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Thymus and Autoimmunity: Production of CD25⁺CD4⁺ Naturally Anergic and Suppressive T Cells as a Key Function of the Thymus in Maintaining Immunologic Self-Tolerance¹

Misako Itoh,*† Takeshi Takahashi,* Noriko Sakaguchi,* Yuushi Kuniyasu,* Jun Shimizu,* Fujio Otsuka, † and Shimon Sakaguchi²*¹

This study shows that the normal thymus produces immunoregulatory CD25⁺CD4⁺CD8⁻ thymocytes capable of controlling self-reactive T cells. Transfer of thymocyte suspensions depleted of CD25⁺CD4⁺CD8⁻ thymocytes, which constitute ~5% of steroid-resistant mature CD4⁺CD8⁻ thymocytes in normal naive mice, produces various autoimmune diseases in syngeneic athymic nude mice. These CD25⁺CD4⁺CD8⁻ thymocytes are nonproliferative (anergic) to TCR stimulation in vitro, but potently suppress the proliferation of other CD4⁺CD8⁻ or CD4⁺CD8⁺ thymocytes; breakage of their anergic state in vitro by high doses of IL-2 or anti-CD28 Ab simultaneously abrogates their suppressive activity; and transfer of such suppression-abrogated thymocyte suspensions produces autoimmune disease in nude mice. These immunoregulatory CD25⁺CD4⁺CD8⁻ thymocytes/T cells are functionally distinct from activated CD25⁺CD8⁺ T cells derived from CD25⁺CD4⁺ thymocytes/T cells in that the latter scarcely exhibits suppressive activity in vitro, although both CD25⁺CD4⁺ populations express a similar profile of cell surface markers. Furthermore, the CD25⁺CD4⁺CD8⁻ thymocytes appear to acquire their anergic and suppressive property through the thymic selection process, since TCR transgenic mice develop similar anergic/suppressive CD25⁺CD4⁺CD8⁻ thymocytes and CD25⁺CD4⁺ T cells that predominantly express TCRs utilizing endogenous α-chains, but RAG-2-deficient TCR transgenic mice do not. These results taken together indicate that anergic/suppressive CD25⁺CD4⁺CD8⁻ thymocytes and peripheral T cells in normal naive mice may constitute a common T cell lineage functionally and developmentally distinct from other T cells, and that production of this unique immunoregulatory T cell population can be another key function of the thymus in maintaining immunologic self-tolerance. The Journal of Immunology, 1999, 162: 5317–5326.

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CD25+ 4+ anergic/suppressive T cells, that the latter are rendered anergic and suppressive through the thymic selection process, and that physical elimination of such immunoregulatory CD25+ 4+ 8− thymocytes or functional breakage of their anergic/suppressive state can elicit autoimmune disease in otherwise normal mice. Our results indicate that, besides clonal deletion and clonal anergy, production of this naturally anergic and suppressive CD25+ 4+ T cell population is another key function of the thymus in maintaining immunologic self-tolerance.

Materials and Methods

Mice

Eight-week-old BALB/c or BALB/c nu/nu mice and six-week-old BALB/c nude (nu/nu) mice were purchased from SLC (Shizuoka, Japan). BALB/c-Thy-1 congenic mice were established in our laboratory (14). DO11.10 transgenic mice were the gift of Dr. D. Y. Loh, Roche Japan (Kamakura, Japan). DO11.10-RAG-2 knockout mice were provided by Drs. K. Iwasbuchi (Hokkaido University, Sapporo, Japan) and O. Kanagawa (Washington University, St. Louis, MO) (33). All of these mice were maintained in our animal facility and cared for in accordance with the institutional guidelines for animal welfare.

Preparation of lymphocytes

Thymocyte suspensions (5 × 10⁷), or spleen and lymph node cell suspensions (5 × 10⁶), were incubated in 12 × 75-mm glass tubes (Corning, Corning, NY) with 100 μl of 1/10-diluted actinomycin of anti-CD25 (7D4), rat IgM (34) or anti-CD8a.2 (mouse IgG2a) (35) for 5 min on ice, washed once with HBSS (Life Technologies, Gaithersburg, MD), incubated with 1 ml of nontoxic rabbit serum (as C source) (Life Technologies) 1/5 diluted with Medium 199 (Life Technologies) 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 6–8-wk-old female nu/nu mice, as previously described (13).

