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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-Tolerance[1]

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This study shows that the normal thymus produces immunoregulatory $CD25^+4^+8^-$ thymocytes capable of controlling self-reactive T cells. Transfer of thymocyte suspensions depleted of $CD25^+4^+8^-$ thymocytes, which constitute ~5% of steroid-resistant mature $CD4^+8^-$ thymocytes in normal naive mice, produces various autoimmune diseases in syngeneic athymic nude mice. These $CD25^+4^+8^-$ thymocytes are nonproliferative (anergic) to TCR stimulation in vitro, but potently suppress the proliferation of other $CD4^+8^-$ or $CD4^-8^+$ 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 $CD25^+4^+8^-$ thymocytes/T cells are functionally distinct from activated $CD25^+4^+$ T cells derived from $CD25^-4^+$ thymocytes/T cells in that the latter scarcely exhibits suppressive activity in vitro, although both $CD25^+4^+$ populations express a similar profile of cell surface markers. Furthermore, the $CD25^+4^+8^-$ thymocytes appear to acquire their anergic and suppressive property through the thymic selection process, since TCR transgenic mice develop similar anergic/suppressive $CD25^+4^+8^-$ thymocytes and $CD25^+4^+$ 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 $CD25^+4^+8^-$ 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.**

The thymus clonally deletes self-reactive T cells with high avidity TCRs for self Ags expressed in the thymus (1, 2). It may also render some self-reactive T cells anergic (3–5). Although our understanding of the mechanism of clonal deletion or anergy has substantially advanced recently, these mechanisms alone may not be sufficient for controlling self-reactive T cells, especially those that react with self Ags expressed outside the thymus. For example, there is mounting evidence that potentially pathogenic self-reactive T cells are present in the periphery of normal individuals, and that they can be activated by immunization with self constituents along with potent adjuvant or by repeated stimulation with self Ags in vitro (6, 7). Furthermore, T cell-mediated autoimmune diseases (such as insulin-dependent diabetes, thyroiditis, and gastritis) can be produced in normal rodents by simply eliminating a peripheral $CD4^+$ T cell subpopulation defined by an expression level of a particular cell surface molecule such as CD5, CD45RB/C, or CD25 (8–15). There is also accumulating evidence in various models of autoimmune disease that development of disease can be prevented by inoculating peripheral $CD4^+$ T cells from normal histocompatible animals (14, 16–20). Normal thymocytes, $CD4^+8^-$ thymocytes in particular, also bear this autoimmune-preventive activity (16, 21). These findings when taken together suggest that the thymus may play another essential role in maintaining natural self-tolerance by producing an immunoregulatory population of T cells (22, 23).

We have shown previously that elimination of $CD25^+$ peripheral T cells, which constitute 5–10% of peripheral $CD4^+$ T cells and less than 1% of $CD8^+$ T cells in normal naive mice, produces various autoimmune diseases in otherwise normal mice; and the reconstitution of the eliminated population prevents the autoimmune development (13, 14). These naturally present immunoregulatory $CD25^+4^+$ T cells are unique in that 1) they are anergic to TCR stimulation in vitro (24), if one defines anergy as a reversible antiproliferative state (25); 2) upon TCR stimulation, however, they potently suppress the activation/proliferation of other $CD4^+$ T cells and $CD8^+$ T cells in an Ag-nonspecific manner (24); furthermore, 3) in contrast to other regulatory T cells exerting cytokine-mediated control on autoimmune T cells (26–30), the $CD25^+4^+$ T cells suppressively control other T cells on the surface of APC through a cognate cellular interaction (24, 31). It remains to be determined, however, where and how the $CD25^+4^+$ T cells acquire the anergic, suppressive, and autoimmune-preventive property.

In this study, we attempt to determine the origin of these naturally anergic and suppressive $CD25^+4^+$ T cells, and to examine their role in maintaining immunologic self-tolerance. We demonstrate that the normal thymus is continuously generating and releasing not only pathogenic self-reactive T cells, but also the

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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/+* mice and six-week-old BALB/c nude (*nu/nu*) mice were purchased from SLC (Shizuoka, Japan). BALB/c-*Thy-1*^{a} congenic mice were established in our laboratory (14). DO11.10 transgenic mice were the gift of Dr. D. Y. Loh, Roche Japan (Kamakura, Japan) (32). DO11.10-RAG-2 knockout mice were provided by Drs. K. Iwabuchi (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 (5×10^7), or 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 (7D4, rat IgM) (34) or anti-CD8a.2 (mouse IgG2a) (35) for 45 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, 1×10^6 cells were incubated with FITC-labeled or biotinylated mAbs, with PE-streptavidin (BioMeda, 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-Phycoerythrin (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) (3E2) (36), CD5 (53-7.3) (37), CD8 (53-6.7) (37), CD11a/CD18 (LFA-1) (2D7) (38), CD24 (heat-stable Ag) (M1/69), CD44 (IM7) (39), CD45RB (16A) (40), CD62L (L-selectin) (Mel-14) (41), CD69 (H1.2F3) (42), CD90.2 (Thy-1.2) (30-H12), CD2 (RM2.5), or TCR Vβ8.1, 8.2, 8.3 (F23.1) (43) were purchased from PharMingen (San Diego, CA). Anti-CD122 (IL-2R β-chain) (TM-β1) (44) and anti-DO11.10 clonotypic Ab (KJ1-26) (45) were the gifts of Dr. T. Tanaka (Osaka University, Osaka, Japan) and Dr. O. Kanagawa (Washington University, St. Louis, MO), respectively.

