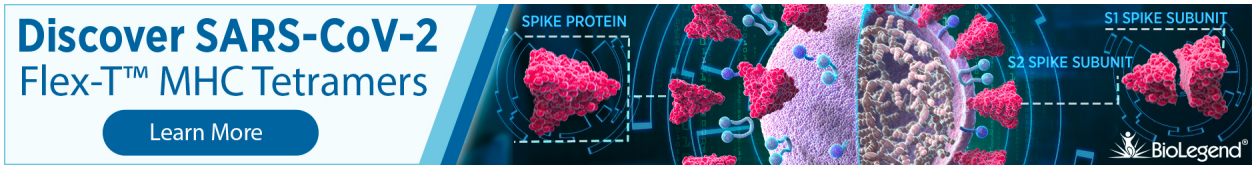

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Induction of Tumor Immunity by Removing $CD25^+CD4^+$ T Cells: A Common Basis Between Tumor Immunity and Autoimmunity[1]

Jun Shimizu,* Sayuri Yamazaki,† and Shimon Sakaguchi[2]*†

This study shows that removal of a T cell subpopulation can evoke effective tumor immunity in otherwise nonresponding animals. Elimination of CD25-expressing T cells, which constitute 5–10% of peripheral $CD4^+$ T cells in normal naive mice, elicited potent immune responses to syngeneic tumors in vivo and eradicated them. The responses were mediated by tumor-specific $CD8^+$ CTLs and tumor-nonspecific $CD4^-8^-$ cytotoxic cells akin to NK cells. Furthermore, in vitro culture of $CD25^+4^+$ T cell-depleted splenic cell suspensions prepared from tumor-unsensitized normal mice led to spontaneous generation of similar $CD4^-8^-$ cytotoxic cells capable of killing a broad spectrum of tumors; reconstitution of $CD25^+4^+$ T cells inhibited the generation. In this culture, self-reactive $CD25^-4^+$ T cells responding to self peptides/class II MHC complexes on APCs spontaneously proliferated upon removal of $CD25^+4^+$ T cells, secreting large amounts of IL-2. The IL-2 thus produced appeared to be responsible for the generation of $CD4^-8^-$ NK cells as lymphokine-activated killer cells, because direct addition of an equivalent amount of IL-2 to the culture of $CD4^-8^-$ cells generated similar lymphokine-activated killer/NK cells, whereas coculture of normal $CD4^-8^-$ cells with $CD25^-4^+$ T cells from IL-2-deficient mice did not. Thus, removal of immunoregulatory $CD25^+4^+$ T cells can abrogate immunological unresponsiveness to syngeneic tumors in vivo and in vitro, leading to spontaneous development of tumor-specific effector cells as well as tumor-nonspecific ones. This novel way of evoking tumor immunity would help to devise effective immunotherapy for cancer in humans. ***The Journal of Immunology*****, 1999, 163: 5211–5218.**

There are accumulating demonstrations that many tumor Ags recognized by autologous CTLs are antigenically normal self-constituents (reviewed in ref. 1, 2). Furthermore, immunotherapy of cancer by vaccination with tumor Ags or transfusion of ex vivo propagated cytotoxic lymphocytes often leads to the appearance of autoimmunity because of antigenic cross-reactions between tumor Ags and normal tissue Ags (3–5). These findings indicate that a part of tumor immunity can be an autoimmunity, and suggest the possibility that the mechanisms maintaining immunologic tolerance to self-constituents may impede generation of effective immunity against autologous tumor cells. If it is the case, a particular way of breaking immunologic self-tolerance may evoke effective tumor immunity in otherwise nonresponding individuals.