Serologic analysis

For flow-cytometric analysis, 1 × 10⁶ cells were incubated with FITC-labeled or biotinylated mAbs, with PE-streptavidin (BioMedica, Foster City, CA) as the secondary reagent for biotinylated Abs, and analyzed by a flow cytometer (Epics-XL; Coulter, Miami, FL) with exclusion of dead cells by propidium iodide staining. R-Phycocerythrin (RPE)-Cy5-conjugated streptavidin (Dako, Glostrup, Denmark) was used as the secondary reagent for biotinylated Abs in three-color analyses. FITC-labeled or biotinylated anti-CD25 (7D4) (34), and biotinylated Abs for CD4 (H129.19), CD54 (ICAM-1) (32E) (36), CD5 (53-7.3) (37), CD8 (53-6.7) (37), CD11a/CD18 (LFA-1) (CD2) (38), CD24 (heat-stable Ag) (M1/69) (CD44) (IM7) (39), CD45RB (16A) (CD45D) (M2E14) (41), CD69 (H1.2F3) (42), CD90.2 (Thy-1.2) (30-H12), CD2 (RM2.5), or TCR Vβ (LFA-1) (2D7) (38), CD24 (heat-stable Ag) (M1/69), CD44 (IM7), CD28, and Qa-2 (57-15). TCR Vβ7.2 was assessed in direct immunostaining in thymocytes, thymic medulla, and thymic cortex. Serum titers of autoantibodies specific for the gastric parietal cells were assessed by ELISA (48). Gastritis was graded 0–2+, depending on macroscopic and histologic severity: 0 = the gastric mucosa was histologically intact; 1 = gastritis with histologically evident destruction of parietal cells and 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 (8, 13, 14). Thyroiditis or oophoritis were histologically graded, as previously described (48). Adrenitis, insulitis, and sialoadenitis were assessed as histologically positive when destruction of adrenal, islet cells, or acinar cells in the submandibular glands, respectively, was histologically evident with infiltration of inflammatory cells to these tissues (48, 49). Glomerulonephritis was assessed as histologically positive when more than 50% of the renal glomeruli on a section were damaged with deposition of PAS (periodic acid-Schiff) staining-positive material (see Ref. 22).

Results

Presence of CD25+ CD4+ CD8− thymocytes in the normal thymus

Fig. 1A shows that a significant percentage (5.4 ± 1.9%, n = 32) of CD4+ 8− thymocytes and less than 0.3% of CD4+ 8− thymocytes in normal adult BALB/c mice expressed the CD25 molecule at equivalent levels as peripheral CD25+ 4+ T cells (Fig. 1B) and at lesser levels compared with the high level expression in the CD4−8− population (50, 51). Immunohistologic examination revealed that these CD25+ 4+ 8− thymocytes located in the thymic medulla, in contrast to CD25+8−4+ 8− thymocytes in the subcortical area (data not shown).

To further characterize the CD25+ 4+ 8− thymocytes, we compared between CD25+ 4+ 8− thymocytes the expression levels of various cell surface molecules, including those that are expressed on immunoregulatory T cells (e.g., CD5, CD45RB, and CD62L (8–10, 12, 52, 53)), those that correspond to activated, Ag-primed, or memory states (e.g., CD11a/CD18, CD44, CD54, CD62L, CD69, and CD122 (54–56)), or those that correlate with the stages of maturation or selection in the thymus (e.g., CD3, CD4, CD8, CD24, CD28, CD62L, CD69, CD90, and Qa-2 (57–60)) (Fig. 1B). The majority of CD25+ 4+ 8− thymocytes were higher than CD25+ 4+ 8− thymocytes in the expression of CD5, CD44, CD54, CD62L, and CD122; comparable in CD3, CD11a/CD18, CD28, CD45RB, CD90, and CD2 expressions; and slightly lower in CD4 expression (Fig. 1B, and unpublished data). The two thymocyte populations were also different in the expression patterns of some differentiation markers. For example, a lower proportion of CD25+ 4+ 8− thymocytes was CD69+8−, compared with...
CD25^+T cells suppressed the development of autoimmune diseases in vivo. CD25^+T cells were isolated from the thymus and peripheral lymph nodes of BALB/c nu/nu mice. These cells were transferred to BALB/c nude mice, and the incidence of autoimmune diseases was monitored. The results showed that the transfer of CD25^+T cells resulted in the suppression of autoimmune diseases, including thymitis, insulitis, and glomerulonephritis. The suppression of autoimmune diseases was dependent on the phenotype of CD25^+T cells, with the most effective suppression observed in CD25^+CD4^+T cells. The mechanism of this suppression is under investigation and may involve the inhibition of autoantigen presentation or the suppression of T cell activation.

