

Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor α -Chains (CD25)

Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases¹

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Approximately 10% of peripheral CD4⁺ cells and less than 1% of CD8⁺ cells in normal unimmunized adult mice express the IL-2 receptor α -chain (CD25) molecules. When CD4⁺ cell suspensions prepared from BALB/c *nu/+* mice lymph nodes and spleens were depleted of CD25⁺ cells by specific mAb and C, and then inoculated into BALB/c athymic nude (*nu/nu*) mice, all recipients spontaneously developed histologically and serologically evident autoimmune diseases (such as thyroiditis, gastritis, insulinitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis); some mice also developed graft-vs-host-like wasting disease. Reconstitution of CD4⁺CD25⁺ cells within a limited period after transfer of CD4⁺CD25⁻ cells prevented these autoimmune developments in a dose-dependent fashion, whereas the reconstitution several days later, or inoculation of an equivalent dose of CD8⁺ cells, was far less efficient for the prevention. When *nu/nu* mice were transplanted with allogeneic skins or immunized with xenogeneic proteins at the time of CD25⁻ cell inoculation, they showed significantly heightened immune responses to the skins or proteins, and reconstitution of CD4⁺CD25⁺ cells normalized the responses. Taken together, these results indicate that CD4⁺CD25⁺ cells contribute to maintaining self-tolerance by down-regulating immune response to self and non-self Ags in an Ag-nonspecific manner, presumably at the T cell activation stage; elimination/reduction of CD4⁺CD25⁺ cells relieves this general suppression, thereby not only enhancing immune responses to non-self Ags, but also eliciting autoimmune responses to certain self-Ags. Abnormality of this T cell-mediated mechanism of peripheral tolerance can be a possible cause of various autoimmune diseases. *The Journal of Immunology*, 1995, 155: 1151–1164.

One of the characteristics of spontaneous autoimmune diseases (especially organ-specific ones) in humans and animals is that more than one autoimmune disease (or autoimmunity) of a common spectrum tends to develop in affected individuals; e.g., insulin-de-

pendent diabetes mellitus (IDDM),³ thyroiditis, and gastritis with pernicious anemia in humans (1, 2), dogs (3), chickens (4), rats (5, 6), and mice (7, 8). Non-organ-specific (i.e., systemic) autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, also tend to overlap each other in humans and animals (9–11). Furthermore, associative development of organ-specific and systemic autoimmune diseases has been noted in humans (12–14), dogs (15), and mice (16). These findings collectively indicate that various autoimmune diseases, whether organ-specific or systemic, in humans or animals, might arise from breakdown of a common mechanism of immunologic self-tolerance.

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³ Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; GVHD, graft-vs-host disease; HA, heat-aggregated.

There is accumulating evidence from humans and animals that CD4⁺ helper/inducer type T cells, which conduct cell-mediated tissue destruction or help B cells to form autoantibodies, are the key mediators of various organ-specific and systemic autoimmune diseases (17–21). Hence, a critical issue in elucidating the pathogenetic mechanism of autoimmune disease would be to determine what mechanism of immunologic tolerance is responsible for controlling the pathogenic CD4⁺ self-reactive T cells. Although the cause and mechanism of spontaneous autoimmune diseases in humans and animals are largely unclear at present, it is of note that similar autoimmune diseases can be produced in normal rodents by simply removing a particular T cell subpopulation from the immune system, without exogenous immunization with self-Ags in adjuvant (22–26). For example, elimination of a peripheral CD4⁺ subpopulation expressing the CD5 molecule at high levels, or the CD45RB/C molecule at low levels, elicited spontaneous activation/expansion of self-reactive T cells from the remaining CD4 population, leading to the development of organ-specific autoimmune diseases, graft-vs-host disease (GVHD)-like wasting disease, or both, in selected strains of normal mice or rats; reconstitution of the eliminated population prevented the autoimmune development (22–28). These findings indicate that potentially pathogenic CD4⁺ self-reactive T cells are present in the periphery of normal individuals; their activation/expansion is controlled by other CD4⁺ cells; and these two CD4⁺ populations can be distinguished by the expression levels of particular cell surface molecules.

To further characterize CD4⁺ self-reactive T cells in a dormant state and CD4⁺ T cells normally controlling them, we have searched for a T cell surface molecule that is more specific than CD5 or CD45RB/C in differentiating the two CD4 subpopulations in the normal immune system. Here we demonstrate that elimination of a peripheral CD4⁺ subpopulation expressing the IL-2 receptor α -chains (IL-2R α) (CD25) produces a wide spectrum of organ-specific autoimmune diseases, systemic autoimmune diseases, and GVHD-like wasting disease in normal mice; and that reconstitution of CD4⁺CD25⁺ T cells prevents these autoimmune developments. The result indicates that CD25-expressing “activated” CD4 cells actively participate in maintaining self-tolerance in the periphery, and that abnormality of this T cell-mediated immunoregulation can be a common mechanism of various autoimmune diseases.

Materials and Methods

Mice

Female BALB/c *nu*⁺ and BALB/c *nu/nu* mice (6- to 8-wk old) were purchased from Japan SLC, Inc., Shizuoka, Japan,⁴ and maintained in a

specific pathogen-free animal facility in The Institute of Physical and Chemical Research (RIKEN). To obtain BALB/c *nu*⁺ or *nu/nu* mice, *nu*⁺ males and females were mated in the facility.

Treatment of lymphocytes with Ab and complement

Lymphocyte suspensions (5×10^7) prepared from spleens and inguinal, axillar, brachial, and mesenteric lymph nodes were incubated in 12×75 -mm glass tubes (Corning, Corning, NY) with 100 μ l of 1:10-diluted ascites of anti-CD25 (7D4, rat IgM) (29), anti-L3T4 (CD4) (GK1.5, rat IgG2b) (30), anti-Lyt-2.2 (CD8) (mouse IgG2a) (31), or anti-CD5 mAb (mouse IgG2a) (32), for 45 min on ice. They were then washed once with HBSS (Life Technologies, Grand Island, NY), incubated with 1.0 ml of nontoxic rabbit serum (Life Technologies) diluted 1:5 with Medium 199 (Life Technologies) for 30 min in a 37°C water bath with occasional shaking, with 100 μ g of DNase I (Sigma Chemical Co., St. Louis, MO) added for the last 5 min of incubation. After washing twice with HBSS, the suspensions were then i.v. injected into 6- to 8-wk-old female *nu/nu* mice (22). Hybridomas secreting these mAbs were purchased from the American Type Culture Collection (Rockville, MD). To remove CD4⁺ cells completely after anti-L3T4 plus C treatment, the treated cells were incubated for 1 h at 4°C on plastic dishes precoated overnight with 100 μ g/ml concentration of affinity-purified anti-rat IgG (Cappel-Organon Teknika, West Chester, PA), and nonadherent cells were collected (33). To check by FACS the effectiveness of anti-CD25 (7D4) plus C treatment, the treated cells were incubated with culture supernatant of anti-CD25 (PC61, rat IgG1) (34), and then with FITC-labeled F(ab')₂ anti-rat IgG (Jackson ImmunoResearch, West Grove, PA); after blocking with normal rat serum, they were incubated with PE-conjugated-anti-CD4 (Fig. 1B). Less than 1% of lymphocytes after the anti-Lyt-2.2 plus C or anti-L3T4 plus C and panning treatment were positive for FITC-labeled rat anti-Lyt-2 (CD8) (53–6.7) (35) (Becton Dickinson, Mountain View, CA) or FITC-anti-rat IgG (Jackson ImmunoResearch) staining, respectively.

Flow cytometric analysis

Lymphocytes (1×10^6) were incubated with FITC-labeled or biotinylated mAbs, and analyzed by a FACScan flow cytometer (Becton Dickinson) with exclusion of dead cells by propidium iodide staining. PE-streptavidin (Biomedex, Foster City, CA) was used as the secondary reagent for biotinylated Abs. From Pharmingen (San Diego, CA), we purchased FITC-labeled anti-CD3 (145-2C11) (36), FITC-anti- $\alpha\beta$ TCR (H57-597) (37), FITC-anti-CD5 (53-7.3) (35), FITC- or biotinylated anti-CD25 (7D4) (29), FITC-anti-CD44 (Pgp-1) (38), FITC-anti-L-selectin (Mel-14) (39), FITC-anti-CD69 (40), and biotinylated anti-CD45RB (16A) (41). FITC- or PE-labeled rat anti-L3T4 (CD4) (GK1.5) (30) was purchased from Becton Dickinson. Biotinylated rat anti-IL-2R β -chain (IL-2R β) mAb (TM- β 1) was generously donated by Dr. M. Miyasaka of Osaka University (42).