Cell sorting

Spleen and lymph node suspensions or thymocyte suspensions prepared from 8-wk-old BALB/c mice were stained with FITC-conjugated anti-CD25 (7D4) (PharMingen) and PE-conjugated anti-CD4 (H129.19) (PharMingen), and sorted by a FACS (Epics-Elite; Coulter), as previously described (14). Purity of the CD25$^+$ and CD25$^-$CD4$^+$ populations was >90 and ~99%, respectively.

In vitro proliferation assay

Along with RBC-lysed and mitomycin C-treated BALB/c spleen cells ($5\sim10 \times 10^4$) as APCs, thymocytes or lymph node/spleen cells ($2\sim2.5 \times 10^4$), sorted as described above, were cultured for 3 days in 96-well round-bottom plates (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 10% FCS (Life Technologies), penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μM 2-ME (24). Anti-CD3 Ab (145-2C11) (46) (Cedarlane Laboratories, Hornby, Ontario, Canada) at a final concentration of 10 μg/ml, Con A at 1 μg/ml, or OVA peptides (residue 323–339) (27) at 0.3 μM were added to the culture for stimulation (24). Incorporation of [^{3}H]thymidine ([^{3}H]TdR) (1 μCi/well) by proliferating lymphocytes during the last 6 h of the culture was measured. In expressing the degree of suppression exerted by CD25$^+$4$^+$8$^-$ thymocytes on CD25$^-$4$^+$8$^-$ thymocytes, percentage of suppression was defined as 100 × [(cpm of CD25$^-$4$^+$8$^-$ thymocytes − cpm of the mixed population)/cpm of CD25$^-$4$^+$8$^-$ thymocytes].

Murine rIL-2 (3.89×10^6 U/mg) was a gift of Shionogi (Osaka, Japan). Anti-CD28 mAb (37.51) (47) was purchased from PharMingen. To prepare Con A blasts for in vivo transfer, thymocytes (5×10^6/ml) were cultured with 5 μg/ml Con A for 3 days.

Steroid treatment of mice

Eight-week-old BALB/c mice were i.p. injected with 2.5 mg of hydrocortisone acetate (Sigma) 2 days before use.

Intrathymic injection

A 25 μl volume of CD4$^-$CD8$^-$ thymocytes (1×10^5) purified by FACS from BALB/c thymocyte suspensions stained with PE anti-CD4 Ab and FITC anti-CD8 Ab, as described above, was injected into each lobe of the thymus exposed by incision of the sternum. The thymic region of the recipient BALB/c-*Thy-1*^{a} mice was irradiated at 6 Gy before injection by covering extremities with lead plates (15).

Histology and serology

Stomachs and other organs were fixed with 10% Formalin and processed for hematoxylin and eosin staining. 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 mucus-secreting cells (8, 13, 14). Thyroiditis and oophoritis were histologically graded, as previously described (48). Adrenalitis, insulitis, and sialoadenitis were assessed as histologically positive when destruction of adrenocorical cells, Langerhans 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. 1*A* 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. 1*B*) 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^{high}4$^-$8$^-$ thymocytes in the subcortical area (data not shown).

To further characterize the CD25$^+$4$^+$8$^-$ thymocytes, we compared between CD25$^+$ or 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. 1*B*). 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. 1*B*, 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^{high}, compared with