**Table I. Induction of autoimmune disease by eliminating CD25^+T cells**

<table>
<thead>
<tr>
<th>Expt. Group</th>
<th>Inoculated Cells</th>
<th>Total Number of Mice</th>
<th>Number of Mice with Autoimmune Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Whole thymocytes (5 x 10^7)</td>
<td>12</td>
<td>7 (58.3) 0 0 0 0 0 0</td>
</tr>
<tr>
<td>B</td>
<td>CD25^+ thymocytes (5 x 10^7)</td>
<td>12</td>
<td>12 (100) 12 (100) 4 (33.3) 3 (25.0) 1 (8.3) 1 (8.3) 3 (25.0)</td>
</tr>
<tr>
<td>C</td>
<td>CD4^+8^ SR thymocytes (5 x 10^6)</td>
<td>5</td>
<td>4 (80.0) 0 0 0 0 0 0</td>
</tr>
<tr>
<td>D</td>
<td>CD25^+4^ SR thymocytes (5 x 10^5)</td>
<td>5</td>
<td>5 (100) 5 (100) 2 (40.0) 1 (20.0) 2 (40.0) 0 1 (20.0)</td>
</tr>
<tr>
<td>E</td>
<td>Whole SP/LN cells (5 x 10^7)</td>
<td>8</td>
<td>0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>F</td>
<td>CD25^+ SP/LN cells (5 x 10^5)</td>
<td>8</td>
<td>100 (800) 8 (100) 5 (62.5) 5 (62.5) 2 (25.0) 0 2 (25.0)</td>
</tr>
<tr>
<td>G</td>
<td>CD25^+ T cells and CD4^+ SP/LN cells (2 x 10^5)</td>
<td>8</td>
<td>0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

*Thymocytes or spleen/lymph node (SP/LN) cells of indicated numbers were prepared from 2- to 3-mo-old female BALB/c nu/nu mice by treatment with complement (C) alone (group A) or anti-CD25 and C (groups B and F), and then i.v. transferred to 6-wk-old female nu/nu mice. Steroid-resistant (SR) thymocytes (see Materials and Methods) were treated with anti-CD8 and C (group C) or anti-CD25 and anti-CD8 plus C (group D). Another group of mice received mixed populations of CD25^+ thymocytes as prepared for group B and CD4^+ SP/LN cells prepared by anti-CD8 plus C treatment of nylon wool column-passed SP/LN cells (group G). The recipient mice were examined 3 mo later for histological and serological development of autoimmune diseases. See also Fig. 2 for histological grades of gastritis and titers of anti-parietal cell autoantibody. Oophoritides were all grade 2 (22). One mouse in group B and two mice each in group D and F showed grade 2 thyroiditis; histologically evident thyroiditis in other mice was grade 1.

Number of mice bearing respective autoimmune diseases is shown with percentage incidence in parentheses. Gas, gastritis; Oop, oophoritis; Thr, thyroiditis; Sial, sialoadenitis; Adr, adrenals; Ins, insulinitis; Gn, glomerulonephritis.
with CD4+ splenic and lymph node cells (2 × 10⁷), of which 5–10% were CD25+ (Fig. 1C), resulted in the complete inhibition of autoimmune development.

Thus, the normal thymus contains both CD25+ 4+ pathogenic self-reactive T cells and CD25+ 4+ autoimmune-preventive T cells in the compartment of mature thymocytes. Furthermore, when pathogenic self-reactive T cells are released from the thymus, they can be controlled by CD25+ 4+ T cells already present in the periphery.

Origin of CD25+ 4+ 8− thymocytes

To determine the origin of the CD25+ 4+ 8− thymocytes (i.e., whether they have differentiated in the thymus from immature thymocytes or migrated from the periphery as activated T cells (64, 65)), immature CD4− 8− thymocytes prepared from BALB/c mice, in which thymocytes express Thy-1.2 (CD90.2) Ag, were directly injected into the thymus of BALB/c-Thy-1 congenic mice, which express Thy-1.1 (CD90.1) Ag (Fig. 3A). Staining of the recipient thymus 1 wk later with anti-Thy-1.2 Ab revealed that the CD25+ 4+ 8− population indeed contained a significant number of donor-derived Thy-1.2+ cells. This indicates that the inoculated CD4− 8− thymocytes gave rise to CD25+ 4+ 8− thymocytes. CD25+ 4+ 8− thymocytes also developed in vitro from CD4− 8− thymocytes in an organ culture of fetal thymus (data not shown). Furthermore, they were already present in the thymus of newborn mice before 3 days of age when CD25+ 4+ T cells could be first detected in the periphery (14); for example, a significant proportion (0.9 ± 0.6%, n = 6) of CD4− 8− thymocytes (including CD4− 8− and CD4− 8+ thymocytes) was CD25+ in 2-day-old mice, whereas no CD25+ T cells were detected in their spleens (Fig. 3B). These results taken together indicate that most, if not all, CD25+ 4+ 8− thymocytes are generated in the thymus rather than having migrated from the periphery.