Cell sorting

For separating CD4⁺ T cells by the expression levels of CD25, we enriched spleen and lymph node cell suspensions for CD4⁺ cells by incubating the cells with the mixture of culture supernatants of J11D (rat IgM) (43) and anti-CD8 (35), and then panning them on plastic dishes precoated overnight with affinity-purified anti-rat IgG (Cappel). The nonadherent cells (>90% of which were CD4⁺) were labeled with FITC-anti-CD25, and fractionated into CD25⁺ or CD25⁻ fraction on a

⁴ To examine whether autoimmune development may be influenced by genetic differences among substrains of BALB/c *nu*⁺ or *nu/nu* mice, endogenous

viruses, or conditions under which the mice are maintained, four colonies of BALB/c *nu/nu* mice (i.e., one used in the present experiment, two colonies from Japan (Clea and Charles River strains), and one from the National Institutes of Health, Bethesda) were compared for autoimmune induction by transferring CD25⁻ peripheral lymphocytes from respective BALB/c *nu*⁺ mice. Experiments with the former three *nu/nu* strains were performed at RIKEN, Japan, and those with the National Institutes of Health strain at the University of California, San Diego, CA. The incidence of organ-specific autoimmune diseases was comparable among these three strains and the one used in this report. There were variations in the incidence of glomerulonephritis, the severity of GVHD-like wasting disease, and the degree of splenomegaly; the *nu/nu* mice used in this report and those from National Institutes of Health were more susceptible than the other two colonies.

FACStar^{Plus} flow cytometer with the Consort 30 program (Becton Dickinson). The brighter stained 50% of CD25⁺ cells and duller stained 50% of CD25⁻ cells were collected as the CD4⁺CD25⁺ and CD4⁺CD25⁻ populations, respectively. In some experiments (Table III), CD25⁺ cells were enriched from the CD4⁺ cell-enriched suspensions described above by panning on plastic dishes precoated with purified 7D4 mAb (100 μ g/ml). CD25⁺ cells in the adherent fraction were 60 to 70% pure.

Serologic analysis

Detailed descriptions exist of the ELISA (using alkaline phosphatase-conjugated secondary Ab and *p*-nitrophenyl disodium hexahydrate as the substrate) for detecting autoantibodies against the gastric parietal cell Ags, thyroglobulins, ss or dsDNA, or anti-trinitrophenyl hapten Abs (16, 44). Serum IgG concentration was assayed by the single radial immunodiffusion method according to the manufacturer's instructions (The Binding Site, Birmingham, England).

Skin grafting

From the tail skin of C57BL/6 or BALB/c mice, we cut 5 mm \times 5 mm-pieces, transplanting a piece of each to opposite sides of the backs of BALB/c *nu/nu* mice. The grafted sites were wrapped for 1 wk with gauze and bandages (45). The grafts were observed daily and a record was kept of the time taken for necrosis and sloughing of the grafted skins.

Humoral immune responses to heat-aggregated BSA

BSA (type V, Sigma Chemical Co.) was heated at 75°C for 30 min and 1 mg of the HA-BSA was injected subcutaneously. Serum titers of IgG isotype anti-BSA Abs were assessed by ELISA, in which the ELISA plates coated overnight with 1 mg/ml of BSA in PBS were incubated with 1:40-diluted test sera and with alkaline phosphatase-conjugated anti-mouse IgG (Southern Biologic Technology, Birmingham, AL) as the secondary reagent. Anti-BSA titers were expressed as units when the absorbance of 1:40-diluted standard pooled serum from HA-BSA-immunized BALB/c mice was arbitrarily assumed to be 100 U.

Clinical and histologic grading of autoimmune disease

Tissues and organs (thyroid, salivary glands, lung, pancreas, stomach, adrenal gland, kidney, and ovaries) were fixed in 10% formaline and processed for hematoxylin and eosin or periodic acid Schiff staining. Grading of clinical and histologic severity of autoimmune diseases was previously described (8, 16, 22).

Results

Induction of autoimmune disease in BALB/c athymic *nu/nu* mice by inoculating *nu/+* T cell suspensions free of CD25⁺ cells

Based on the finding that elimination of CD5^{high} or CD45RB^{low} peripheral T cells elicited autoimmune disease and/or GVHD-like wasting disease in normal rodents (22–25, 27, 28), we have searched for T cell surface molecules in which expression levels correlate positively with the levels of CD5 expression, but inversely with CD45RB expression, or vice versa. Figure 1 shows that peripheral CD3⁺ cells expressing CD25 were CD5-high, but CD45RB-negative. Approximately 10 to 15%, 0 to 1%, and ~1% of CD4⁺ cells, CD8⁺ cells, and non-T (CD3⁻) cells, respectively, in the spleen, lymph nodes, and peripheral blood expressed CD25 in normal adult mice (including BALB/c, A, C3H, and C57BL/6 strains, whether the mice were raised under specific pathogen-free or conven-

tional conditions); e.g., in the lymph nodes of 3-month-old BALB/c mice, $9.0 \pm 3.3\%$ of CD3⁺ cells, $11.3 \pm 2.1\%$ of CD4⁺ cells, $0.8 \pm 0.8\%$ of CD8⁺ cells, and $1.2 \pm 0.5\%$ of CD3⁻ cells were CD25⁺ ($n = 7$) (Fig. 1A). By flow cytometric analysis, few CD25⁺ cells expressed high levels of IL-2R β . The majority (~90%) of CD25⁺ cells were CD44^{high}, ~70% being L-selectin^{low}, ~25% being CD69^{high}.

Treatment of spleen and lymph node cell suspensions with anti-CD25 mAb (7D4) plus C completely eliminated CD25⁺ cells (Fig. 1B): in every treatment, <0.1% of CD4⁺ cells after 7D4 plus C-treatment were stained with PC61, another anti-CD25 mAb recognizing a different epitope, or anti-rat Ig, which detected nonlyzed CD25⁺ cells coated with 7D4 Abs. Anti-CD5 plus C-treatment eliminated lymphocytes expressing relatively high levels of CD5, and reduced the number of CD4⁺ T cells to one-fifth and CD25⁺ cells to <0.1% of CD4⁺ cells.

To determine whether elimination of CD25⁺ cells from normal mice causes autoimmune disease, 1×10^8 , 5×10^7 , or 1×10^7 spleen and lymph node cell suspensions (~30% of which were CD3⁺) prepared from 2- to 3-month-old female BALB/c *nu/+* or *+/+* mice were depleted of CD25⁺ cells (Fig. 1B), and then inoculated into 6-wk-old female BALB/c *nu/nu* mice (Table I). In 3 mo, depending on the inoculated cell dosage, the *nu/nu* mice developed inflammatory lesions in various organs and circulating autoantibodies specific for the organs; e.g., gastritis, oophoritis, thyroiditis, sialoadenitis, adrenalitis, and insulinitis, in that order of incidence. Proteinuria due to glomerulonephritis (see Table I legend for histologic severity) was observed in 30 to 40% of the *nu/nu* mice. Some mice (~10%) were afflicted with macroscopically evident arthritis of bilateral fore and/or hind legs (16). One half of the CD25⁻ cell-inoculated *nu/nu* mice showed varying degrees of splenomegaly (see Fig. 2), which became evident within 2 wk after cell transfer. Furthermore, 30% of the *nu/nu* mice that received high doses of CD25⁻ cell inocula (Group C) died within 3 wk of a wasting disease clinically akin to acute GVHD (e.g., hunching posture, diarrhea, skin scaling, and severe splenomegaly).