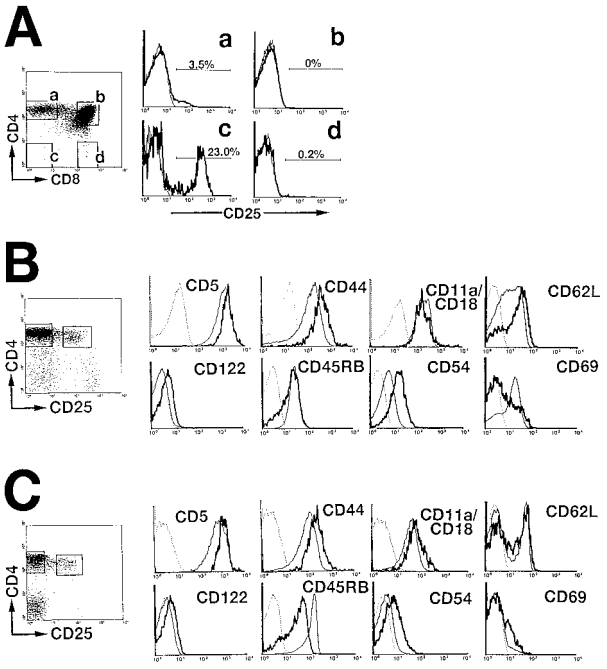

FIGURE 1. $CD25^+4^+8^-$ population in the thymus and periphery. *A*, Thymocyte suspensions prepared from a 2-mo-old BALB/c mouse were stained with PE anti-CD4, FITC anti-CD8, and biotinylated anti-CD25 with R-phycoerythrin (RPE)-Cy5-conjugated streptavidin as the secondary reagent. Expression of CD25 on the thymocytes in fraction (*a–d*) on the *left* is shown on the *right* as a histogram. Percentage of $CD25^+$ cells in each fraction is also shown. To examine expression of various cell surface molecules on *B*, $CD25^+$ or $CD25^-4^+8^-$ thymocytes, or *C*, $CD25^+$ or $CD25^-4^+$ lymph node cells, thymocyte suspensions depleted of $CD8^+$ cells by anti-CD8 and C treatment, or nondepleted lymph node cell suspensions, were stained with PE anti-CD4, FITC anti-CD25, and biotinylated Abs specific for various cell surface molecules. Expression of indicated molecules on $CD25^+$ or $CD25^-CD4^+$ thymocytes/T cells enclosed on the *left* is shown on the *right* as histograms: bold line is for $CD25^+4^+8^-$ thymocytes/T cells, solid line for $CD25^-4^+8^-$ thymocytes/T cells, and dotted line for control staining with an irrelevant Ab. A representative result of five independent experiments is shown in *A–C*.

$CD25^-4^+8^-$ thymocytes (30% vs 60%); and some $CD25^+4^+8^-$ thymocytes (~20%) were Qa-2^+, compared with <1% of Qa-2^+ cells among $CD25^-4^+8^-$ thymocytes (data not shown). Taken together, the phenotype of $CD25^+CD4^+8^-$ thymocytes (e.g., their $CD5^{high}$, $CD11a/CD18^{high}$, $CD44^{high}$, $CD45RB^{low}$, $CD62L^{high}$, and $CD54^{high}$ expression) suggests that they may be in a more mature, activated, or primed state than $CD25^-4^+8^-$ thymocytes (54–56); CD69 and/or Qa-2 expression on some $CD25^+4^+8^-$ thymocytes, together with their no CD24 expression (data not shown), suggests that they may have been recently selected (57–60); and $CD5^{high}$, $CD45RB^{low}$, and $CD62L^{high}$ phenotype of $CD25^+4^+8^-$ thymocytes is similar to the phenotype of immunoregulatory T cell populations previously reported by us and others (8–10, 12–16, 52, 53).

These expression patterns of cell surface molecules were similar between $CD25^+4^+8^-$ thymocytes and $CD25^+4^+$ T cells in the lymph nodes (Fig. 1, *B* and *C*). Furthermore, both $CD25^+4^+8^-$ thymocytes and $CD25^+4^+$ T cells did not express NK1.1 Ag in C57BL/6 mice (data not shown), indicating that they are different from NKT cells (61, 62).

Induction of autoimmune disease by eliminating $CD25^+4^+8^-$ thymocytes

To determine whether $CD25^+4^+8^-$ thymocytes suppressively control pathogenic self-reactive thymocytes/T cells, we removed $CD25^+$ thymocytes from thymocyte suspensions prepared from normal BALB/c *nu/+* mice by in vitro treatment with anti-CD25 mAb and C, and then transferred the remaining cells (5×10^7) to BALB/c athymic *nu/nu* mice (Table I, Fig. 2). In 3 mo, the transfer produced histologically and serologically evident autoimmune diseases at higher incidences and in a wider spectrum of organs (such as the gastric mucosa, thyroid glands, salivary glands, adrenal glands, and ovaries (see Refs. 8 and 22 for histology)) than the transfer of nondepleted thymocyte suspensions, which produced only autoimmune gastritis in some nude mice, but no other autoimmune diseases. Glomerulonephritis that developed in some of the $CD25^-$ thymocyte-transferred nude mice was due to the deposition of immune complexes in the renal glomeruli, as previously described (13, 22). Hemolytic anemia or inflammatory bowel disease as reported in CD25 gene-knockout mice was not observed in the thymocyte-transferred nude mice (63).

Transfer of $CD25^-4^+8^-$ thymocytes prepared from steroid-resistant thymocytes, in which a similar proportion (5.9 ± 2.1%, $n = 5$) of $CD4^+8^-$ thymocytes was $CD25^+$ as in normal thymuses (see above), also produced a wide spectrum of autoimmune diseases in nude mice as with the transfer of $CD25^-$ peripheral T cells (13). On the other hand, transfer of $CD25^-$ thymocytes (5×10^7) mixed

Table I. *Induction of autoimmune disease by eliminating $CD25^+$ thymocytes*[a]

Expt. Group	Inoculated Cells	Total Number of Mice	Number of Mice with Autoimmune Disease[b]						
			Gas	Oop	Thr	Sial	Adr	Ins	Gn
A	Whole thymocytes (5×10^7)	12	7 (58.3)	0	0	0	0	0	0
B	$CD25^-$ thymocytes (5×10^7)	12	12 (100)	12 (100)	4 (33.3)	3 (25.0)	1 (8.3)	1 (8.3)	3 (25.0)
C	$CD4^+8^-$ SR thymocytes (5×10^6)	5	4 (80.0)	0	0	0	0	0	0
D	$CD25^-4^+$ SR thymocytes (5×10^6)	5	5 (100)	5 (100)	2 (40.0)	1 (20.0)	2 (40.0)	0	1 (20.0)
E	Whole SP/LN cells (5×10^7)	8	0	0	0	0	0	0	0
F	$CD25^-$ SP/LN cells (5×10^7)	8	8 (100)	8 (100)	5 (62.5)	5 (62.5)	2 (25.0)	0	2 (25.0)
G	$CD25^-$ thymocytes (5×10^7) and $CD4^+$ SP/LN cells (2×10^7)	8	0	0	0	0	0	0	0

[a] Thymocytes or spleen/lymph node (SP/LN) cells of indicated numbers were prepared from 2- to 3-mo-old female BALB/c *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 *nu/nu* 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 thyroiditides in other mice were grade 1.