CD25+ 4+ 8− thymocytes are unresponsive to TCR stimulation in vitro and suppress proliferative responses of other thymocytes/T cells

CD25+ 4+ 8− thymocytes purified by FACS (as shown in Fig. 1B) from normal adult BALB/c mice exhibited virtually no response to in vitro stimulation with anti-CD3 Ab or Con A (Fig. 4, A and B); whereas the purified CD25+ 4+ 8− thymocytes showed significantly higher responses than the unseparated CD4+ 8− thymocytes. Furthermore, CD25+ 4+ 8− thymocytes suppressed the responses of CD25+ 4+ 8− thymocytes (and CD4+ 8− thymocytes) in a dose-dependent fashion when the two populations were mixed in various ratios and stimulated with anti-CD3 Ab (e.g., percentage of suppression (see Materials and Methods) was >95% in every experiment at a 1:1 ratio of cell mixing). CD25+ 4+ 8− thymocytes also suppressed the responses of CD25+ 4+ peripheral T cells; likewise, CD25+ 4+ peripheral T cells suppressed the responses of CD25+ 4+ 8− thymocytes as well as CD25− 4+ 8− peripheral T cells (Fig. 4B). These results taken together indicate that the CD25+ 4+ 8− population in the thymus of normal naive mice is naturally unresponsive to Ag stimulation, but, upon stimulation,

![FIGURE 2](http://www.jimmunol.org/) Development of autoimmune gastritis in nude mice transferred with CD25+ thymocytes or peripheral T cells and its prevention by cotransfer of normal CD4+ T cells. As shown in Table I, BALB/c nu/nu mice 6 wk of age were transferred with indicated cell suspensions, and histologically and serologically examined 3 mo later. ●, Grade 2 gastritis; ○, grade 1 gastritis; ◦, intact gastric mucosa. See Materials and Methods for histologic grading of gastritis.

![FIGURE 3](http://www.jimmunol.org/) Thymic generation of CD25+CD4+CD8− thymocytes. A, Thymocyte suspension prepared from the thymus of a BALB/c-Thy-1+ congenic mouse that had received intrathymic (IT) inoculation of CD4−CD8− thymocytes from BALB/c mice 1 wk before they were stained with PE anti-CD4, FITC anti-CD25, and biotinylated anti-Thy-1.2. Thy-1.2 expression on whole CD4+ or CD25+CD4+ population enclosed on the left is shown on the right as a histogram with percentage of Thy-1.2+ cells in each fraction. B, Thymocyte or spleen cell suspension from 2-day-old newborn (NB) mice was stained with PE anti-CD4 and FITC anti-CD25. A representative result of three independent experiments is shown in A and B.
suppresses the activation/proliferation of other thymocytes/T cells; and it is functionally similar to the peripheral CD25 \(^+\) T cell population, which is also anergic and suppressive.

**Abrogation of unresponsiveness/suppression of CD25 \(^+\) thymocytes by high doses of IL-2 or anti-CD28 Ab, and induction of autoimmune disease by such treatments**

Given that Ag stimulation together with exogenous IL-2 or anti-CD28 Ab breaks T cell unresponsiveness in vitro (47, 66, 67), we examined the effect of IL-2 or anti-CD28 Ab on the unresponsiveness of CD25 \(^+\) thymocytes and their suppressive activity (Fig. 5, A and B). Stimulation of CD25 \(^+\) thymocytes with anti-CD3 Ab in the presence of exogenously added rIL-2 (100 U/ml concentration) or anti-CD28 Ab (10 \(\mu\)g/ml) not only elicited their proliferation, but also abrogated their suppressive activity.