Among CD25⁻ lymphocytes, CD4⁺CD25⁻ T cells displayed the disease-inducing activity and apparently did not need a CD8⁺ population for autoimmune induction: inoculation of CD4⁺CD25⁻ cells, without CD8⁺ cells, induced autoimmune diseases in *nu/nu* mice, whereas inoculation of CD8⁺CD25⁻ cells, without CD4⁺ cells, did not (Group F vs G). CD4⁺CD25⁻ cells did not require co-transfer of either donor B cells or donor APCs for efficient autoimmune induction: all four *nu/nu* mice transferred with CD4⁺CD25⁻ cell suspensions completely free of B cells, non-T, non-B cells, and CD8⁺ cells by the panning method and FACS developed gastritis, oophoritis and/or other autoimmune diseases. These results, however, do not exclude the possibility that CD8⁺ cells might also play an

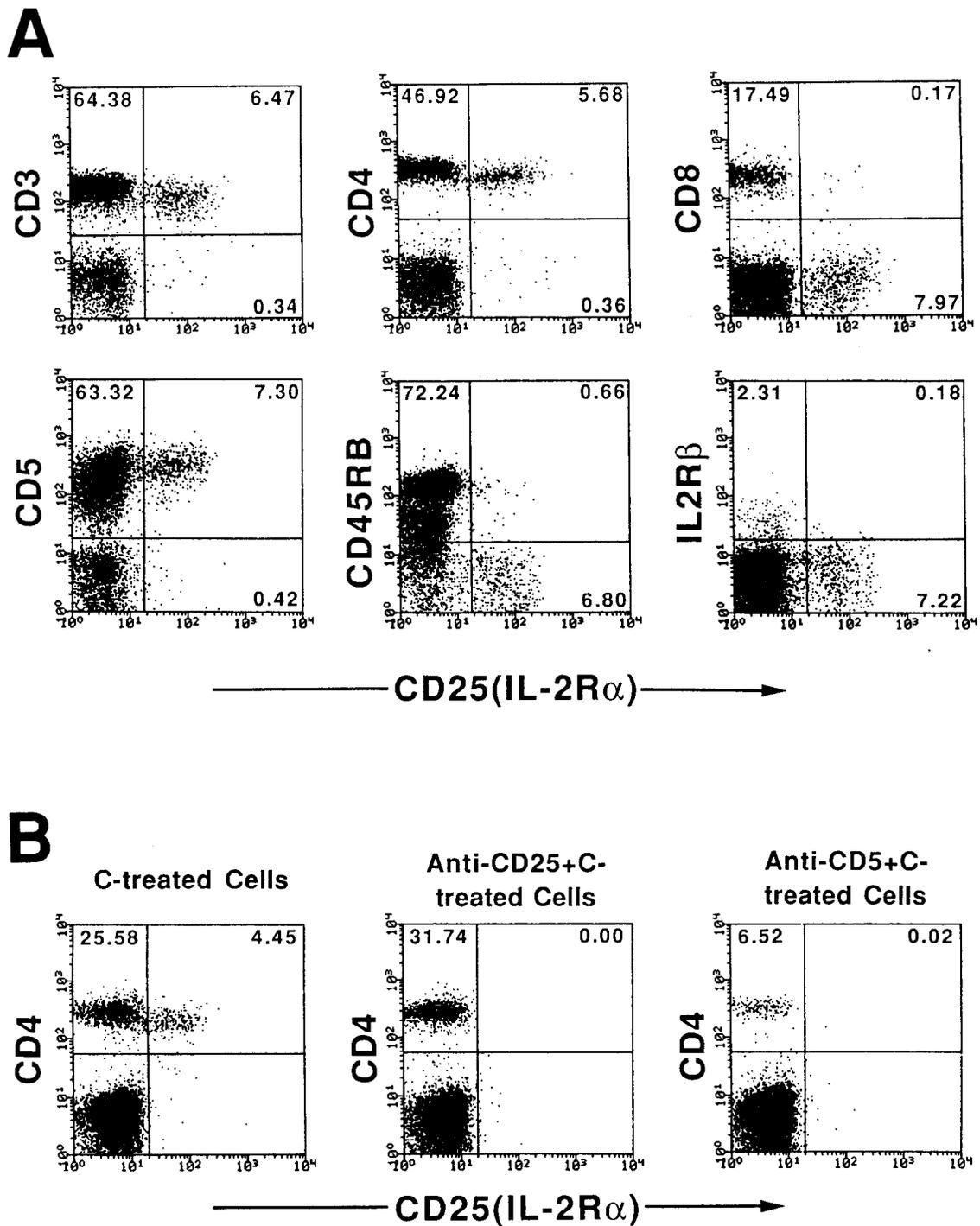


FIGURE 1. A, Expression of CD25 on mouse peripheral T cells. Lymph node cells of a 2-mo-old BALB/c mouse were stained with PE-anti-CD25 (abscissa) and FITC-anti-CD3, anti-CD4, anti-CD8, anti-CD5, anti-CD45RB, or anti-IL2R β (ordinate). A representative staining of seven mice is shown. B, Expression of CD25 on CD4⁺ spleen and lymph node cells treated with C only, anti-CD25 (7D4) plus C, or anti-CD5 plus C. The treated cells were stained with anti-CD25 (PC61) and subsequently with FITC-anti-rat Ig (abscissa) and PE-anti-CD4 (ordinate). Percentage of cells in each quadrant is shown for each staining. A representative staining in one of five experiments is shown.

effector role when help from CD4⁺ effector cells is available, since the spectrum of autoimmune diseases in the CD4⁺ CD25⁻ cell-inoculated *nu/nu* mice was slightly nar-

rower and their incidence somewhat lower compared with those inoculated with CD25⁻ cell suspensions, which contained CD8⁺CD25⁻ cells (Group D vs G).

Table I. Induction of autoimmune disease in *nu/nu* mice by inoculating *nu/+* T cell subpopulations^a

Expt. Group	Treatment of Cells	Total No. of Mice	No. of Mice with Autoimmune Disease ^b							
			Gas	Oop	Thyr	Sial	Adr	Ins	Glom	Arth
A	C (5 × 10 ⁷)	18	0	0	0	0	0	0	0	0
B	C (1 × 10 ⁷)	10	0	0	0	0	0	0	0	0
C	Anti-CD25 + C (1 × 10 ⁸)	12 ^c	12 (100)	12 (100)	9 (75.0)	9 (75.0)	4 (33.3)	1 (8.3)	5 (41.7)	1 (8.3)
D	Anti-CD25 + C (5 × 10 ⁷)	22	22 (100)	21 (95.5)	16 (72.7)	10 (45.1)	7 (31.8)	2 (9.1)	7 (31.8)	2 (9.1)
E	Anti-CD25 + C (1 × 10 ⁷)	10	8 (80.0)	6 (60.0)	4 (40.0)	4 (40.0)	0	0	0	1 (10.0)
F	Anti-CD25 + C Anti-CD4 (5 × 10 ⁷)	10	0	0	0	0	0	0	0	0
G	Anti-CD25 + C Anti-CD8 (5 × 10 ⁷)	16	14 (87.5)	13 (81.3)	7 (43.8)	5 (31.3)	2 (12.3)	0	3 (18.8)	0
H	Anti-CD5 + C Anti-CD8 (5 × 10 ⁷)	12	6 (50.0)	5 (41.7)	2 (16.7)	3 (25.0)	1 (8.4)	0	0	0

^a Spleen and lymph node cells of indicated numbers were prepared from 2- to ~3-mo-old female BALB/c *nu/+* mice and treated with indicated antisera and C, and then i.v. transferred to 6-wk-old female *nu/nu* mice. The recipient *nu/nu* mice were examined 3 mo later for histologic and serologic development of autoimmune diseases. See Figure 2 for histologic grades of gastritis and thyroiditis in group D mice. Glomerulonephritides of two mice in group D was grade 3, and of four mice was grade 2; all of these mice showed proteinuria (>300 mg/dl). Two mice showed anasarca. One mouse in group D with severe insulinitis had glucosuria (>2 g/dl). Mice in groups C, D, and G histologically showed periarterial accumulation of mononuclear cells (especially in the kidney), interstitial pneumonitis, mononuclear cell infiltration into the portal area of the liver, and/or exocrine pancreatitis; for example, 36.4%, 31.8%, 18.2%, and 9.1% of group D mice showed respective lesions. Abbreviations: gas, gastritis; oop, oophoritis; thyr, thyroiditis; sial, sialoadenitis; adr, adrenalitis; ins, insulinitis; glom, glomerulonephritis; arth, arthritis.

^b Number of mice with histologically evident autoimmune disease. Percentage of incidence shown in parentheses.

^c Among 16 mice at the beginning of the experiment, 5 (31.3%) died of wasting disease within 3 wk after cell transfer.

Transfer of CD4⁺CD5^{low} cells prepared from the same number of lymphocytes as the CD4⁺CD25⁻ cell preparation produced similar organ-specific autoimmune diseases as well, although at lower incidences and with few systemic autoimmune diseases (Group H). Judging from the dependency of the autoimmune incidences on CD25⁻ cell doses (Groups C, D, and E), the lower incidence and narrower spectrum may be due to a smaller number of CD25⁻ cells in the CD4⁺CD5^{low} cell inocula (Fig. 1B).

