Naturally anergic and suppressive CD25⁺CD4⁺ T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation

Yuhshi Kuniyasu¹,², Takeshi Takahashi¹,³, Misako Itoh¹, Jun Shimizu¹, Gotaroh Toda² and Shimon Sakaguchi¹,³

¹Department of Immunopathology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan
²Department of Medicine, Jikei Medical University, Tokyo 305-0006, Japan
³Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

Keywords: anergy, autoimmune disease, immunoregulation, self-tolerance

Abstract

A CD4⁺ T cell subpopulation defined by the expression levels of a particular cell surface molecule (e.g., CD5, CD45RB, CD25, CD62L or CD38) bears an autoimmune-preventive activity in various animal models. Here we show that the expression of CD25 is highly specific, when compared with other molecules, in delineating the autoimmune-preventive immunoregulatory CD4⁺ T cell population. Furthermore, although CD25 is an activation marker for T cells, the following findings indicate that immunoregulatory CD25⁺CD4⁺ T cells are functionally distinct from activated or anergy-induced T cells derived from CD25⁻CD4⁺ T cells. First, the former are autoimmune-preventive in vivo, naturally unresponsive (anergic) to TCR stimulation in vitro and, upon TCR stimulation, able to suppress the activation/proliferation of other T cells, whereas the latter scarcely exhibit the in vivo autoimmune-preventive activity or the in vitro suppressive activity. Second, such activated or anergy-induced CD25⁻ spleen cells produce various autoimmune diseases when transferred to syngeneic athymic nude mice, whereas similarly treated normal spleen cells, which include CD25⁺CD4⁺ T cells, do not. Third, upon polyclonal T cell stimulation, CD25⁺CD4⁺ T cells express CD25 at higher levels and more persistently than CD25⁻CD4⁺ T cell-derived activated T cells; moreover, when the stimulation is ceased, the former revert to the original levels of CD25 expression, whereas the latter lose the expression. These results collectively indicate that naturally anergic and suppressive CD25⁺CD4⁺ T cells present in normal naive mice are functionally and phenotypically stable, distinct from other T cells, and play a key role in maintaining immunologic self-tolerance.

Introduction

There is accumulating evidence that, besides clonal deletion or anergy, T cell-mediated dominant control of self-reactive lymphocytes also contributes to the maintenance of immunologic self-tolerance (1–3). For example, development of T cell-mediated autoimmune diseases [such as thyroiditis, gastritis and insulin-dependent diabetes mellitus (IDDM)] can be prevented in various animal models by inoculating CD4⁺ T cells or a subpopulation of CD4⁺ T cells (such as CD5high, CD45RBlow, CD62Lhigh or CD25⁻CD4⁺ T cells) prepared from histocompatible normal animals (4–17). Furthermore, various autoimmune diseases including thyroiditis, gastritis and IDDM can be de novo produced in normal rodents by simply removing a CD5high, CD45RBlow or CD25⁺ subpopulation of CD4⁺ T cells without exogenous immunization with self-antigens, and reconstitution of the removed cell population prevented the development of these autoimmune diseases (4–11). It remains obscure, however, whether such an immunoregulatory CD4⁺ T cell population is functionally and phenotypically a single entity of regulatory T cells irrespective of various cell surface phenotypes reported to date for the
population(s); whether they are functionally distinct from other immunoregulatory T cells, such as anergy-induced suppressive T cells or regulatory T cells secreting a particular immunoregulatory cytokine (18–24); and whether they are functionally and phenotypically stable in the normal immune system if they are engaged in the maintenance of immunologic self-tolerance at all.

We have previously shown that CD25+ T cells, which constitute 5–10% of CD4+ T cells and <1% of CD8+ T cells in the periphery of normal naive mice, are able to prevent autoimmune disease in vivo and suppress immune responses to non-self antigens in general (9–11). Interestingly, the CD25+CD4+ T cells are naturally unresponsive (anergic) to TCR stimulation in vitro (25–27), if one defines anergy as a reversible anti-proliferative state (25). Furthermore, upon TCR stimulation, they potently suppress the activation/proliferation of other CD4+ T cells and CD8+ T cells presumably through inhibiting IL-2 formation (25–27). This in vitro CD25+CD4+ T cell-mediated suppression depends on cell–cell interactions on antigen-presenting cells (APC) and does not appear to be mediated by far-reaching or long-lasting humoral factors or apoptosis-inducing signals (25, 27). Similar findings were also made with CD38+CD45RBlowCD4+ T cells (28). Furthermore, the normal thymus is continuously producing CD25+CD4+ T cells as a functionally mature immunoregulatory T cell subpopulation (26).

In this report, we have attempted to further characterize the immunoregulatory CD4+ T cells concerned with the maintenance of immunologic self-tolerance. We show that, compared with other markers (such as CD45RB, CD62L or CD38), the expression of CD25 is highly specific for this regulatory cell population, which plays a key role in maintaining immunologic self-tolerance and controlling immune responses in general.

