS treatment) was always smaller than that of CD4\(^+\)CD25\(^-\) cells (85–90%), suggesting possible resistance of CD25\(^+\) T cells to Ab-mediated deletion. This possibility was supported by analysis of an intracellular Bcl-xL molecule in pre- and post-ALS CD25\(^+\) and CD25\(^-\) T cells. Naive CD4\(^+\)CD25\(^-\) T cells showed two distinct peaks with 20–50% of the cells being Bcl-X\(_L\)\(^{hi}\) and the remaining cells being Bcl-X\(_L\)\(^{lo}\). In contrast, only a small fraction (0.2–3%) of naive CD4\(^+\)CD25\(^-\) T cells were Bcl-X\(_L\)\(^{hi}\). The difference in Bcl-X\(_L\) expression between naive CD25\(^+\) and CD25\(^-\) T cells was small, albeit statistically significant. Thus, the marked difference in the proportion of Bcl-X\(_L\)\(^{hi}\) cells between naive CD25\(^+\) and CD25\(^-\) T cells may be due to up-regulation of Bcl-X\(_L\) in CD25\(^+\) cells that occurred at the translational rather than the transcriptional level. Translational regulation of Bcl-X\(_L\) and Bcl-2 has been reported during cell rescue by
been reported that naive CD4+ cells (four mice at each time point) were separated by a microbeads method.

FIGURE 4. Expression of Bcl-xL by CD25+ and CD25− T cells. a, MoFlo-sorted naive CD25+ and CD25− T cells were analyzed for Bcl-xL and Bcl-2 expression by real-time PCR. Results are expressed by mean ± SD of the ratio (relative expression in CD25+ cells divided by that in CD25− cells for each experiment, n = 10). b, Pre- and post-ALS changes in Bcl-xL gene expression. Spleen and lymph node T cells pooled from four to six B6 mice for each time point were separated into CD25+ and CD25− cells by a MoFlo cell sorter and analyzed for Bcl-xL gene expression by real-time PCR. c, Sorted naive and post-ALS CD25+ and CD25− T cells were analyzed for the presence of intracellular Bcl-xL molecule. Pooled T cells (four mice at each time point) were separated by a microbeads method.

FIGURE 5. Expression of intracellular Bcl-xL and Foxp3 by CD25+ and CD25− T cells. Naive and post-ALS (1 wk) CD4+ T cells were stained for CD4, CD25, and Bcl-xL, and Foxp3. CD25+ and CD25− T cells were analyzed for the presence of the Bcl-xL and Foxp3 molecules.
when they were adoptively transferred along with diabetogenic T cells (15). Since we have shown that autoimmunity is relatively weaker than alloimmunity (38), concomitant deletion of autoimmune effector T cells and preservation of autoimmune Treg cells by ALS favors the shifting of balance between the effector cells and regulatory cells toward a Treg cell-dominant condition, resulting in long-lasting prevention of recurrence of autoimmune diabetes.

Disclosures

The authors have no financial conflict of interest.

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6. Bonnefoy-Berard, N., C. Vincent, and J. P. Revillard. 1991. Antibodies against antigen-presenting cells (15). Since we have shown that autoimmunity is relatively weaker than alloimmunity (38), concomitant deletion of autoimmune effector T cells and preservation of autoimmune Treg cells by ALS favors the shifting of balance between the effector cells and regulatory cells toward a Treg cell-dominant condition, resulting in long-lasting prevention of recurrence of autoimmune diabetes.

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References