Immunopathology of autoimmune diseases

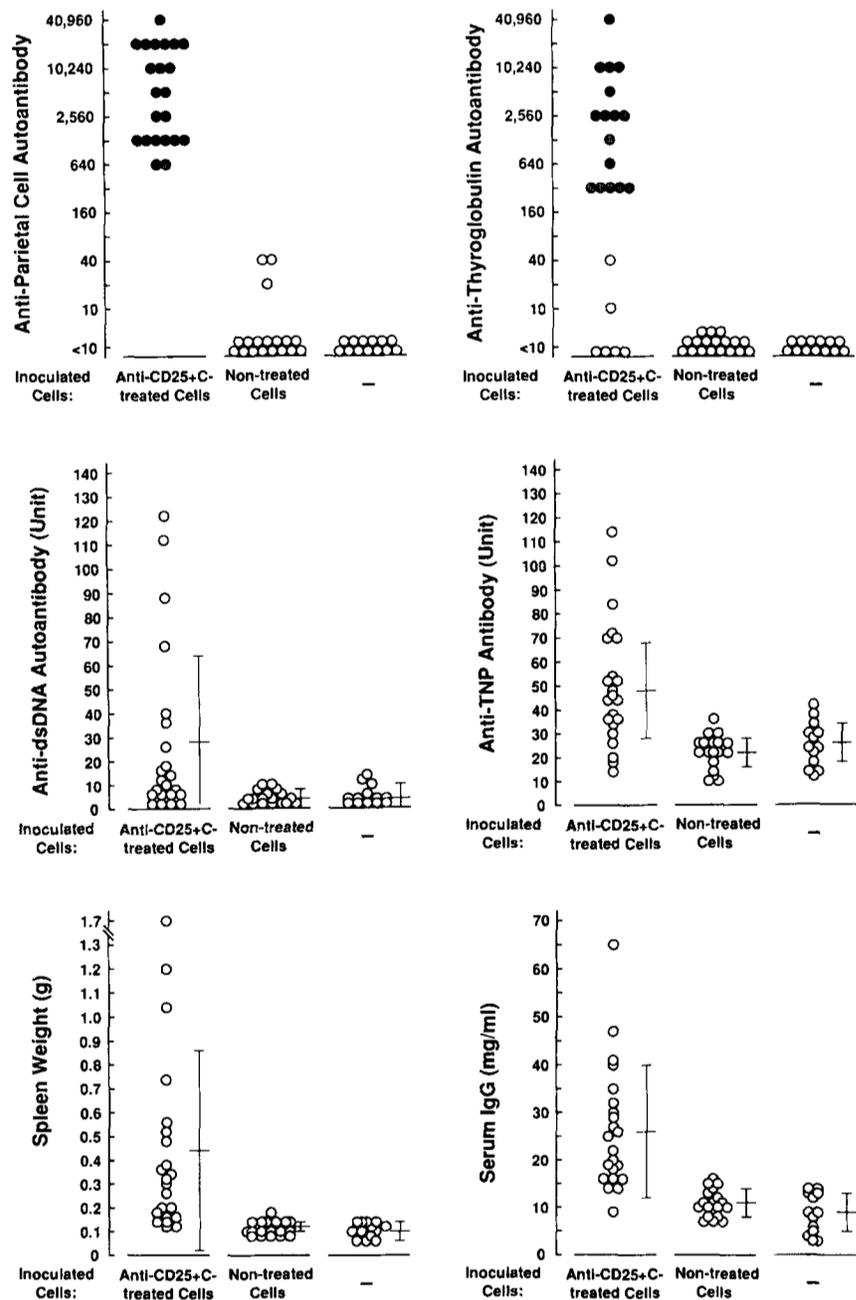
Macroscopic and microscopic views of autoimmune diseases in the CD25⁻ cell-inoculated BALB/c *nu/nu* mice (Table I) were similar to those previously reported (8, 16, 22, 44). These autoimmune diseases were accompanied by specific autoantibodies in the circulation, such as those specific for the gastric parietal cells or thyroglobulins in autoimmune gastritis or thyroiditis, respectively, and IgG isotype anti-dsDNA autoantibodies characteristic of lupus-like systemic autoimmune disease (Fig. 2). Autoantibodies specific for the oocytes, adrenocortical cells, or ductal cells of the salivary glands were also detected by indirect immunofluorescence test in the sera of mice with histologically evident oophoritis, adrenalitis, and sialoadenitis, respectively (16, 44). Control nontreated *nu/nu* mice or *nu/nu* mice inoculated with whole lymphocytes did not develop these autoantibodies. The CD25⁻ cell-inoculated

nu/nu mice showed hypergammaglobulinemia and spontaneous development of anti-trinitrophenyl Abs, whereas the two control groups did not, suggesting polyclonal B-cell activation in the former. In contrast to increased spleen weights of the CD25⁻ cell-inoculated *nu/nu* mice, normal spleen weights of control *nu/nu* mice transferred with whole lymphocytes indicated no significant histoincompatibility between the *nu/nu* mice and the *nu/+* mice; this suggests that the wasting disease, splenomegaly, and autoimmunity in the CD25⁻ cell-inoculated *nu/nu* mice were not due to an allogeneic GVH reaction (11).

Prevention of autoimmune disease in *nu/nu* mice by restoring normal T cells, especially CD4⁺CD25⁺ cells

Graded numbers of *nu/+* spleen and lymph node cells were either co-transferred with a fixed number of CD25⁻ lymphocytes or inoculated 10 days after CD25⁻ cell transfer to determine the dose of normal lymphocytes and the time of cell inoculation required for preventing the spectrum of autoimmune diseases (Table II), in particular, the histologic/serologic development of gastric autoimmunity (Fig. 3). Co-transfer of 1 × 10⁷ or 5 × 10⁷ normal lymphocytes (~30% of which were CD3⁺ cells) with CD25⁻ lymphocytes prepared from 5 × 10⁷ lymphocytes effectively prevented histologic and serologic autoimmune development in a dose-dependent fashion. In contrast, an

FIGURE 2. Various parameters of autoimmune diseases in *nu/nu* mice inoculated with *nu/+* spleen and lymph node cells treated with anti-CD25 plus C (CD25⁻ lymphocytes) ($n = 22$), or C only (whole lymphocytes) ($n = 18$), as shown in Table I as the experimental groups D and A, respectively, or in age-matched non-treated *nu/nu* mice ($n = 13$). Filled (●) or shaded (◐) circles indicate grade 2 or grade 1 gastritis, respectively, in antiparietal cell autoantibody assay, and grade 2 or grade 1 thyroiditis, respectively, in antithyroglobulin autoantibody assay; open circles (○) indicate histologically intact gastric mucosa or thyroid gland (see Refs. 22 and 44 for histologic grading). Vertical bars indicate mean \pm SD.



even larger number of normal lymphocytes (e.g., 1×10^8) failed to prevent gastritis and oophoritis when the cells were inoculated 10 days after the CD25⁻ cell transfer, although they were effective against other autoimmune diseases including systemic ones and GVHD-like diseases. The less effective prevention could not be attributed to less “space” in the immune system for the lymphocytes inoculated later, since the number of peripheral T cells in this group of *nu/nu* mice at the time of death was greater than the T cell number in other groups (data not shown).