[b] Number of mice bearing respective autoimmune diseases is shown with percentage incidence in parentheses. Gas, gastritis; Oop, oophoritis; Thr, thyroiditis; Sial, sialoadenitis; Adr, adrenalitis; Ins, insulitis; Gn, glomerulonephritis.

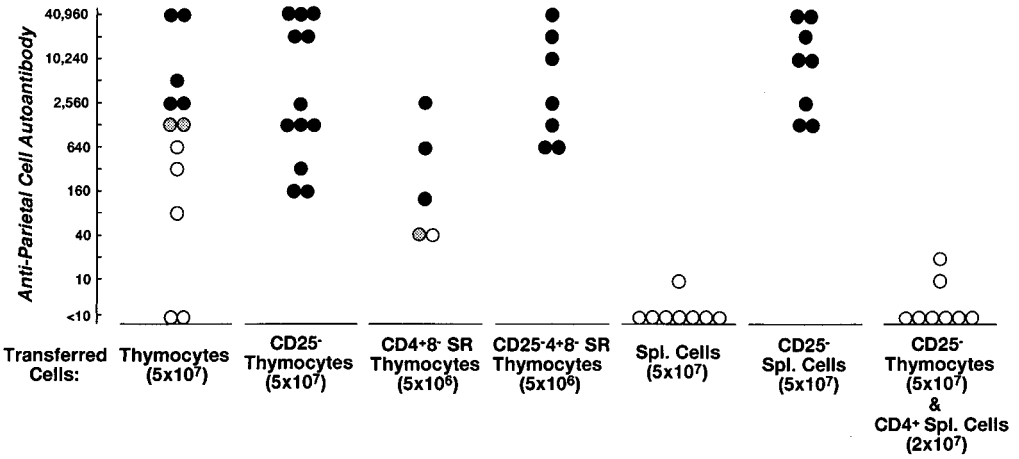

FIGURE 2. Development of autoimmune gastritis in nude mice transferred with CD25$^-$ thymocytes or peripheral T cells and its prevention by cotransfer of normal CD4$^+$ T cells. As shown in Table I, BALB/c *nu/nu* mice 6 wk of age were transferred with indicated cell suspensions, and histologically and serologically examined 3 mo later. ●, Grade 2 gastritis; ◍, grade 1 gastritis; ○, intact gastric mucosa. See *Materials and Methods* for histologic grading of gastritis.

with CD4$^+$ splenic and lymph node cells (2×10^7), of which 5–10% were CD25$^+$ (Fig. 1*C*), 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^a* congenic mice, which express Thy-1.1 (CD90.1) Ag (Fig. 3*A*). 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$^+$CD4$^+$8$^-$ 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 ($0.9 \pm 0.6\%$, $n = 6$) of CD4$^+$ 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. 3*B*). 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. 1*B*) 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$^-$4$^+$ peripheral T cells (Fig. 4*B*). 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,

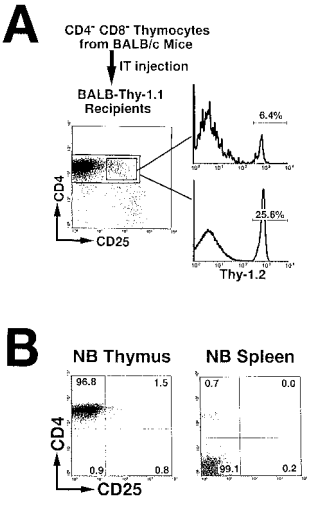

FIGURE 3. Thymic generation of CD25$^+$CD4$^+$CD8$^-$ thymocytes. *A*, Thymocyte suspension prepared from the thymus of a BALB/c-*Thy-1^a* congenic mouse that had received intrathymic (IT) inoculation of CD4$^-$CD8$^-$ thymocytes from BALB/c mice 1 wk before they were stained with PE anti-CD4, FITC-anti-CD25, 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*.