**Methods**

**Mice**

Both 8-week-old BALB/c or and 6-week-old BALB/c athymic nude (nu/nu) mice were purchased from SLC (Shizuoka, Japan). To obtain BALB/c nu/+ or +/+ mice, BALB/c nu/+ mice were mated in our animal facility. BALB/c-Thy-1+ congenic mice were established in our laboratory as previously described (10). DO11.10 transgenic mice expressing ovalbumin (OVA)-specific transgenic TCR were the kind gift of Dr. D. Y. Loh (Hoffman-La Roche (29). All these mice were maintained in our animal facility and cared for in accordance with the institutional guidelines for animal welfare.

**Preparation of lymphocytes**

To eliminate CD25+ cells, spleen and lymph node cell suspensions (5×10^7) were incubated in 12×75 mm glass tubes (Corning, Corning, NY) with 100 µl of 1:10-diluted ascites of anti-CD25 antibody (7D4, rat IgM) (30) or anti-CD8 antibody (3.155) (rat IgM) for 45 min on ice, washed once with HBSS (Gibco/BRL, Gaithersburg, MD), incubated with 1.0 ml of non-toxic rabbit serum [as complement (C) source] (Cederlane, Hornby, Ontario, Canada) 1:5-diluted with Medium 199 (Gibco) for 30 min in a 37°C water bath with occasional vigorous shakings, with 100 µg of DNase I (Sigma, St. Louis, MO) added for the last 5 min of the incubation, washed twice with HBSS, and then i.v. injected into 6- to 8-week-old female nude mice (9). To eliminate CD8+ cells or CD4+ cells, cell suspensions (5×10^7) were treated with anti-CD4 antibody (RL172.4) or anti-CD8 antibody (3.155) as previously described (9).

**Serological analysis**

For flow cytometric analysis, 1×10^6 cells were incubated with FITC-labeled or biotinylated mAb, with phycoerythrin (PE)-streptavidin (BioMeda, Foster City, CA) as the secondary reagent for biotinylated antibody and analyzed by a flow cytometer (Epics XL; Coulter, Miami, FL) with exclusion of dead cells by propidium iodide staining. FITC-labeled or biotinylated anti-CD25 antibody (7D4), and biotinylated antibody for CD4 (H129.19), CD8 (53-6.7), CD45RB (16A) (31), CD62L (L-selectin) (Mel-14) (32), CD90.2 (Thy-1.2) (30-H12) and CD90.1 (Thy-1.1) were purchased from PharMingen (San Diego, CA). Biotinylated anti-CD38 antibody was a gift from Dr. K. Miyake (Saga Medical School, Saga, Japan (33).

**Cell sorting**

Spleen and lymph node cell suspensions or thymocyte suspensions prepared from 8-week-old BALB/c mice were stained with FITC-conjugated anti-CD25 antibody (7D4) and PE-conjugated anti-CD4 antibody (H129.19), and sorted by a FACS (Epics Elite; Coulter), as previously described (25). Purity of the CD25+ cells and CD25+CD4+ populations was >90% and ~99% respectively. In some experiments, CD4+ T cells were first enriched from spleen and lymph node cells by removing B cells, CD8+ T cells and adherent cells by panning on antibody-coated plastic dishes, as previously described (9), and then stained with FITC–anti-CD25 antibody along with biotinylated anti-CD62L, anti-CD45RB or anti-CD38 antibody with PE–streptavidin as the secondary reagent. CD25+CD4+ T cells thus enriched were sorted into a CD62Llow/high or CD62Llow CD45RBlow/high or CD45RBlow or CD38high population (26).

**Cell culture**

Lymph node and spleen cells (2.0–2.5×10^7), sorted as described above, and red blood cell-lysed, X-irradiated (20 Gy)-treated BALB/c spleen cells (5×10^6) as APC were cultured for 3 days in 96-well round-bottom plates (Costar) in RPMI 1640 medium supplemented with 10% FCS (Gibco/BRL), penicillin (100 U/ml) (Gibco/BRL), streptomycin (100 µg/ml) (Gibco/BRL) and 50 µM 2-mercaptoethanol (Sigma) (25, 26). Anti-CD3 antibody (145-2C11) (34) (Cederlane) at a final concentration of 10 µg/ml, Con A at 1.0 µg/ml or OVA peptides (residue 323–339) (29) at 0.3 µM, were added to the culture for stimulation (25). Incorporation of...
[\textsuperscript{3}H]thymidine (1 \muCi/well) by proliferating lymphocytes during the last 6 hours of the culture was measured. Recombinant murine IL-2 (rIL-2) (3.89 \times 10\textsuperscript{6} U/mg) was a gift of Shionogi (Osaka, Japan) (25). To prepare Con A blasts for \textit{in vivo} transfer, spleen and lymph node cells (5 \times 10\textsuperscript{5}/ml) were cultured with 5 \mug/ml of Con A for 3 days and washed twice with HBSS.