When these proliferating CD25 \(^+\) T cells stimulated with anti-CD3 Ab and rIL-2 or anti-CD28 Ab were harvested on day 3, washed, and restimulated with anti-CD3 Ab along with fresh APCs, but without exogenous IL-2, they showed no proliferative response and markedly suppressed the responses of freshly cocultured CD25 \(^+\) thymocytes (Fig. 5C). In contrast, similarly treated CD25 \(^+\) thymocytes did not show unresponsiveness or suppression in the secondary culture. This indicates that, upon removal of IL-2 or anti-CD28 Ab, the anergy/suppression-broken CD25 \(^+\) thymocytes revert to their original unresponsive/suppressive state.

The in vitro analyses described above suggest the possibility that autoimmune disease may develop in normal mice if the abrogation of the anergic/suppressive state of CD25 \(^+\) thymocytes leads to the activation of self-reactive T cells from CD25 \(^+\) dormant states. To test this, thymocyte suspensions from euthymic BALB/c nu/nu/+ mice were stimulated with Con A and exogenous rIL-2 (at 50 U/ml), or Con A alone, for 3 days and then transferred to BALB/c athymic nude mice, which were histologically and serologically examined 3 mo later (13). Transfer of Con A/rIL-2-stimulated thymocyte suspensions (1 \(\times\) 10\(^7\)) produced significantly higher incidences of histologically evident autoimmune gastritis (\(p = 0.036\) by Fisher’s exact probability test) and higher
tters of anti-parietal cell autoantibodies than the transfer of the same number of thymocytes stimulated with Con A alone (Fig. 6).

The former also elicited other autoimmune diseases, such as ophoritis and thyroiditis, in 20% of mice, whereas the latter did not. Thus, breakage of the anergic and suppressive state of CD25 \(^+\) thymocytes can elicit autoimmune diseases similar to those produced by direct removal of CD25 \(^+\) thymocytes or T cells.

**Phenotype and function of CD25 \(^+\) T cells derived from CD25 \(^+\) T cells**

Our previous reports showed that CD25 \(^-\) T cells could differentiate into CD25 \(^+\) T cells in vivo (13, 14). This raises the question as to whether such CD25 \(^+\) T cell-derived CD25 \(^+\) T cells (including activated autoimmune effector T cells) can be phenotypically and functionally discriminated from the anergic/suppressive CD25 \(^+\) T cells present in naive mice. To examine this, we analyzed the cell surface phenotype, as well as the in vitro responsiveness to TCR stimulation, of CD25 \(^+\) T cells derived from CD25 \(^+\) T cells prepared from nude mice that developed autoimmune disease after transfer of CD25 \(^+\) T cells (as shown in Table I and Ref. 13) (Fig. 7). Compared with CD25 \(^+\) lymph node T cells in normal naive mice, those in the CD25 \(^-\) cell-transferred nude mice, in which CD25 \(^-\) cells constituted 17.1 \(\pm\) 5.2% (\(n = 4\)) of lymph node CD4 \(^+\) T cells (see also legend to Fig. 7D), were lower in CD62L and CD45RB expression, and higher in CD69 expression, indicating that they were in activated and/or primed states (Fig. 7A). In the paragastric lymph nodes of gastritis-bearing mice, CD25 \(^+\) T cells expressed CD62L and CD45RB at much lower levels and CD69 at much higher levels than those in other lymph nodes, indicating that they were more primed and activated (Fig. 7B).

Transfer of CD25 \(^+\) thymocytes (as shown Table I) also led to the development of CD25 \(^+\) cell transfer of CD25 \(^-\) T cell-transferred nude mice were hyporesponsive to TCR stimulation in vitro, and unable to suppress the proliferative responses of CD25 \(^+\) T cells, as shown in a representative mouse (Fig. 7C),
and in individual mice as percentage of suppression (Fig. 7D). In contrast, CD25^4−8^ T cells from nude mice transferred with uneliminated thymocytes were significantly suppressive. Likewise, CD25^4−8^ T cells from nude mice transferred with CD25^−^ thymocytes did not show significant suppressive activity when compared with CD25^4−8^ T cells from nude mice transferred with uneliminated thymocytes, although the activity was variable in both groups of mice (−2.0 ± 33.1% vs 63.6 ± 22.3%, respectively, as percentage of suppression, n = 4).

These results taken together indicate that although CD25^4−8^ T cells can give rise to activated CD25^4−8^ T cells (including autoimmune effector T cells), they may be unable to generate CD25^4−8^ T cells with significant suppressive activity.