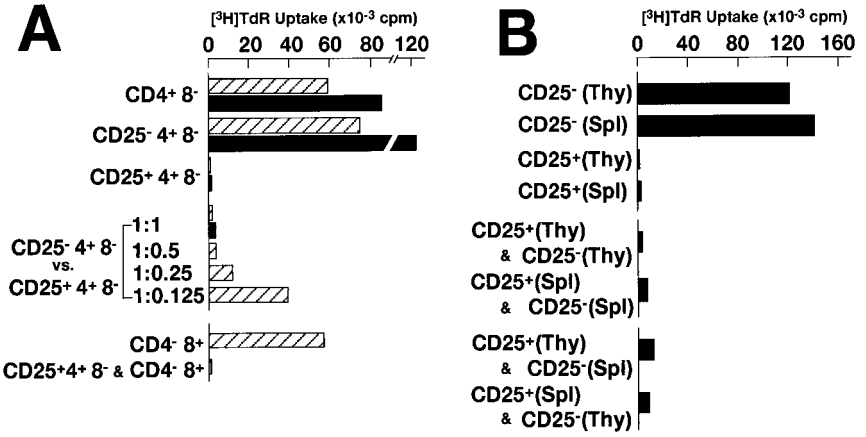

FIGURE 4. $CD25^+4^+8^-$ thymocytes are unresponsive to TCR stimulation and suppress other thymocytes and T cells. *A*, $CD25^+$ or $CD25^-4^+8^-$ thymocytes purified by FACS (as enclosed in Fig. 1*B*) from 2-mo-old BALB/c mice, or these two populations mixed at various ratios, were cultured for 3 days with anti-CD3 mAb (hatched bars) or Con A (black bars), along with mitomycin C-treated spleen cells as APC. $CD4^-8^+$ thymocytes purified by FACS were similarly stimulated alone or after being mixed with an equal number of $CD25^+4^+8^-$ thymocytes. *B*, $CD25^+$ or $CD25^-4^+8^-$ thymocytes (designated as $CD25^+$ (Thy) or $CD25^-$ (Thy), respectively) or $CD25^+$ or $CD25^-4^+$ spleen cells (designated as $CD25^+$ (Spl) or $CD25^-$ (Spl), respectively) were purified by FACS from 2-mo-old BALB/c mice. They were then mixed in indicated combinations at an equal ratio and stimulated with anti-CD3 Ab along with APCs. A representative result of more than five independent experiments is shown in *A* and *B*. In these experiments (including those shown in Figs. 5, 7, and 8 below), background counts in the wells with APC only were <2000 cpm. The means of duplicate cultures are shown in each, and the SEs of the mean were all within 10% of the mean in Fig. 4 and Figs. 5, 7, and 8.

suppresses the activation/proliferation of other thymocytes/T cells; and it is functionally similar to the peripheral $CD25^+4^+$ T cell population, which is also anergic and suppressive.

Abrogation of unresponsiveness/suppression of $CD25^+4^+8^-$ 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 $CD25^+4^+$ thymocytes and their suppressive activity (Fig. 5, *A* and *B*). Stimulation of $CD25^+4^+8^-$ thymocytes with anti-CD3 Ab in the presence of exogenously added rIL-2 (100 U/ml concentration) or anti-CD28 Ab (10 μg/ml) not only elicited their proliferation, but also abrogated their suppressive activity.

When these proliferating $CD25^+4^+$ 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 $CD25^-4^+8^-$ thymocytes (Fig. 5*C*). In contrast, similarly treated $CD25^-4^+8^-$ 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 $CD25^+4^+8^-$ 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 $CD25^+4^+8^-$ thymocytes leads to the activation of self-reactive T cells from $CD25^-$ dormant states. To test this, thymocyte suspensions from euthymic BALB/c *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 (1×10^7) produced significantly higher incidences of histologically evident autoimmune gastritis ($p = 0.036$ by Fisher's exact probability test) and higher titers 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 oophoritis and thyroiditis, in 20% of mice, whereas the latter did not. Thus, breakage of the anergic and suppressive state of $CD25^+4^+8^-$ thymocytes can elicit autoimmune diseases similar to those produced by direct removal of $CD25^+4^+8^-$ thymocytes or T cells.

Phenotype and function of $CD25^+4^+$ T cells derived from $CD25^-4^+$ T cells

Our previous reports showed that $CD25^-4^+$ T cells could differentiate into $CD25^+$ T cells in vivo (13, 14). This raises the question as to whether such $CD25^-4^+$ T cell-derived $CD25^+4^+$ T cells (including activated autoimmune effector T cells) can be phenotypically and functionally discriminated from the anergic/suppressive $CD25^+4^+$ 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 $CD25^+$ or $CD25^-4^+$ lymph node 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 $CD25^+4^+$ lymph node T cells in normal naive mice, those in the $CD25^-$ cell-transferred nude mice, in which $CD25^+$ cells constituted 17.1 ± 5.2% ($n = 4$) of lymph node $CD4^+$ T cells (see also legend to Fig. 7*D*), were lower in CD62L and CD45RB expression, and higher in CD69 expression, indicating that they were in activated and/or primed states (Fig. 7*A*). In the paragastric lymph nodes of gastritis-bearing mice, $CD25^+4^+$ 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. 7*B*). Transfer of $CD25^-$ thymocytes (as shown in Table I) also led to the development of $CD25^+$ or $CD25^-4^+$ T cells with similar expression patterns of cell surface molecules (data not shown).