To render normal spleen cells anergic \textit{in vitro}, BALB/c spleen cells were cultured with 1 \muM ionomycin (IM; Sigma) overnight, as described by Jenkins et al. (35).

\textbf{Histology and serology}

Stomachs and other organs were fixed with 10% formalin and processed for hematoxylin & eosin staining. Serum titers of autoantibodies specific for the gastric parietal cells were assayed by ELISA (36). Gastritis was graded 0 to 2+ depending on macroscopic and histological severity: 0, the gastric mucosa was histologically intact; 1+, gastritis with histologically evident destruction of parietal cells and chief cells with cellular infiltration of the gastric mucosa; 2+, severe destruction of the gastric mucosa accompanying the formation of giant rugae due to compensatory hyperplasia of mucous-secreting cells (4,9).

\textbf{Results}

\textit{Cell surface phenotype of naturally anergic and suppressive CD25\textsuperscript{+}CD4\textsuperscript{+} T cells in normal naive mice}

We have previously shown that CD25\textsuperscript{+}CD4\textsuperscript{+} T cells present in normal naive mice contained cells expressing various levels of CD45RB or CD62L, although the expression levels of these molecules might change depending on the environment under which the mice were maintained (26). Others have also shown that autoimmune-preventive CD4\textsuperscript{+} T cells can be CD38\textsuperscript{+}CD45RB\textsuperscript{low} (28). To determine whether the anergic property or the suppressive activity, or both, of the CD25\textsuperscript{+}CD4\textsuperscript{+} population in normal naive mice can be attributed to a subpopulation included in it, the CD25\textsuperscript{+}CD4\textsuperscript{+} population was further dissected into smaller populations by expression levels of CD45RB, CD62L, or CD38 and each population was assessed \textit{in vitro} for proliferative response to Con A stimulation or for suppressive activity on the activation/proliferation of co-cultured CD25\textsuperscript{−}CD4\textsuperscript{+} T cells. Figure 1 shows that, when the CD25\textsuperscript{+}CD4\textsuperscript{+} T cell population was dissected to a CD45RB\textsuperscript{high} or CD45RB\textsuperscript{low} (Fig. 1B), CD62L\textsuperscript{high} or CD62L\textsuperscript{low} (Fig. 1C), or CD38\textsuperscript{high} or CD38\textsuperscript{low} subpopulation (Fig. 1D) by cell sorting, every subpopulation was virtually non-proliferative to the stimulation and potent suppress the proliferation of co-cultured CD25\textsuperscript{−}CD4\textsuperscript{+} T cells. The result indicates that the anergic and suppressive property of the CD25\textsuperscript{+}CD4\textsuperscript{+} T cell population cannot be reduced to a smaller subpopulation defined by an expression level of CD45RB, CD62L or CD38. Furthermore, high proliferative responses of CD25\textsuperscript{−}CD4\textsuperscript{+} T cells (Fig. 1A) imply that CD38\textsuperscript{high} or CD38\textsuperscript{low}, CD62L\textsuperscript{high} or CD62L\textsuperscript{low}, or CD45RB\textsuperscript{high} or CD45RB\textsuperscript{low} cells included in the CD25\textsuperscript{−}CD4\textsuperscript{+} T cell population (Fig. 1B–D) may not be sufficiently suppressive, if at all, even if such a subpopulation should contain suppressive T cells. Thus, expression of CD25 is highly specific for naturally anergic and suppressive CD4\textsuperscript{+} T cells present in normal naive mice.

\textit{Intensity and the pattern of CD25 expression are different between CD25\textsuperscript{+}CD4\textsuperscript{+} T cells in normal naive mice and those activated from CD25\textsuperscript{−}CD4\textsuperscript{+} T cells}