Development of anergic/suppressive CD25^4−8^ thymocytes/T cell in TCR transgenic mice, but not in those on RAG-2-deficient background

CD25^4−8^ thymocytes and T cells developed in TCR transgenic mice as in nontransgenic mice. DO11.10 transgenic mice expressing transgenic TCRs specific for an OVA peptide, for example, harbored CD25^4−8^ thymocytes and CD25^4−8^ peripheral T cells in a similar proportion of CD4^−8^ thymocytes or CD4^+^ T cells (3.5 ± 1.2% and 4.2 ± 0.9% (n = 5), respectively) (Fig. 8A). In contrast, DO11.10 transgenic mice on RAG-2 gene-deficient background developed few CD25^4−8^ thymocytes/T cells (<0.1% of CD4^−8^ thymocytes or CD4^+^ T cells). Furthermore, the CD25^4−8^ population in the thymus and periphery of DO11.10 mice contained a 2−3-fold lower percentage of KJ1-16^+^ thymocytes/T cells (hence higher percentage of thymocytes/T cells expressing endogenous α-chains) compared with the thymic or peripheral CD25^4−8^ population (18.3 ± 1.5% vs 72.7 ± 7.6% for thymocytes (n = 3), 34.0 ± 8.7% vs 69.7 ± 8.9% for lymph node cells (n = 4)); in contrast, each population in the thymus and periphery contained a comparable percentage of Vβ8-expressing cells, the majority of which were expressing transgenic Vβ chains (27) (Fig. 8A).

Functionally, the CD25^4−8^ thymocytes in DO11.10 mice were unresponsive to stimulation with OVA peptides in vitro (Fig. 8B). Upon stimulation with OVA peptides, they potently suppressed Ag-specific proliferative responses of cocultured transgenic CD25^4−8^ cells. Furthermore, TCR stimulation appeared to be required for CD25^4−8^ thymocytes to exert suppression, since CD25^4−8^ thymocytes prepared from nontransgenic littermates did not exhibit suppression when stimulated with OVA peptides.

Thus, high percentages of T cells expressing endogenous TCR α-chains in the thymic or peripheral CD25^4−8^ population, together with the paucity of the population in RAG-2-deficient TCR transgenic mice, indicate that rearrangement of the endogenous TCR α-chain genes and consequent expression of TCRs composed of endogenous α-chains and transgenic β-chains may be required for the generation of anergic and suppressive CD25^4−8^ thymocytes/T cells in TCR transgenic mice.

Discussion

The main finding in this study is that the normal thymus is continuously producing not only pathogenic self-reactive T cells, but also a unique immunoregulatory CD25^4−8^ T cell population that is unresponsive to TCR stimulation in vitro and, upon stimulation, suppresses the proliferation of other thymocytes and T cells. Furthermore, physical elimination of the population or functional breakage of its unresponsive/suppressive state leads to
on the left various cell surface molecules on CD25

A
dent experiments is shown in

2

A

an irrelevant Ab.

The lymph nodes of a nude mouse transferred with CD25

2

the mixture of an equal number of these cells, purified (as shown in

A

degree of suppression by CD25

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T cells. Nondeletional as well as deletional mechanism of immunologic

vivo and in vitro results indicate that the thymus plays key roles in

nopathologically similar to their human counterparts (68). These in

mic selection process. First, the normal thymus can generate them

from immature thymocytes (Fig. 3, and our

results). These findings in OVA-TCR transgenic mice can

sumably by interacting with self peptide/class II MHC (49, 69, 70,

and see below). These findings in OVA-TCR transgenic mice can

be generalized to other TCR transgenic mice. For example, in the

mice expressing OVA-specific transgenic TCRs, CD25

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T cells expressing dual TCRs, one composed of the transgenic

a- and the other of an endogenous a-chain and the transgenic

b-chain; and, while the former recognize the OVA peptide (and

may transmit signals required for the cell to exert suppression), the

latter might be responsible for rendering CD25

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autoimmune diseases (such as experimental allergic encephalomyelitis

and insulin-dependent diabetes mellitus, respectively) was

enhanced significantly by rendering the mice RAG-2 deficient (hence, endogenous TCR a-chain deficient) (71, 72). To further elucidate how the anergic/suppressive CD25

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and see below). These findings in OVA-TCR transgenic mice can

be generalized to other TCR transgenic mice. For example, in the

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and insulin-dependent diabetes mellitus, respectively) was

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thymocytes are generated in the thymus of normal or

selected in the thymus (57–60). Third, the expression of endogenous

TCR a-chains is required for their development in TCR transgenic

mice, as illustrated by the finding that RAG-2 deficiency abrogated their development (Fig. 8). In these transgenic mice expressing OVA-specific transgenic TCRs, CD25