Functionally, $CD25^+4^+$ lymph node T cells from $CD25^-$ T cell-transferred nude mice were hyporesponsive to TCR stimulation in vitro, and unable to suppress the proliferative responses of $CD25^-4^+$ T cells, as shown in a representative mouse (Fig. 7*C*),

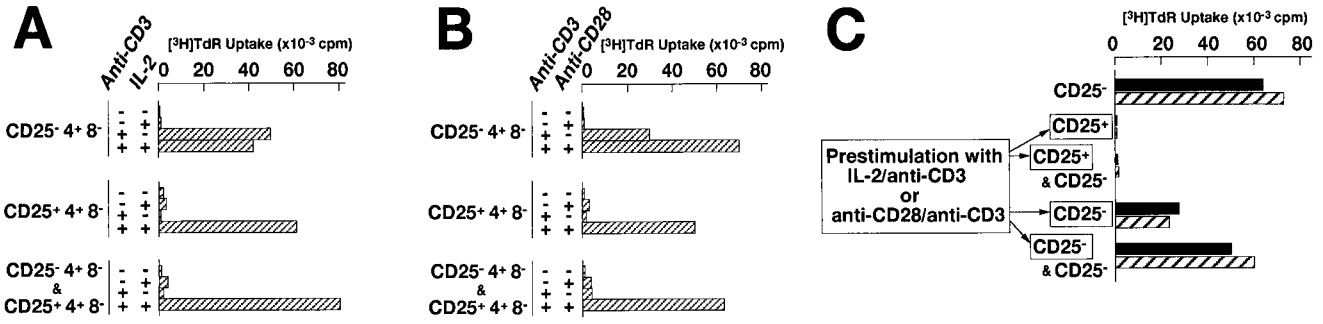

FIGURE 5. Breaking unresponsiveness and abrogating suppressive activity of CD25$^+$4$^+$ T cells by T cell stimulation along with exogenous IL-2 or anti-CD28 Ab and their reversal to the unresponsive/suppressive state after removing IL-2 or anti-CD28 Ab. *A* and *B*, CD25$^+$ or CD25$^-$4$^+$8$^-$ thymocytes, or the two populations mixed at an equal ratio, were prepared from normal 2-mo-old BALB/c mice and stimulated with anti-CD3 Ab with rIL-2 (100 U/ml) or anti-CD28 Ab (10 μg/ml). *C*, CD25$^+$ or CD25$^-$4$^+$8$^-$ thymocytes prestimulated with anti-CD3 Ab and rIL-2, or with anti-CD3 Ab and anti-CD28 Ab, for 3 days were washed and restimulated with anti-CD3 Ab along with fresh APCs, but without exogenous rIL-2 or anti-CD28 Ab, for 3 days (closed bars, anti-CD3/IL-2 prestimulation; hatched bars, anti-CD3/anti-CD28 prestimulation). These prestimulated CD25$^+$4$^+$ T cells were also mixed with freshly prepared CD25$^-$4$^+$8$^-$ thymocytes at an equal ratio and stimulated with anti-CD3. A representative result of three independent experiments is shown in *A–C*.

and in individual mice as percentage of suppression (Fig. 7*D*). In contrast, CD25$^+$4$^+$ T cells from nude mice transferred with uneliminated spleen cells were significantly suppressive. Likewise, CD25$^+$4$^+$ T cells from nude mice transferred with CD25$^-$ thymocytes did not show significant suppressive activity when compared with CD25$^+$4$^+$ T cells from nude mice transferred with uneliminated thymocytes, although the activity was variable in both groups of mice ($-2.0 \pm 33.1\%$ vs $63.6 \pm 22.3\%$, respectively, as percentage of suppression, $n = 4$).

These results taken together indicate that although CD25$^-$4$^+$ T cells can give rise to activated CD25$^+$4$^+$ T cells (including autoimmune effector T cells), they may be unable to generate CD25$^+$4$^+$ 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$^+$4$^+$ peripheral T cells in a similar proportion of CD4$^+$8$^-$ thymocytes or CD4$^+$ T cells ($3.5 \pm 1.2\%$ and $4.2 \pm 0.9\%$ ($n = 5$), respectively) (Fig. 8*A*). 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 ($18.3 \pm 1.5\%$ vs $72.7 \pm 7.6\%$ for thymocytes ($n = 3$), $34.0 \pm 8.7\%$ vs $69.7 \pm 8.9\%$ 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. 8*A*).

Functionally, the CD25$^+$4$^+$8$^-$ thymocytes in DO11.10 mice were unresponsive to stimulation with OVA peptides in vitro (Fig. 8*B*). Upon stimulation with OVA peptides, they potently suppressed Ag-specific proliferative responses of cocultured transgenic CD25$^-$4$^+$ 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$^+$4$^+$ 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.

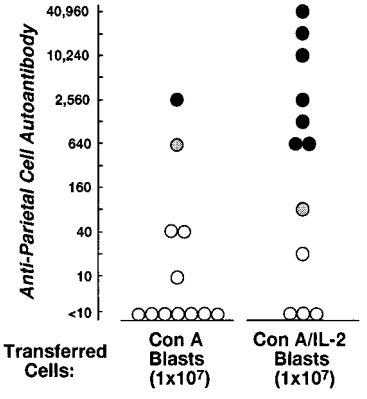

FIGURE 6. Induction of autoimmune disease in BALB/c nude mice by transferring BALB/c thymocytes stimulated with Con A and IL-2. Thymocyte suspensions from BALB/c *nu*/+ mice were stimulated with Con A and rIL-2, or Con A alone, for 3 days, and 1×10^7 blastic cells were i.v. transferred to BALB/c athymic nude mice. The recipients were histologically examined 3 mo later, and titers of anti-parietal cell autoantibodies were assessed by ELISA. ●, ◍, ○; see Fig. 2 for pathologic grading of gastritis.