Since CD25 is generally expressed on activated T cells (30,37), we examined the intensity and the pattern of CD25 expression on CD25\textsuperscript{−} cell-depleted or non-depleted splenic CD4\textsuperscript{+} T cells prepared from normal naive BALB/c mice when the cells were stimulated with Con A (Fig. 2A). Staining of stimulated (day 3 or 7) or pre-stimulated (day 0) spleen cells with FITC-anti-CD4 antibody and PE-anti-CD25 antibody showed that the CD25\textsuperscript{+}CD4\textsuperscript{+} T cells present before stimulation increased their CD25 expression levels by day 3 and retained higher CD25 expression levels than the CD25\textsuperscript{−}CD4\textsuperscript{+} T cell-derived Con A blasts (or CD8\textsuperscript{+} Con A blasts) even on day 7 when the CD25 expression levels on the CD25\textsuperscript{−}CD4\textsuperscript{+} T cell-derived cells had already declined from the maximum levels (as judged from their mean fluorescent intensity). Furthermore, with a decline of the stimulation, the CD25 expression levels on the CD25\textsuperscript{+}CD4\textsuperscript{+} T cell-derived blasts reverted to the pre-stimulation levels, whereas the CD25\textsuperscript{−}CD4\textsuperscript{+} T cell-derived blasts virtually lost CD25 expression. This difference in the time course and the intensity of CD25 expression was confirmed by preparing CD25\textsuperscript{+}CD4\textsuperscript{+} T cells or CD25\textsuperscript{−}CD4\textsuperscript{+} T cells from Thy-1-congenic strains of mice (Fig. 2B), i.e. CD25\textsuperscript{+}CD4\textsuperscript{+} T cells prepared from BALB/c-Thy-1\textsuperscript{+} congenic mice (which express CD90.1) and CD25\textsuperscript{−}CD4\textsuperscript{+} T cells from BALB/c mice (which express CD90.2) were mixed at 1:9 ratio as in normal mice [in which ~10% of CD4\textsuperscript{+} T cells are CD25\textsuperscript{+} (Fig. 1A)] and stimulated with Con A (Fig. 2B). Indeed, the stimulated CD25\textsuperscript{+}CD4\textsuperscript{+} (CD90.1\textsuperscript{+}) T cells showed higher levels of CD25 expression during the stimulation than the CD25\textsuperscript{−}CD4\textsuperscript{+} (CD90.2\textsuperscript{−}) T cell-derived blasts. Thus, CD25\textsuperscript{−}CD4\textsuperscript{+} T cells present in normal naive mice are distinct from CD25\textsuperscript{−} T cell-derived activated T cells in intensity, stability and pattern of CD25 expression when stimulated.

Con A-activated T cells from normal CD4\textsuperscript{+} T cells can prevent autoimmune disease but those from TCR transgenic mice or activated CD8\textsuperscript{+} T cells cannot

CD4\textsuperscript{+} T cells, especially CD25\textsuperscript{+}CD4\textsuperscript{+} T cells, in normal naive mice bear the autoimmune-preventive activity (9–11). To determine whether Con A-activated CD4\textsuperscript{+} T cells, the majority of which express CD25 as shown in Fig. 2, can prevent autoimmune disease as well, we transferred to BALB/c athymic nude mice the cell mixtures containing a fixed number of CD25\textsuperscript{−} cells and graded numbers of CD4\textsuperscript{+} T cell blasts prepared by Con A treatment of normal BALB/c spleen cells. The mice were examined 3 months later for the incidence of histologically evident autoimmune disease in various organs (Table 1), for the histological severity of gastritis and for the titers of anti-parietal cell autoantibody (Fig. 3). Con A blasts from CD4\textsuperscript{+} T cells were able to prevent the development of autoimmune diseases in a dose-dependent fashion, but an equivalent dose of Con A blasts from CD8\textsuperscript{+} T cells did not, although the preventive activity of the CD4\textsuperscript{+} blasts was slightly lower when compared with the same number of unstimulated CD4\textsuperscript{+} splenic T cells. By contrast, Con
A blasts from CD25+CD4+ T cells showed no autoimmune-preventive activity at all.

DO11.10 TCR transgenic mice [in which the majority of CD4+ T cells express TCR specific for an OVA peptide (29)] harbor CD25+CD4+ T cells in the thymus and periphery, although their proportion is slightly smaller (~5% of the CD4+ population) than non-transgenic littermates (~10%) (26). To determine then whether CD4+ T cells activated by a specific antigen can prevent autoimmune disease, we transferred to BALB/c nude mice normal CD25+ splenic cells mixed with splenic T cells from DO11.10 transgenic mice and subsequently immunized the nude mice with OVA (Table 2 and Fig. 4). A group of nude mice received the mixture of CD25- cells and the transgenic T cells that had been activated in vitro with OVA peptides. The inoculated transgenic T cells showed no significant autoimmune-preventive activity in terms

Fig. 1. Cell surface phenotype of naturally anergic and suppressive CD4+ T cells. (A) CD25+ or CD25-CD4+ T cells (enclosed with rectangle) purified by FACS from BALB/c spleen and lymph node cells stained with FITC-anti-CD4 (ordinate) and PE-anti-CD25 (abscissa) as shown in the upper figure or these two populations mixed at various ratios were cultured for 3 days with anti-CD3 mAb along with X-irradiated BALB/c spleen cells as APC and the degree of proliferation was assessed as incorporation of [3H]thymidine (lower figure). In (B), (C) and (D), spleen and lymph node cells enriched for CD4+ T cells by panning (see Methods) were stained with FITC-anti-CD25 (ordinate) and PE-anti-CD45RB (abscissa), PE-anti-CD62L, or PE-anti-CD38 respectively. Among CD25+ cells, CD45RBlow or CD45RBhigh, CD62Llow or CD62Lhigh, CD38high or CD38low cells (enclosed with rectangles) in the CD25+ population were purified by FACS and stimulated with anti-CD3 antibody as described above. Percentage of each population among enriched CD4+ T cells (~90% purity) is also shown. Each purified population was mixed with CD25-CD4+ T cells (prepared by FACS as in Fig. 1A) at an equal ratio and stimulated. A representative result of three independent experiments is shown in (A–D). In these experiments (including those shown in Figs 3–6 below), background counts in the wells with APC only were <1500 c.p.m. The means of duplicate cultures are shown and the SEMs were all within 10% of the mean.
IM-induced anergic T cells are unable to exert suppressive activity