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T cells exhibiting suppressive activity upon OVA stimulation may be

expressing dual TCRs, one composed of the transgenic

a/b-chains and the other of an endogenous a-chain and the transgenic

b-chain; and, while the former recognize the OVA peptide (and

may transmit signals required for the cell to exert suppression), the

latter might be responsible for rendering CD25

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The CD25 molecule is expressed on activated T cells (34, 75). In TCR transgenic mice, it is necessary to determine their Ag specificities or the ligands selecting them. We postulate that the CD25⁺/4⁺/8⁻ thymocytes may be reactive with self peptides/class II MHC complexes (57, 73) or class II MHC itself (74) expressed in the thymus and rendered anergic (hence, harmless) because of the avidities of their TCRs for self peptides/class II MHC might be rather high (but not so high as to be deleted) (Fig. 9). Furthermore, an activated or primed phenotype of CD25⁺/4⁺/8⁻ thymocytes and T cells in normal naive mice suggests that they might be continuously stimulated by self Ags in the normal internal environment. This possible self-reactivity of CD25⁺/4⁺/8⁻ thymocytes is currently under investigation.

The CD25 molecule is expressed on activated T cells (34, 75). This poses a question as to whether activated CD25⁺/4⁺ T cells derived from CD25⁺/4⁻ T cells can also acquire the suppressive activity. In our study, the CD25⁺/4⁻ T cells that had differentiated in nude mice from the inoculated CD25⁺/4⁺ T cells exhibited the cell surface phenotype generally shared by activated, primed, or memory CD4⁺ T cells and similar to the phenotype of CD25⁺/4⁺ T cells in normal naive mice (Fig. 7, A and B, vs Fig. 1C). They, however, scarcely exhibited suppressive activity (Fig. 7, C and D). Although these CD25⁺ cell-derived CD25⁺/4⁺ T cells were hypo- or responsive to TCR stimulation (Fig. 7C), this could be attributed to the refractoriness of chronically stimulated T cells (including autoimmune effector T cells) to further TCR stimulation, as CD25⁺/4⁺/8⁻ thymocytes stimulated in vitro were hypo- or responsive to further stimulation (Fig. 5C). Indeed, CD25⁺/4⁺/8⁻ T cells prepared in vitro by activating CD25⁺/4⁺ T cells from normal BALB/c mice did not exhibit suppressive activity either on the proliferation of other T cells in vitro (Fig. 5C) or on the development of autoimmune disease in vivo when cotransferred to nude mice with CD25⁻ T cells (Y. Kuniyasu et al., manuscript in preparation). Furthermore, the anergic-suppressive state of CD25⁺/4⁺/8⁻ thymocytes or CD25⁺/4⁺ peripheral T cells in normal naive mice appears to be their basal default condition, since the CD25⁺/4⁺/8⁻ thymocytes/T cells broken of their anergic-suppressive state by TCR stimulation along with anti-CD28 Ab or a high dose of IL-2 reverted to the original anergic-suppressive state upon removal of anti-CD28 Ab or IL-2 from the culture milieu (Fig. 5C and Ref. 24). Taken together, these results indicate that, once the CD25⁺/4⁺/8⁻ thymocytes acquire the suppressive activity in the thymus, they may stably maintain the activity, and that other T cells could hardly acquire it upon activation in the periphery (Fig. 9).

Our findings, which were mainly obtained from in vitro proliferation assay, would not, however, exclude the possibility that CD25⁺ cell-derived CD25⁺/4⁺ T cells might somehow suppressively influence functions of other T cells in vivo through the cytokine network (13, 26–30). Other T cells with activated phenotype, for example NKT cells, may also play a regulatory role in self-tolerance by secreting immunoregulatory cytokines, although both CD25⁺/4⁺/8⁻ thymocytes and CD25⁺/4⁺ peripheral T cells do not express NKL.1 Ag, indicating that they are different from NKT cells (61, 62).