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

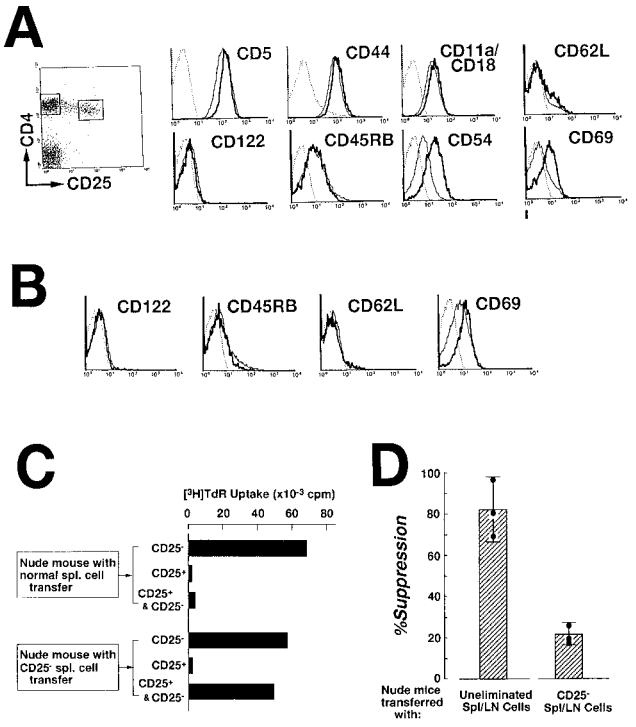

FIGURE 7. Phenotypic and functional characterization of $CD25^+4^+$ T cells in nude mice transferred with $CD25^-4^+$ T cells. *A*, Expression of various cell surface molecules on $CD25^+$ or $CD25^-4^+$ lymph node cells from nude mice inoculated with $CD25^-4^+$ T cells 3 mo before (Table I, group F). Lymph node cells were stained with PE anti-CD4, FITC anti-CD25, and biotinylated Abs specific for various cell surface molecules. Expression of indicated molecules on $CD25^+$ or $CD25^-4^+$ cells enclosed on the *left* is shown on the *right* as a histogram: bold line is for $CD25^+4^+$ cells, solid line for $CD25^-4^+$ cells, and dotted line for control staining with an irrelevant Ab. *B*, Cells from paragastric lymph nodes of gastritis-bearing nude mice were stained as in *A*. A representative result of three independent experiments is shown in *A* and *B*. *C*, $CD25^+$ or $CD25^-4^+$ T cells, or the mixture of an equal number of these cells, purified (as shown in *A*) from the lymph nodes of a nude mouse transferred with $CD25^-$ spleen cells (group F in Table I) or uneliminated spleen cells (group E in Table I) 3 mo before were stimulated with anti-CD3 Ab and APCs for 3 days. *D*, The degree of suppression by $CD25^+4^+$ T cells on the proliferation of $CD25^-4^+$ T cells was calculated as percentage of suppression (see *Materials and Methods*) and shown for individual *nu/nu* mice transferred with $CD25^-$ or uneliminated spleen cells, as shown in Table I ($n = 3$ each). Percentage of $CD25^+$ cells among lymph node $CD4^+$ cells in these nude mice transferred with $CD25^-$ or uneliminated spleen cells was 18.3 ± 5.6% and 19.5 ± 1.4%, respectively. Vertical bars denote SDs.

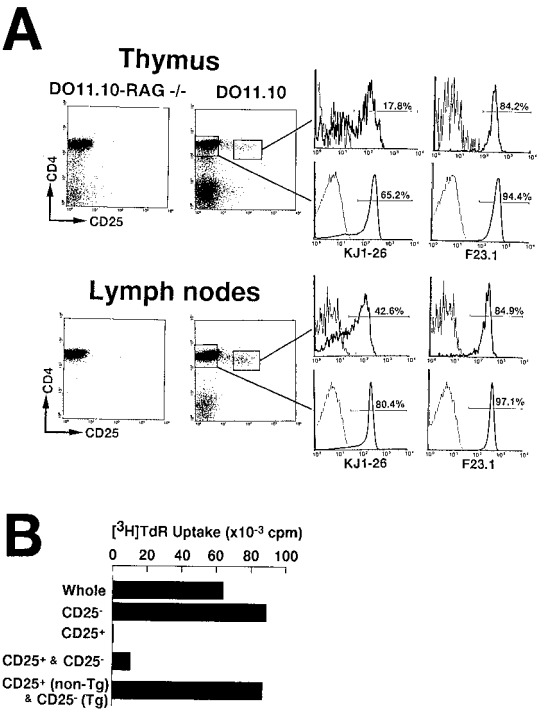

FIGURE 8. Development of anergic/suppressive $CD25^+4^+8^-$ thymocytes/T cells in TCR transgenic mice, but not in those on RAG-2-deficient background. *A*, Thymocytes or lymph node cells from DO11.10 transgenic mice or DO11.10 mice on RAG-2 gene-deficient background were stained with PE anti-CD4 and FITC anti-CD25. Expression of TCRs detected by KJ1.26 clonotype-specific Ab or F23.1 specific for Vβ8.1, 8.2, 8.3 on $CD25^+$ or $CD25^-4^+8^-$ thymocytes or lymph node cells (enclosed on the *left*) is shown on the *right side* as histograms. *B*, $CD25^+$ or $CD25^-4^+8^-$ thymocytes, or the mixture of the two in equal numbers, from DO11.10 mice were stimulated with OVA peptides for 3 days. $CD25^-4^+8^-$ thymocytes from DO11.10 mice ($CD25^-$ (Tg)) were also mixed with an equal number of $CD25^+4^+8^-$ thymocytes from nontransgenic littermates ($CD25^+$ (non-Tg)) and stimulated with OVA peptides. A representative result of three independent experiments is shown.