CD25⁺CD4⁺ T cells present in normal naive mice are anergic to TCR stimulation (Fig. 1). To determine then whether any anergic T cells are suppressive to other T cells, we rendered CD25⁻CD4⁺ T cells anergic by treating them with IM overnight and examined their in vitro suppressive activity on anti-CD3 antibody-stimulated proliferation of freshly prepared CD25⁺CD4⁺ T cells (Fig. 5A) and their in vivo autoimmune-suppressive activity (Fig. 6 and Table 3, experiment G). Such anergy-induced T cells derived from CD25⁺CD4⁺ T cells failed to suppress the responses of CD25⁺CD4⁺ T cells in vitro or to prevent the development of autoimmune disease in vivo. Exogenously added IL-2 partially restored the response of IM-treated CD25⁺CD4⁺ T cells, exerting no significant effects on their suppressive activity (Fig. 5B). Furthermore, transfer of IM-treated CD25⁺ spleen cells to nude mice produced autoimmune diseases at similar incidences and with similar severities as the transfer of non-treated CD25⁺ spleen cells (Table 3, experiment D). The results indicate that the anergic state of IM-treated cells may be limited in duration and hardly alter the autoimmune-inducing activity of the CD25⁺CD4⁺ population.

Besides IM treatment, we attempted to induce anergy in purified CD25⁺CD4⁺ T cells (or T cell lines derived from CD25⁺CD4⁺ T cells) by culturing them on anti-CD3 antibody-bound plates (38). The anergy thus induced was, however, transient and incomplete when compared with IM treatment, and not significantly suppressive on the activation/proliferation of CD25⁺CD4⁺ T cells (data not shown).

CD25⁺ cells activated with Con A alone are suppressive in vivo and in vitro but those activated with Con A and IL-2 are not

In contrast with the treatment of CD25⁺CD4⁺ T cells with Con A alone (Fig. 1A), treatment of CD25⁻CD4⁺ T cells with Con A and a high dose of IL-2 broke their anergic state and simultaneously abrogated their suppressive activity in vitro (Fig. 5B) (25). Furthermore, BALB/c splenic cells similarly treated with Con A and IL-2 not only failed to prevent autoimmune disease in nude mice when co-transferred with CD25⁺ spleen cells (Table 3, experiment F); they themselves also produced autoimmune disease in nude mice (Table 3, experiment C, and Fig. 6) (25). As the controls of these experiments, spleen cells treated with Con A alone elicited few autoimmune diseases (Table 3, experiment B), whereas Con A-treated CD25⁺ spleen cells efficiently produced various autoimmune diseases at high incidences (data not shown). Thus, treatment of normal spleen cells, especially CD4⁺ T cells, with a high dose of IL-2 along with TCR stimulation can abrogate not only the in vitro suppressive activity but also the in vivo autoimmune-preventive activity of CD25⁺CD4⁺ T cells.

Discussion

The immunoregulatory CD4⁺ T cells concerned with the maintenance of natural immunologic self-tolerance appear to
Table 1. Autoimmune-preventive activity of CD4+ T cell blasts prepared from normal splenic T cells

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Inoculated cells</th>
<th>Total no. of mice</th>
<th>No. of mice with autoimmune disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>CD25− spleen cells (2.5 × 10^7)</td>
<td>16</td>
<td>16 (100) 12 (75.0) 7 (43.8) 6 (37.5) 3 (18.8) 3 (18.8)</td>
</tr>
<tr>
<td>B</td>
<td>CD25− spleen cells (2.5 × 10^7) mixed with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4+ T cells (2.5 × 10^7)</td>
<td>8</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>CD4+ T cells (0.5 × 10^7)</td>
<td>9</td>
<td>4 (44.4) 1 (11.1) 0 0 0 0</td>
</tr>
<tr>
<td>C</td>
<td>CD25− spleen cells (2.5 × 10^7) mixed with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4+ Con A blasts (2.5 × 10^7)</td>
<td>8</td>
<td>1 (12.5) 0 0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>CD4+ Con A blasts (0.5 × 10^7)</td>
<td>9</td>
<td>7 (77.8) 2 (22.2) 1 (11.1) 1 (11.1) 0 0</td>
</tr>
<tr>
<td></td>
<td>CD8+ Con A blasts (2.5 × 10^7)</td>
<td>9</td>
<td>9 (100) 7 (77.8) 2 (22.2) 1 (11.1) 2 (22.2) 2 (22.2)</td>
</tr>
<tr>
<td>D</td>
<td>CD25− spleen cells (2.5 × 10^7) mixed with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4+ Con A blasts (2.5 × 10^7) from CD25− spleen cells</td>
<td>5</td>
<td>5 (100) 4 (80.0) 2 (40.0) 0 0 1 (20.0)</td>
</tr>
</tbody>
</table>