To determine whether autoimmune preventive activity is in normal CD4⁺ cells, especially in CD4⁺CD25⁺ cells,

as suggested by CD25⁺ cell subtraction experiments in Table I, graded numbers of CD4⁺ cells, CD4⁺CD25⁺ cells, or CD8⁺ cells were co-transferred with CD25⁻ cells; the extent and severity of autoimmune development in the recipient *nu/nu* mice were assessed 3 mo later (Table III and Fig. 4). Co-transfer of 2×10^6 CD4⁺CD25⁺ cell-enriched suspensions (60–70% of which were CD25⁺ cells), equivalent with 1 to 2×10^7 CD4⁺ cells in the number of contained CD25⁺ cells, effectively prevented CD25⁻ cell-induced autoimmune disease in terms of the spectrum of affected organs and incidence/severity of particular autoimmune diseases, whereas co-transfer of $2 \times$

Table II. Dose of normal lymphocytes and time of lymphocyte inoculation required for preventing CD25⁻ cell-induced autoimmune disease in nu/nu mice^a

Expt. Group	Inoculated Lymphocytes	Total No. of Mice	No. of Mice with Autoimmune Disease ^b							
			Gas	Oop	Thyr	Sial	Adr	Ins	Glom	Arth
A	CD25 ⁻ cells (5 × 10 ⁷)	8	8 (100)	8 (100)	4 (50.0)	3 (37.5)	2 (25.0)	0	2 (25.0)	0
B	CD25 ⁻ cells (5 × 10 ⁷) + normal cells (1 × 10 ⁷)	8	2 (25.0)	0	0	1 (12.5)	0	0	0	0
C	CD25 ⁻ cells (5 × 10 ⁷) + normal cells (5 × 10 ⁷)	8	0	0	0	0	0	0	0	0
D	CD25 ⁻ cells (5 × 10 ⁷) + normal cells (1 × 10 ⁸) 10 days later	8	6 (75.0)	2 (25.0)	0	1 (12.5)	0	0	0	0

^a Anti-CD25 plus C-treated cell suspensions (designated as CD25⁻ cells) of indicated numbers were prepared from 2- to ~3-mo-old female BALB/c nu/+ mice spleens and lymph nodes and transferred with or without nontreated spleen and lymph node cell suspensions of indicated numbers prepared from the same cell source. A group of mice were inoculated with normal lymphocyte suspensions 10 days after transfer of CD25⁻ cells (group D). The recipient nu/nu mice were examined 3 mo later for histologic and serologic development of autoimmune diseases.

^b Number of mice with histologically evident autoimmune disease is shown. Percentage of incidence shown in parentheses. For definition of abbreviations, see Table I, footnote ².

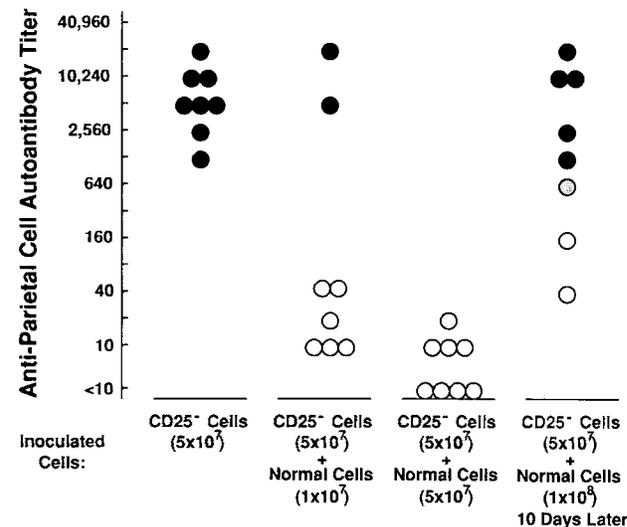


FIGURE 3. Titers of antiparietal cell autoantibodies in the nu/nu mice that had 3 mo earlier received transfer of CD25⁻ cells (prepared from 5 × 10⁷ nu/+ spleen and lymph node cells), cotransfer of CD25⁻ cells and normal lymphocytes of indicated numbers, or transfer of CD25⁻ cells and then normal lymphocytes 10 days later (see Table II). ○, ◐, ●. See legend for Figure 2. Combined result of two independent experiments is shown.

10⁶ whole CD4⁺ cells did not. Compared with CD4⁺ cells, co-inoculation of a comparable dose (2 × 10⁷) of CD8⁺ cells was far less effective in preventing gastritis and oophoritis, although effective prevention of other autoimmune diseases by the CD8⁺ cells suggested that normal

CD8⁺ cells might bear a low level of autoimmune-preventive activity. The activity could be attributed to CD8⁺CD25⁺ cells (constituting ~1% of CD8⁺ cells (Fig. 1A)), since CD8⁺CD25⁻ cells appeared to rather enhance autoimmune development in collaboration with CD4⁺CD25⁻ cells (Table I, Group D).

Induction of autoimmune disease in naive nu/nu mice by transferring CD4⁺ cells from autoimmune nu/nu mice

Three months after inoculation of anti-CD25 plus C-treated cells prepared from 5 × 10⁷ nu/+ peripheral lymphocytes, CD3⁺, CD4⁺, or CD8⁺ cells comprised 37.6 ± 8.7%, 21.8 ± 6.1%, and 12.2 ± 5.0%, respectively, of lymph node cells in the recipient nu/nu mice (n = 7) (CD4⁺ or CD8⁺ cells in age-matched control nu/nu mice (n = 4) were 2.1 ± 0.4% and 5.4 ± 0.4%, respectively). CD3⁺ cells, especially CD4⁺ cells, expressed CD25 in these nu/nu mice; i.e., 19.5 ± 2.4% of CD3⁺ cells, 36.2 ± 2.5% of CD4⁺ cells, 2.8 ± 2.1% of CD8⁺ cells, and 2.0 ± 1.0% of non-T (CD3⁻) cells in the lymph nodes (n = 7) (Fig. 5). The majority (~90%) of CD25⁺ cells did not express IL-2Rβ-chains assessed by flow cytometry. More than 95% of CD25⁺ cells in the disease-bearing nu/nu mice were CD5^{high}, CD45RB^{low}, and CD44^{high}; ~90% were L-selectin^{low}, and ~50% were CD69^{high}. These results indicate that a fraction of the inoculated CD25⁻ nu/+ lymphocytes, especially CD4⁺CD25⁻

Table III. Prevention of CD25⁻ cell-induced autoimmune disease in *nu/nu* mice by cotransfer of CD25⁺ cells^a

Expt. Group	Inoculated Lymphocytes	Total No. of Mice	No. of Mice with Autoimmune Disease ^b							
			Gas	Oop	Thyr	Sial	Adr	Ins	Glom	Arth
A	CD25 ⁻ cells (5 × 10 ⁷)	8	8 (100)	7 (87.5)	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)	3 (37.5)	0
B	CD25 ⁻ cells (5 × 10 ⁷) + CD4 ⁺ cells (2 × 10 ⁶)	8	4 (50.0)	1 (12.5)	0	0	0	0	0	0
C	CD25 ⁻ cells (5 × 10 ⁷) + CD4 ⁺ cells (2 × 10 ⁷)	8	0	0	0	0	0	0	0	0
D	CD25 ⁻ cells (5 × 10 ⁷) + CD8 ⁺ cells (2 × 10 ⁷)	8	6 (75.0)	3 (37.5)	0	1 (12.5)	0	0	0	0
E	CD25 ⁻ cells (5 × 10 ⁷) + CD4 ⁺ CD25 ⁺ cells (2 × 10 ⁶)	6	1 (16.7)	0	0	0	0	0	0	0

^a Anti-CD25 plus C-treated cells (CD25⁻ cells) prepared from spleen and lymph nodes (5 × 10⁷) of 2- to ~3-mo-old female BALB/c *nu/+* mice were cotransferred with CD4⁺, CD8⁺, or CD4⁺CD25⁺ cells of indicated numbers enriched from the same cell source by C-dependent cytotoxic treatment and panning of Ab-coated dishes. Purity of CD4⁺ or CD8⁺ cell suspensions were >90%; that of CD4⁺CD25⁺ cells 60 to 70%. The recipient *nu/nu* mice were examined 3 mo later for histologic and serologic development of autoimmune diseases.

^b Number of mice with histologically evident autoimmune disease is shown. Percentage of incidence shown in parentheses. For definition of abbreviations, see Table I, footnote ^a.

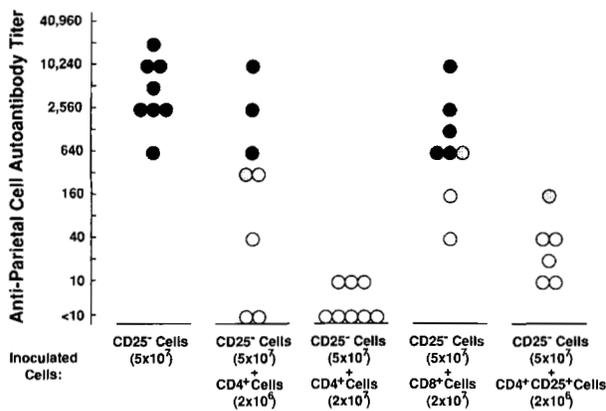


FIGURE 4. Titers of antiparietal cell autoantibodies in the *nu/nu* mice inoculated 3 mo earlier with CD25⁻-cell suspensions only, or CD25⁻-cell suspensions mixed with CD4⁺, CD4⁺CD25⁺, or CD8⁺ cell-enriched suspensions of indicated numbers (see Table III). ○, ◐, ●. See legend for Figure 2. Combined result of two independent experiments is shown.

cells, gave rise to CD25⁺-activated T cells, which were similar in the cell surface phenotype to CD4⁺CD25⁺ peripheral lymphocytes in normal mice (Fig. 1A).