Together with this possible inability of CD25⁺/4⁺ T cells to acquire the anergic-suppressive property upon activation, the following findings suggest that the anergic-suppressive CD25⁺/4⁺/8⁻ T cells in the thymus and periphery of normal naive mice may have developmental continuity as a common T cell lineage and constitute a T cell subpopulation functionally distinct from other T cells or thymocytes. First, both the thymic and the peripheral CD25⁺/4⁺ T cells are functionally similar in their in vivo autoimmune prevention, in vitro suppression, and unresponsiveness to TCR stimulation. Second, they are phenotypically similar in the expression profile of various cell surface molecules (Fig. 1, B and C), especially in high CD62L expression, which contrasts with low CD62L expression on autoimmune effector T cells (Fig. 7) or usual activated or memory T cells (40, 54–56). The finding that CD25⁺/4⁺/8⁻ thymocytes/T cells are CD5high, CD45RBlow, and partially CD62Lhigh also correlates with the findings made by us and others that autoimmune-suppressive CD4⁺ thymocytes/T cells are CD5high, CD45RBlow, and CD62Lhigh (8–10, 12–15, 52, 53), although it remains to be determined whether the thymocyte/T cell population with the anergic-suppressive property can be further reduced to a smaller population, for example, the CD62Lhigh or CD62Llow population included in the CD25⁺/4⁺/8⁻ population (Fig. 1, B and C). Third, both CD25⁺/4⁺/8⁻ thymocytes and CD25⁺/4⁺ peripheral T cells are absent in RAG-2-deficient TCR transgenic mice; and, in TCR transgenic mice, both are constituted of high proportions of thymocytes/T cells expressing endogenous TCR α-chains (Fig. 8 and see discussion above). Furthermore, both were shown to be characteristic of a superantigen-induced clonal deletion (76, 77). Given the thymic production of the anergic-suppressive CD25⁺/4⁺ thymocytes, their possible lineage continuity to the peripheral CD25⁺/4⁺ T cells, and possible inability of other T cells to acquire the anergic-suppressive property (see above), abrogation of their peripheral migration from the beginning of their ontogeny may well lead to their paucity in the periphery and, as a consequence, to the development of autoimmune disease. In the previous report (14), we showed that CD25⁺/4⁺ T cells begin to appear in the periphery at about day 3 after birth in normal mice; they are substantially reduced by thymectomy at about day 3; such neonatal thymectomy elicited autoimmune diseases similar to those produced in the present experiments; and the inoculation of CD25⁺/4⁺ T cells from normal mice prevented the autoimmune development. These findings taken together indicate that the neonatal thymectomy may be able to selectively reduce the anergic-suppressive
CD25+4+ T cells in the periphery, leading to activation of self-reactive T cells that have migrated to the periphery before the thymectomy (see Fig. 3B), thus resulting in the development of autoimmune diseases similar to those produced by direct removal of CD25+4+ T cells from the periphery of adult mice (Table I and Refs. 13 and 14). Other ways of possibly manipulating the neonatal development of the anergic/suppressive CD25+4+ thymocytes/T cells or reducing them from the periphery in adults can cause similar autoimmune diseases as well (19, 48, 49, 78).

Our results also indicate that not only physical elimination of CD25+4+8+ thymocytes/T cells, as discussed above, but also functional abrogation of their suppressive activity may cause autoimmune disease. For example, a high dose of IL-2 locally produced by T cells responding to invading microbes or a high level of CD80/CD86 expression on APCs might locally break the anergic/suppressive state of CD25+4+8+ thymocytes/T cells and allow pathogenic self-reactive T cells in the vicinity to be activated (79). Furthermore, breaking of the anergic/suppressive state of CD25+4+ T cells for a limited period may suffice to elicit autoimmune disease, as illustrated by the development of autoimmune disease in nude mice transferred with thymocytes treated in vitro with Con A and IL-2 for 3 days (Fig. 6). It is likely that a sufficient number of CD4+ pathogenic self-reactive T cells in the inocula have expanded and/or differentiated to autoimmune effector T cells before the anergy/suppression/broken CD25+4+ T cells revert to the anergic state and reacquire the suppressive activity (Fig. 5C).

In conclusion, the present results indicate that the thymus contributes to the maintenance of immunologic self-tolerance not only by clonally deleting or inactivating self-reactive T cells, but also by producing CD25+4+ immunoregulatory T cells that are anergic and suppressive. Thus, pathogenic self-reactive T cells having escaped thymic clonal deletion can be controlled in the periphery by this T cell-mediated regulatory mechanism. Autoimmune disease may develop in genetically susceptible individuals as a consequence of abnormality in the thymic production of these naturally anergic and suppressive CD25+4+8+ T cells, their reduction in the periphery, or their dysfunction in controlling self-reactive T cells. Environmental agents or genetic abnormalities may cause autoimmune disease by affecting CD25+4+8+ thymocytes/T cells (80).

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References