the spontaneous activation and expansion of self-reactive thymocytes/T cells, which mediate various autoimmune diseases immunopathologically similar to their human counterparts (68). These in vivo and in vitro results indicate that the thymus plays key roles in the nondeletional as well as deletional mechanism of immunologic self-tolerance (Fig. 9). Indeed, when pathogenic self-reactive T cells that have escaped thymic clonal deletion are released from the thymus, they can be controlled in the periphery by $CD25^+4^+$ T cells that have been already produced by the thymus (Table I, Fig. 4*B*).

For the following reasons, the anergic and suppressive $CD25^+4^+8^-$ thymocytes appear to be generated through the thymic selection process. First, the normal thymus can generate them in vivo and in vitro from immature thymocytes (Fig. 3, and our unpublished data). Second, the phenotype of cell surface molecules (such as CD69 or Qa-2) suggests that they may be recently selected in the thymus (57–60). Third, the expression of endogenous TCR α-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, $CD25^+4^+$ T cells exhibiting suppressive activity upon OVA stimulation may be expressing dual TCRs, one composed of the transgenic α/β-chains and the other of an endogenous α-chain and the transgenic β-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 $CD25^+4^+8^-$ thymocytes anergic and suppressive through the thymic selection process presumably 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 self-reactive transgenic TCRs specific for the myelin basic protein or a pancreatic islet cell Ag, the development of autoimmune diseases (such as experimental allergic encephalomyelitis and insulin-dependent diabetes mellitus, respectively) was enhanced significantly by making the transgenic mice RAG-2 deficient or TCR Cα deficient (hence, endogenous TCR α-chain deficient) (71, 72). To further elucidate how the anergic/suppressive $CD25^+4^+8^-$ thymocytes are generated in the thymus of normal or

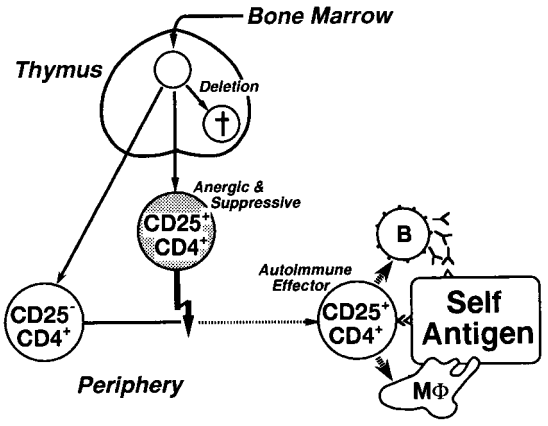

FIGURE 9. Thymic production of anergic/suppressive CD25$^+$4$^+$ T cells as a key function of the thymus in maintaining immunologic self-tolerance. While the normal thymus deletes T cells highly reactive with self Ags expressed in the thymus, it continuously produces some potentially pathogenic self-reactive CD4$^+$ T cells, which persist in the periphery at CD25$^-$ quiescent state. The normal thymus also continuously produces anergic and suppressive CD25$^+$4$^+$ T cells, which are suppressing the activation and expansion of CD4$^+$ self-reactive T cells from CD25$^-$ dormant state. When the immunoregulatory CD25$^+$4$^+$ T cells are eliminated or substantially reduced, or their regulatory function is impaired, CD25$^-$ self-reactive T cells become activated, expand, and differentiate to CD25$^+$ activated autoimmune effector T cells (dotted arrow), which may help B cells to form autoantibodies (Th2 response) or conduct cell-mediated immune responses by recruiting inflammatory cells, including activated macrophages (Mϕ) (Th1 response).

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 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. 1*C*). They, however, scarcely exhibited suppressive activity (Fig. 7, *C* and *D*). Although these CD25$^-$ cell-derived CD25$^+$4$^+$ T cells were hyporesponsive to TCR stimulation (Fig. 7*C*), 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 hyporesponsive to further stimulation (Fig. 5*C*). Indeed, CD25$^+$4$^+$ T cells prepared in vitro by activating CD25$^-$ T cells from normal BALB/c mice did not exhibit suppressive activity either on the proliferation of other T cells in vitro (Fig. 5*C*) 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. 5*C* and Ref. 24). Taken together, these results indicate that, once the CD25$^+$CD4$^+$8$^-$ 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$^+$ activated 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 NK1.1 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 CD5^{high}, CD45RB^{low}, and partially CD62L^{high} also correlates with the findings made by us and others that autoimmune-suppressive CD4$^+$ thymocytes/T cells are CD5^{high}, CD45RB^{low}, and CD62L^{high} (8–10, 12–16, 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 CD62L^{high} or CD62L^{low} 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 characteristically resistant to 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. 3*B*), 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, breakage 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. 5*C*).

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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