Autoimmune diseases (including insulinitis/diabetes mellitus, adrenalitis, and arthritis) in CD25⁻ cell-inoculated *nu/nu* mice could be adoptively transferred to syngeneic naive *nu/nu* mice by T cells (22, and S. Sakaguchi, manuscript in preparation). To determine whether the CD4⁺CD25⁺ cells

in the disease-bearing *nu/nu* mice contained autoimmune effector T cells, 1 × 10⁶ CD4⁺CD25⁺ cells were enriched from the regional (paragastric, hepatic, paraaortic, and mesenteric) lymph nodes of the *nu/nu* mice with gastritis and oophoritis and then transferred to naive *nu/nu* mice; the *nu/nu* mice were histologically and serologically examined 2 mo later (Table IV, Expt. A). Cell suspensions enriched for CD4⁺CD25⁺ cells, as well as those for CD4⁺ cells, adoptively transferred autoimmune diseases in a disease-specific fashion. Transfer of the same number of the CD4⁺CD25⁻-enriched cell suspensions from the same *nu/nu* mice also produced similar autoimmune diseases, although at lower incidences.

To examine whether the CD25⁻ T cell populations in these donor *nu/nu* mice with gastritis and oophoritis contain self-reactive T cells capable of causing other autoimmune diseases, 5 × 10⁷ spleen cells treated with anti-CD25 plus C, or C only as control, were transferred to naive *nu/nu* mice (Expt. B). In 3 mo, both transfers produced the same autoimmune diseases as in the donors. The transfer of CD25⁻ cells also induced thyroiditis de novo, whereas the control transfer did not.

These results taken together indicate that the CD4⁺CD25⁺ population in *nu/nu* mice with autoimmune disease contained autoimmune effector T cells; the CD4⁺CD25⁻ population in such mice also contained potentially pathogenic self-reactive T cell clones with the same or other Ag specificities; furthermore, the CD4⁺CD25⁺ population (including autoimmune

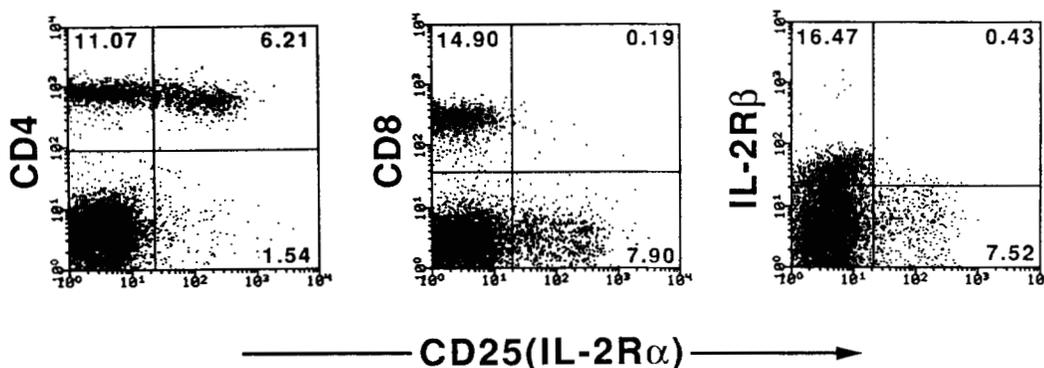


FIGURE 5. Expression of CD25 on lymph node cells of *nu/nu* mice transferred with anti-CD25 plus C-treated lymphocytes 3 months earlier. Each cell suspension was co-stained with PE-anti-CD25 (abscissa) and FITC-anti-CD4, anti-CD8, or anti-IL2R β (ordinate). Percentage of cells in each quadrant is shown for each staining. A representative staining of seven mice similarly treated is shown.

Table IV. Adoptive transfer of autoimmune disease by CD4⁺CD25⁺ cells and de novo autoimmune induction by CD4⁺CD25⁻ cells from *nu/nu* mice with autoimmune disease

Expt. Group	Autoimmune Disease in Donor Mice	Transferred Cells	Autoimmune Disease in Recipient <i>nu/nu</i> Mice ^c		
			Gastritis	Oophoritis	Thyroiditis
A ^a	Gastritis	CD4 ⁺ cells (1×10^6)	3/3 (640, 320, 320)	3/3	0/3 (10, <10, <10)
	Oophoritis	CD4 ⁺ CD25 ⁺ cells (1×10^6)	3/3 (320, 320, 160)	3/3	0/3 (<10, <10, <10)
		CD4 ⁺ CD25 ⁻ cells (1×10^6)	2/3 (320, 160, <10)	1/3	0/3 (<10, <10, <10)
B ^b	Gastritis	C-treated cells (5×10^7)	5/5 (2560, 2560, 1280, 1280, 640)	5/5	0/5 (40, 20, 20 <10, <10)
	Oophoritis	Anti-CD25 + C-treated cells (5×10^7)	5/5 (2560, 1280, 1280, 1280, 640)	5/5	2/5 (640, 320, 40, 20, <10)

^a In Expt. A, CD4⁺, CD4⁺CD25⁺, or CD4⁺CD25⁻ cells of indicated numbers were prepared by panning on Ab-coated dishes and FACS from the regional (paragastric, hepatic, mesenteric, and paraaortic) lymph nodes of individual *nu/nu* mice that had developed indicated autoimmune diseases after transfer of CD25⁻ *nu/+* cells 3 mo before (Tables I-III); each cell suspension was i.v. transferred to 6-wk-old female *nu/nu* mice, which were histologically and serologically examined 2 mo later. One mouse each of CD4⁺ or CD4⁺CD25⁺ cell-transferred *nu/nu* mice had grade 1 gastritis; other histologically evident gastritis was grade 2.

^b In Expt. B, spleen cell suspensions of indicated numbers were prepared from the individual CD25⁻ *nu/+* cell-inoculated *nu/nu* mice with indicated autoimmune diseases, treated with anti-CD25 plus C or C only, and then transferred to 6-wk-old female *nu/nu* mice to examine 3 mo later for histologic and serologic autoimmune development. All histologically evident gastritides were grade 2; all histologically evident thyroiditides were grade 1 (see Refs. 22 and 44 for histologic grading of gastritis and thyroiditis).

^c Number of *nu/nu* mice with histologically evident autoimmune disease among the *nu/nu* mice transferred with each cell fraction. Titers of antiparietal cell autoantibodies or antithyroglobulin autoantibodies assessed by ELISA are shown in parentheses. Total results of three experiments in Expt. A, and five independent experiments in Expt. B are shown.

effector T cells) may down-regulate further expansion/activation of the CD25⁻ dormant self-reactive T cells, even in *nu/nu* mice with particular autoimmune diseases.