Indicated numbers of CD25− spleen cells were prepared from 2- to 3-month-old female BALB/c nu/− or +/+ mice by treatment with anti-CD25 antibody and rabbit complement, and i.v. transferred to 6-week-old female nu/nu mice (group A). Such CD25− spleen cells were also co-transferred with indicated numbers of CD4+ normal spleen cells (group B), CD4+ or CD8+ Con A blasts prepared from normal spleen cells (Group C), or CD4+ Con A blasts prepared from CD25− spleen cells (group D). The recipient nude mice were examined 3 months later for histological and/or serological development of autoimmune diseases. See also Fig. 3 for histological grades of gastritis and titers of anti-parietal cell autoantibody. Oophoritides were all grade 2 (9). Three mice in group A showed grade 2 thyroiditis; histologically evident thyroiditides in other mice were grade 1 (9).

Number of mice bearing respective autoimmune diseases is shown with percentage incidence in parentheses.

![Development of autoimmune gastritis in nude mice transferred with CD25− spleen T cells and its prevention by co-transfer of normal T cells or Con A blasts.](image-url)

**Fig. 3.** Development of autoimmune gastritis in nude mice transferred with CD25− spleen T cells and its prevention by co-transfer of normal T cells or Con A blasts. As shown in Table 1, BALB/c nude mice were transferred with indicated cell suspensions, and histologically and/or serologically examined 3 months later. *CD4+ Con A blasts prepared from CD25− spleen cells. Solid circles, grade 2 gastritis; shaded circles, grade 1 gastritis; open circles, intact gastric mucosa. See Methods for histological grading of gastritis.
Table 2. Inability of activated T cells from TCR transgenic mice to prevent autoimmune diseasea

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Inoculated cells</th>
<th>Total no. of mice</th>
<th>No. of mice with autoimmune disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CD25− spleen cells (2.5 × 10^7)</td>
<td>7</td>
<td>7 (100) 5 (71.4) 2 (28.6) 2 (28.6) 1 (14.3) 0</td>
</tr>
<tr>
<td>B</td>
<td>OVA-TG spleen cells (2.5 × 10^7) and CD25− spleen cells (2.5 × 10^7)</td>
<td>7</td>
<td>7 (100) 6 (85.7) 2 (28.6) 4 (57.1) 1 (14.3) 0</td>
</tr>
<tr>
<td>C</td>
<td>OVA-TG spleen cells (2.5 × 10^7) and CD25− spleen cells (2.5 × 10^7) with in vivo OVA immunization</td>
<td>5</td>
<td>5 (100) 3 (60.0) 2 (40.0) 2 (40.0) 1 (20.0) 0</td>
</tr>
<tr>
<td>D</td>
<td>OVA-Tg spleen cells (2.5 × 10^7) and CD25− spleen cells (2.5 × 10^7) with in vitro OVA stimulation</td>
<td>4</td>
<td>4 (100) 2 (50.0) 1 (25.0) 1 (25.0) 0 0</td>
</tr>
<tr>
<td>E</td>
<td>Normal CD4+ T cells (2.5 × 10^7) and CD25− spleen cells (2.5 × 10^7) with in vivo OVA immunization</td>
<td>4</td>
<td>0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

BALB/c athymic nude mice were transferred with CD25− spleen cells alone (group A) or after mixing with indicated numbers of CD4+ spleen cells from DO11.10 transgenic mice (groups B–D) or normal mice (group E). These nude mice were i.p. immunized with 1 mg of OVA once a week 4 times (groups C and E) or non-immunized (group B). A group of nude mice received CD25− spleen cells from normal mice and DO11.10 CD4+ spleen cells stimulated in vitro for 7 days with OVA (group D). These mice were examined 3 months later for histological and/or serological development of autoimmune diseases. See also Fig. 4 for histological grades of gastritis and titers of anti-parietal cell autoantibody. Oophoritides were all grade 2. Thyroiditides were all grade 1.

aNumber of mice bearing respective autoimmune diseases is shown with percentage incidence in parentheses.