Elimination of CD25⁺ T cells enhances immune responses to non-self Ags

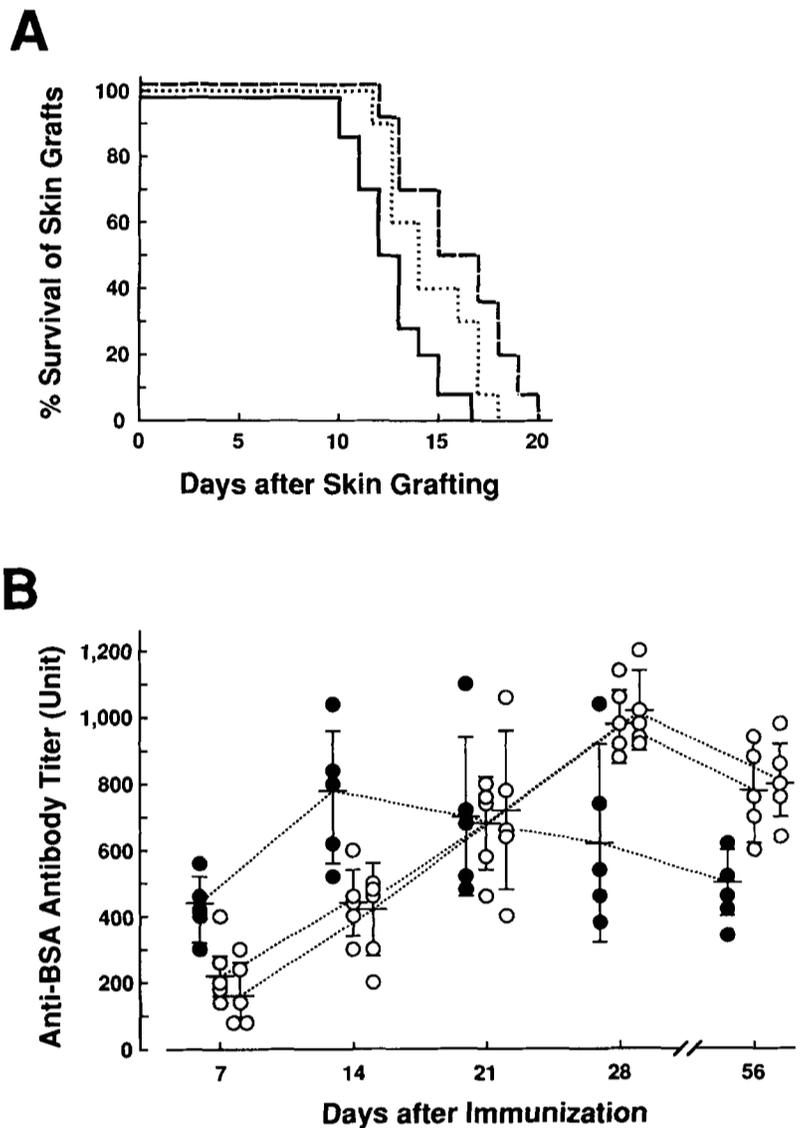
To determine whether CD25⁺ cells down-regulate immune responses to non-self Ags as well, we examined immune responses of *nu/nu* mice to allogeneic skin grafts or HA-BSA immediately after transfer of CD25⁻ lymphocytes, control lymphocytes, or CD25⁻ lymphocytes mixed with normal CD4⁺ lymphocytes (Fig. 6). The CD25⁻ cell-inoculated *nu/nu* mice rejected the allografts significantly faster than those transferred with control lymphocytes. The former also developed significantly higher titers of IgG anti-BSA Abs for the first 2 wk after immunization; the responses, however, waned faster thereafter. Co-inoculation of 2×10^7 CD4⁺ cells with CD25⁻ cells prepared

from 5×10^7 lymphocytes normalized the heightened immune responses to allogeneic skins or HA-BSA. These results taken together indicate that CD25⁺ T cells down-regulate not only autoimmune responses but also both humoral and cellular immune responses to non-self Ags by CD25⁻ cells, although the possibility could not be excluded that CD25⁺ cells might indirectly contribute to normalizing the responses to BSA or allogeneic skins by preventing CD25⁻ cell-induced polyclonal B cell activation or syngeneic GvH-like disease involving skin abnormality, respectively.

Discussion

The present experiments have demonstrated that removal of a particular T cell subpopulation suffices to activate self-reactive T cells and elicit autoimmune disease in an

FIGURE 6. Response to allogeneic skin transplants or HA-BSA by *nu/nu* mice inoculated with CD25⁻ *nu/+* lymphocytes, control *nu/+* lymphocytes, or a mixture of these. **A**, BALB/c *nu/nu* mice were engrafted with tail skins from C57BL/6 mice and inoculated with CD25 plus C-treated cells (solid line), or C-treated cells (dashed line), prepared from 5×10^7 spleen and lymph node cells of BALB/c *nu/+* mice. A group of *nu/nu* mice received a mixture of 5×10^7 CD25 plus C-treated lymphocytes and 2×10^7 normal CD4⁺ cells (dotted line). Percentage of surviving skins (abscissa) on days after transplantation (ordinate) is shown for total result of three independent experiments. Survival of skin grafts of CD25⁻ cell-inoculated group (12.7 ± 2.0 days, $n = 14$) was significantly ($p < 0.005$ by Mann-Whitney *U* test) faster than control group (16.0 ± 2.6 days, $n = 14$) or group inoculated with mixed cell populations (14.7 ± 2.1 days, $n = 10$) ($p < 0.05$). **B**, BALB/c *nu/nu* mice were inoculated with CD25 plus C-treated cells (●), or C-treated cells (○), prepared from 5×10^7 spleen and lymph node cells of BALB/c *nu/+* mice. A group of *nu/nu* mice received a mixture of 5×10^7 CD25 plus C-treated lymphocytes and 2×10^7 normal CD4⁺ cells (◐). At the same time, these mice received s.c. injection of 1 mg HA-BSA in saline. Serum titers of IgG anti-BSA Abs on various days after immunization are shown. The IgG anti-BSA titer of a pooled serum collected from BALB/c mice on day 7 after HA-BSA immunization was arbitrarily designated as 100 units. A representative result of three independent experiments is shown. Anti-BSA titers of CD25⁻ cell-inoculated group on day 7 or 14 were significantly higher ($p < 0.05$ by Mann-Whitney *U* test) than those of groups inoculated with C-treated cells or mixed populations. Titers of control *nu/nu* mice ($n = 3$) with no cell inoculation were < 10 units on day 7 or day 14 after HA-BSA immunization.



environment where the target self-Ags are under normal conditions and in physiologic concentrations. The autoimmune diseases thus induced were similar in immunopathology to human autoimmune diseases, such as autoimmune gastritis with pernicious anemia, Hashimoto's thyroiditis, sialoadenitis in Sjögren's syndrome, Addison's disease, IDDM, autoimmune premature ovarian failure, systemic lupus erythematosus, and rheumatoid arthritis (9). Some of the self-reactive T cells thus demonstrated appeared to be specific for organ-specific self-Ags, since T

cells could adoptively transfer organ-specific autoimmune diseases to naive *nu/nu* mice in a disease-specific fashion with resulting histologically evident destruction of the target organs and the appearance of specific autoantibodies in the circulation (Table IV and Ref. 37). Some T cells inoculated to *nu/nu* mice also polyclonally activated B cells, including autoantibody-forming B cells, which might, in addition to forming autoantibodies, present self-Ags to activated CD4⁺ helper T cells specific for the Ags, thus triggering Ag-driven, T cell-dependent autoimmune responses

and leading to the formation of nephritogenic or vasculitogenic autoantibodies or immune complexes.

As to the pathogenetic mechanism of these murine autoimmune diseases, one may argue that a T cell-immunodeficient state prepared in *nu/nu* mice by partial T cell reconstitution might incur viral infections, which might elicit autoimmune disease, for example, by direct tissue damage resulting in leakage of sequestered self-Ags or antigenic modification of self-constituents, aberrant expression of MHC molecules presenting self-Ags, or immunologic cross-reaction between the virus and self-constituents (47–51). Although our results do not formally exclude such a possibility, the scenario would be unlikely as an initial triggering event of autoimmune disease for the following reasons (see also footnote 4). First, the autoimmune-prone CD25⁻ cell-inoculated *nu/nu* mice were not less immunocompetent, but rather more vigorously responsive to non-self Ags at the initial phase of autoimmune development, than the autoimmune-free *nu/nu* mice inoculated with whole T cells (Fig. 6). This suggests that the former would more easily eliminate invading viruses than the latter. Second, preparation of a simple T cell immunodeficiency in *nu/nu* mice failed to induce autoimmune disease, as illustrated by the lack of any evident autoimmunity in nontreated *nu/nu* mice or *nu/nu* mice inoculated with a small number of normal lymphocytes (Table I and Fig. 2). Third, the development of multiple autoimmune diseases in a single *nu/nu* mouse indicated involvement of multiple self-reactive T cell clones with distinct Ag specificities. This would make an immunologic cross-reaction between viral components and self-constituents unlikely as a triggering mechanism of the autoimmunity, since it would be difficult to envisage a single virus with multiple and distinct cross-reactive epitopes, or simultaneous infections of a single mouse with a number of antigenically different viruses with each epitope cross-reacting with each tissue constituent. A virus might, however, play a role as a target Ag rather than as a triggering agent of autoimmune responses; for example, the heightened immune responses in CD25⁻ cell-inoculated *nu/nu* mice might include humoral responses to endogenous viruses or their products, leading to the formation of pathogenic immune complexes (52).

Cell surface expression or nonexpression of the CD25 molecule could divide peripheral CD4⁺ cells of normal mice into one subpopulation containing dormant self-reactive T cells and another one controlling them. The degree of CD5 or CD45RB/C expression could make similar dissections of CD4⁺ cells, in which autoimmune-preventive CD4⁺ cells were CD5^{high} or CD45RB^{low} (22–28). Compared with being CD5^{high} or CD45RB^{low}, CD25 expression appears to be more specific for the CD4⁺ cells with autoimmune-preventive activity, since depletion of CD25⁺ cells eliminated only a fraction of CD4⁺CD5^{high} or CD4⁺CD45RB^{low} cells (i.e., 20% and 40%, respectively (Fig. 1)) but sufficed to elicit autoimmune disease at

an even higher incidence and in a wider spectrum of organs/tissues than depletion of CD4⁺CD5^{high} cells (Table I) or CD4⁺CD45RB^{low} cells (S. Sakaguchi, unpublished data). Thus, autoimmune induction by eliminating CD4⁺CD5^{high} or CD4⁺CD45RB^{low} cells, reported by us and others (22–28), can be attributed to depletion of CD4⁺CD25⁺ cells.

The CD25 molecule is generally expressed on activated T and B cells (29, 53, 54); and CD4⁺CD25⁺ T cells are present in the peripheral blood of normal healthy individuals (53, 55, 56). This poses the question whether autoimmune inhibition is mediated by any CD4⁺ T cells at a CD25⁻-activated state or by a distinct CD4⁺CD25⁺ T cell subpopulation specialized for the suppressive function. The former seems to be the case for the following reasons. First, the profile of cell surface molecules expressed on CD4⁺CD25⁺ cells in normal mice was akin to the one reported for “activated,” “effector,” or “memory” type T cells in general; i.e., CD45RB^{low}, CD44^{high}, CD5^{high}, and L-selectin^{low} (38, 41, 57, 58). Second, even the CD4⁺CD25⁺ population containing autoimmune effector T cells (indistinguishable from the CD4⁺CD25⁺ population in normal mice in terms of cell surface phenotype (see above) and cytokine-forming pattern (M. Toda and S. Sakaguchi, unpublished data)) appeared to down-regulate in a negative feedback manner further activation/expansion of other autoimmune clones from the CD25⁻ dormant state (Table IV). Furthermore, the activated CD25⁺ self-reactive T cells may also down-regulate immune responses to non-self Ags, as indicated by the initially heightened and then rapidly waning anti-BSA responses by CD25⁻ cell-inoculated autoimmune *nu/nu* mice (Fig. 6). It remains to be determined, however, whether all the CD25⁺ T cells in normal individuals have acquired this phenotype upon activation in the periphery by responding to non-self Ags, since a fraction of CD4⁺CD8⁻ mature thymocytes are also expressing CD25 in normal mice (S. Sakaguchi, unpublished data), and a significant fraction of the cord blood CD4⁺ T cells of healthy newborns free of any infection express CD25 in humans (59). Some of these CD4⁺CD25⁺ T cells/thymocytes in normal individuals might be in an anergic state after encounter with self-Ags (60), as suggested by high levels of CD25 expression on anergic T cells (61, 62).

CD4⁺CD25⁺ cells could inhibit directly, rather than via CD8⁺ cells, the activation/expansion of CD4⁺ autoimmune effector T cells from the CD25⁻ dormant state, although CD4⁺CD25⁺ cells might first act on non-T cells (such as APCs), which in turn control activation of CD4⁺CD25⁻ cells. One plausible mechanism of this T cell-mediated or -dependent immunoregulation would be that CD4⁺CD25⁺ cells might absorb IL-2 (locally produced, for example, upon viral infections of the target organs (48)) as a “cytokine sink” (63), thereby preventing activation of nearby CD25⁻ self-reactive T cells by IL-2. However, such a mechanism, even if operative, cannot

fully account for the CD25⁺ cell-mediated autoimmune inhibition, since the presence of IL-2R β ⁺ cells in the CD25⁻ cell suspensions (Fig. 1A) or the co-transfer of CD8⁺ cells (~30% of which express IL-2R β -chains (42)) with CD25⁻ cells failed to prevent autoimmune disease, irrespective of the higher affinity of IL-2R β (plus IL-2R γ) for IL-2 than CD25 (64). As an alternative mechanism, CD25⁺-activated T cells may constitutively secrete Agnonspecific molecules that raise the activation thresholds of other T cells in a negative feedback manner (see discussion above). Indeed, peripheral CD4⁺CD25⁺ population in normal BALB/c mice predominantly contained cells forming TGF- β , IL-12, IL-4 and/or IL-10, some of which are suppressive on cell-mediated immunity (65, 66) (M. Toda and S. Sakaguchi, unpublished data). Whether cytokines secreted by CD25⁺ cells can control CD25⁻ self-reactive T cells is currently under investigation. It also remains to be determined in our experiments whether CD25⁺-“activated” T cells might more easily proliferate upon transfer to *nu/nu* mice, making less “space” available for expansion of CD25⁻ dormant self-reactive T cells, thereby contributing to the inhibition of autoimmune development (50).

The efficacy of autoimmune prevention by CD25⁺ cells differed for particular autoimmune diseases and depended upon cell doses and time of cell inoculation; e.g., prevention of gastritis required larger numbers of normal T cells within a shorter period after CD25⁻ cell transfer, whereas cell inoculation of a smaller number or at a later time was sufficient for preventing other autoimmune diseases (Table II, III). This finding indicates that the completeness and duration of the CD25⁺ cell elimination significantly influence the incidence and spectrum of autoimmune diseases. It also suggests that even incomplete and/or transient elimination of CD25⁺ cells may suffice to elicit an autoimmune disease in highly susceptible individuals. BALB/c mice indeed developed gastritis when the immune abnormality was mild and/or of short duration (8, 44, 67). With a more severe abnormality over a longer period, they developed not only a high incidence of gastritis but also other autoimmune diseases, such as oophoritis, thyroiditis, sialoadenitis, and adrenalitis, in that order of incidence (44). It should be noted, however, that the degree of immune abnormality itself cannot determine the specificities of the elicited autoimmune responses, since a comparable degree of CD25⁺ cell elimination predominantly induced gastritis in BALB/c mice, oophoritis in a second strain, and very few autoimmune diseases at all in some others (22, 23, and our unpublished data). Host genetic elements (including MHC genes) thus appear to contribute significantly to determining the autoimmune specificities (and the hierarchy of autoimmune susceptibilities); they may, for example, modulate the positive/negative selection of particular self-reactive clones in the thymus or presentation of particular self-Ag to T cells in the periphery, thereby determining which self-reactive clones are more

easily activated, or resistant to activation, upon elimination/reduction of CD25⁺ T cells (8, 44, 67). Possible evolutionary conservation of such genes determining autoimmune phenotype may account for the development of a similar spectrum of autoimmune diseases in CD25⁻ cell-inoculated BALB/c *nu/nu* mice and in other species, including humans (1–16).

In conclusion, the present results from reconstitution of *nu/nu* mice with T cell subpopulations indicate that CD25⁺ lymphocytes, the majority of which are CD4⁺, may sustain immune responses to self and non-self Ags at suppressed levels in normal individuals; and elimination/reduction of CD25⁺ T cells may relieve the suppression, leading to enhancement of immune responses to non-self Ags and elicitation of autoimmune responses to certain self-Ags. An important implication of this autoimmune prevention by CD25⁺ cells is that immune responses to non-self Ags such as viruses and bacteria, and a resulting increase in the number of responding CD25⁺-activated T cells, may simultaneously augment down-regulation of CD25⁻ dormant self-reactive T cells, thereby not only avoiding bystander activation of self-reactive T cells, but also strengthening self-tolerance. Such augmentation of self-tolerance by non-self Ags may account for a protective effect of deliberate or opportunistic viral infection upon autoimmune development (68–70), and may form the basis for developing new preventive measures for autoimmune disease. Another implication of CD4⁺CD25⁺ cell-mediated control is that environmental insults or genetic abnormalities that eliminate/reduce CD4⁺CD25⁺ T cells can be a possible cause of autoimmune disease (71). Indeed, elimination of activated T cells for a limited period as an immunosuppressive treatment elicited autoimmune diseases similar to those shown in this report (44, 67, 72). Further study of the molecular basis of CD25⁺ T cell-mediated immunoregulation and the genetic basis determining autoimmune phenotype will contribute to our understanding of the pathogenetic mechanism of various autoimmune diseases.

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