CD25+CD4+ T cells in normal naive mice are continuously and stably expressing CD25 even in the absence of exogenous T cell stimulation, whereas other T cells lose CD25 when the stimulation is abrogated (Fig. 2).

High expression of CD25 and intermediate to low expression of CD45RB on the immunoregulatory CD25+CD4+ T cells (as shown in Fig. 1) suggest that they might be in an activated or primed state (39–41). The CD25+CD4+ T cells present in normal naive mice are indeed CD5high, CD44high, CD11a/CD18high and CD54high, as observed with activated, primed or memory T cells in general (9,25,26,39–41). However, they appear to be distinct from the usual activated T cells for the following reasons. First, the development of autoimmune disease was prevented by Con A blasts from uneliminated CD4+ T cells, but not by Con A-activated CD4+ T cell blasts prepared from CD25+CD4+ T cells. The latter rather produced severe autoimmune diseases when transferred to athymic nude mice (data not shown). Second, CD25+CD4+ T cells that had differentiated in a few months from the CD25−CD4+ T cells inoculated into nude mice hardly suppressed the in vitro activation/proliferation of CD25−CD4+ T cells prepared from the same nude mice, as previously shown (26). Third,
**CD25⁺CD4⁺ regulatory T cells and control of autoimmunity**

**Fig. 5.** Failure of anergy-broken CD25⁺CD4⁺ T cells or anergized CD25⁺CD4⁺ T cells to suppress the activation/proliferation of other T cells *in vitro*. (A) CD25⁻ spleen cells prepared as described in the legend to Table 1 were treated with IM for 3 days *in vitro* and then stimulated with Con A along with fresh APC. Such IM-treated cells or non-treated cells were also mixed with freshly prepared CD25⁻CD4⁺ T cells at an equal ratio and similarly stimulated. (B) A high dose of rIL-2 (100 U/ml) was also added to the Con A-stimulated culture of freshly prepared CD25⁺CD4⁺ T cells, IM-treated or non-treated CD25⁻ cells, or the mixture of these cells and freshly prepared CD25⁺CD4⁺ T cells. The means of duplicate cultures are shown and the SEMs were all within 10% of the mean. A representative result of two independent experiments is shown.

**Table 3.** Failure of anergy-broken CD25⁺CD4⁺ T cells or anergy-induced CD25⁻CD4⁺ T cells to prevent autoimmune disease

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Inoculated cells</th>
<th>Total no. of mice</th>
<th>No. of mice with autoimmune disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gastritis</td>
</tr>
<tr>
<td>A</td>
<td>CD25⁻ spleen cells (2.5 × 10⁷)</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>B</td>
<td>Con A-treated normal spleen cells (2.5 × 10⁷)</td>
<td>14</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>C</td>
<td>Con A/IL-2-treated normal spleen cells (2.5 × 10⁷)</td>
<td>16</td>
<td>13 (81.3)</td>
</tr>
<tr>
<td>D</td>
<td>IM-treated CD25⁻ spleen cells (2.5 × 10⁷)</td>
<td>6</td>
<td>6 (100)</td>
</tr>
<tr>
<td>E</td>
<td>A and B</td>
<td>12</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>F</td>
<td>A and C</td>
<td>8</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>G</td>
<td>A and D</td>
<td>5</td>
<td>5 (100)</td>
</tr>
</tbody>
</table>

BALB/c athymic nude mice were transferred with CD25⁻ spleen cells (prepared as described in the legend to Table 1) (group A), Con A-stimulated CD4⁺ spleen cells (group B), CD4⁺ blasts prepared by treatment of spleen cells with Con A and a high dose of IL-2 (see Methods) (group C) or IM-treated CD25⁻ spleen cells (group D). CD25⁻ spleen cells were also transferred after mixing with the T cells thus treated (groups E–G). The nude mice were examined 3 months later for histological and/or serological development of autoimmune diseases. See also Fig. 6 for histological grades of gastritis and titers of anti-parietal cell autoantibody. Oophoritides were all grade 2. Thyroiditides were all grade 1.

**Fig. 6.** Effects of breaking the anergic and suppressive state of regulatory T cells, or inducing anergy in other T cells, on induction of autoimmune disease. BALB/c spleen cells treated as in Table 3 were transferred to BALB/c nude mice, which were histologically and serologically examined 3 months later. Solid circles, grade 2 gastritis; shaded circles, grade 1 gastritis; open circles, intact gastric mucosa.
T cell blasts prepared from TCR-transgenic mice, which are homogeneous in terms of TCR specificity, failed to prevent autoimmune disease even when stimulated in vivo or prestimulated in vitro with a specific antigen. The result apparently contrasts with our former finding that DO11.10 transgenic mice harbor CD25+CD4+ T cells, which are anergic and exert potent suppression in vitro upon stimulation with an OVA peptide (25,26). This discrepancy could be attributed in part to a smaller number of OVA-activated CD25+CD4+ regulatory T cells contained in the CD4+ T cell inocula for autoimmune prevention, i.e., the size of the CD25+CD4+ T cell population in DO11.10 was about half that of normal mice. In addition, a sizable number of the transgenic CD25+CD4+ T cells expressed endogenous TCR α chains in association with transgenic TCR β chains, resulting in altered antigen specificities, hence insufficient activation of CD25+CD4+ regulatory T cells with OVA (26). Alternatively, but not exclusively, the discrepancy could be due to a possible failure of OVA-specific CD25+CD4+ regulatory T cells to be efficiently guided to the APC expressing specific self-antigen peptides, hence failure of controlling self-reactive T cells there (25).

Another important characteristic of the immunoregulatory CD25+CD4+ population is that it is naturally anergic to TCR stimulation (Figs 1 and 5) (25–27). This raises the question whether any T cells rendered anergic can also be suppressive on the activation/proliferation of other T cells (18–20). Indeed, recent studies by others have shown that anergic T cells exert suppression not via humoral factors including immunoregulatory cytokines, but through cell–cell interactions on APC apparently in a manner similar to naturally anergic and suppressive CD25+CD4+ T cells (19,20). These experimentally induced anergic T cells are also CD25+ in general (42–44). Our present experiments, however, showed that when CD25+CD4+ T cells were experimentally rendered anergic in vitro by IM treatment, they were ineffective in inhibiting the development of autoimmune disease in vivo and in suppressing the activation/proliferation of other T cells in vitro (Table 3 and Fig. 5). This difference between our results and those of others (18–20) could be attributed, at least in part, to their use of T cell clones, which may be homogenous not only in antigen specificity but also in the state of activation and the pattern of cytokine formation. Alternatively, the difference could be due to different ways of inducing anergy, e.g., our use of IM and their use of specific peptides or plate-bound anti-CD3 antibody (see Results). Furthermore, the following characteristics of CD25+CD4+ naturally anergic/suppressive T cells also make them distinct from experimentally induced anergic and suppressive T cells (18–20). First and foremost, the anergic and suppressive state of the former is the basal and default condition for them, e.g., the CD25+CD4+ T cells whose anergic state had been abrogated by TCR stimulation along with a high dose of IL-2 or anti-CD28 antibody ligating the CD28 molecules spontaneously revert to the original anergic/suppressive state upon removal of IL-2 or anti-CD28 antibody (25,26). Indeed, T cell clones and lines that can be prepared from the CD25+CD4+ T cell population in normal naive mice and maintained by intermittent stimulations with anti-CD3 antibody and IL-2 show the anergic/suppressive property when exogenous IL-2 is withdrawn (25,26) (J. Shimizu and S. Sakaguchi, manuscript in preparation). This markedly contrasts with usual anergic T cells, which will never revert to an anergic state spontaneously once their anergic state is abrogated (42). Second, the suppression by the CD25+CD4+ T cells is antigen-nonspecific in the effector phase (i.e., they suppress not only T cells with the same antigen specificity but also those with other specificities as well, as shown in 25), which is similar to linked suppression (45). In addition, they suppress the activation/proliferation of CD4+ T cells as well as CD8+ T cells (25,26). This contrasts with antigen-specific or -restricted suppression by experimentally induced anergy (18–20). Third, the normal thymus appears to be continuously producing the immunoregulatory CD25+CD4+ T cells which are already anergic and suppressive in the normal thymus (26). Although T cell clones or lines can be prepared which are anergic and suppressive in vitro, it remains to be determined whether normal T cells can be somehow converted to anergic and suppressive T cells in the periphery when exposed to self-antigens without costimulation in vivo (1,18–20).

In conclusion, the present results indicate that naturally anergic and suppressive CD25+CD4+ T cells present in normal naive mice are distinct from other activated, anergic or regulatory T cells in phenotypic and functional characteristics. The phenotype of CD25+CD4+ immunoregulatory T cells, on the other hand, suggests that they themselves may be continuously stimulated with self-antigens, hence in a CD25+ activated state, in the normal internal environment and continuously exerting suppressive control of other T cells (including self-reactive T cells) by raising their activation thresholds (25,26). Further characterization of this unique regulatory T cell population is required to elucidate the mechanism of T cell-mediated dominant immunoregulation and thereby to develop ways for effectively treating or preventing autoimmune disease (9), eliciting tumor immunity (46) or inducing tolerance to allo-transplants (9), by manipulating the mechanism.

Acknowledgements
We thank Dr D. Y. Loh for the TCR transgenic mice, Dr K. Miyake for anti-CD3 antibody and Ms E. Morizumi for preparing the histology. This work was supported by grants-in-aid from the Ministry of Human Welfare and the Organization for Pharmaceutical Safety and Research of Japan.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IM</td>
<td>iomycin</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
</tbody>
</table>

References