Accumulating evidence indicates that not only clonal deletion or anergy, but also T cell-mediated control of self-reactive T cells contributes to the maintenance of immunologic self-tolerance (reviewed in Refs. 6 and 7). Indeed, depletion of a $CD4^+$ T cell subpopulation defined by an expression level of a particular cell surface molecule leads to spontaneous development of various autoimmune diseases in otherwise normal rodents. For example, removal of $CD5^{high}4^+$, $CD45RB/C^{low}4^+$, or $CD25^+4^+$ T cells, without deliberate immunization with self Ags, produced in mice or rats autoimmune gastritis, thyroiditis, insulin-dependent diabetes, and other autoimmune diseases immunopathologically similar to human counterparts; reconstitution of the eliminated population prevented the autoimmunities (8–15). This immunoregulatory activity of $CD5^{high}$ or $CD45RB/C^{low}$ $CD4^+$ T cells can be attributed to $CD25^+4^+$ T cells included in the $CD5^{high}$ or $CD45RB/C^{low}$ 4^+ T cell population (13, 16, 17). Recent studies have shown that the $CD25^+4^+$ population in normal naive mice is nonproliferative (anergic) to antigenic stimulation in vitro, and, upon stimulation, potently suppresses the activation/proliferation of other $CD4^+$ or $CD8^+$ T cells in an Ag-nonspecific manner through cell to cell interactions on APCs (16, 17). Furthermore, removal of $CD25^+4^+$ T cells not only elicits autoimmunity, but also enhances immune responses to non-self Ags such as allogeneic tissue grafts (13). These in vivo and in vitro findings make it likely that removal of the $CD25^+4^+$ immunoregulatory T cells may also evoke effective immune responses to autologous tumor cells in vivo and in vitro by activating effector cells of tumor immunity (18, 19).

In this study, we demonstrate that elimination of $CD25^+$ T cells, which constitute 5–10% of peripheral $CD4^+$ T cells in normal mice and humans (13, 20–23), indeed elicits potent tumor-specific immune responses to syngeneic tumors in vivo and eradicates them. Furthermore, in vitro culture of $CD25^+4^+$ T cell-depleted splenic cell suspensions prepared from tumor-unsensitized normal mice leads to spontaneous generation of active NK cells capable of killing a broad spectrum of tumors. Our results indicate that removal of immunoregulatory $CD25^+4^+$ T cells can break immunological unresponsiveness to autologous tumors in vivo and in vitro, leading to spontaneous development of tumor-specific effector cells as well as tumor-nonspecific ones in otherwise nonresponding individuals. This finding is instrumental in devising effective immunotherapy for cancer in humans.

*Department of Immunopathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; and †Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

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[2] Address correspondence and reprint requests to Dr. Shimon Sakaguchi, Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Shogoin Kawahara-cho 53, Sakyo-ku, Kyoto 606-8507, Japan. E-mail address: shimon@frontier.kyoto-u.ac.jp

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Materials and Methods

Mice

BALB/c, BALB/c-*nu/nu*, C57BL/6 (B6), and C3H/He mice of 6 wk of age were purchased from Japan SLC (Shizuoka, Japan), maintained in our animal facility, and treated in accordance with the institutional guidelines for animal care. IL-2, IL-4, or IFN-γ gene knockout mice (IL-2$^{-/-}$, IL-4$^{-/-}$, and IFN-γ$^{-/-}$ mice, respectively (3)) were purchased from The Jackson Laboratory (Bar Harbor, ME) (24–26). B6-beige (*bg/bg*)[3] mice were purchased from Clea Japan (Tokyo, Japan).

Tumor cells

RL♂1 (BALB/c-derived radiation leukemia) (27–30), B16 (B6-derived melanoma) (31), X5563 (C3H-derived plasmacytoma) (32), and P815 (DBA/2-derived mastocytoma) were gifts from Drs. E. Nakayama (Okayama University, Okayama, Japan), H. Fujiwara (Osaka University, Osaka, Japan), or T. Takahashi (Aichi Cancer Center, Nagoya, Japan).

Monoclonal Abs

FITC- or PE-labeled anti-CD25 (7D4) (33), anti-CD4 (GK1.5), anti-NK1.1 (2B4), anti-α/β-TCR (H57-597), anti-γ/δ-TCR (GL3), anti-CD24 (M1/69), or anti-CD16 Ab (2.4G2) was purchased from PharMingen (San Diego, CA). Hybridomas secreting anti-CD4 (RL172.4), anti-CD8 (3.155), or anti-CD25 (PC61) Ab were purchased from American Type Culture Collection (Manassas, VA) . For three-color staining, R-PE-Cy5-conjugated streptavidin (Dako/Japan, Kyoto, Japan) was used as the secondary reagent for biotinylated Abs. For in vivo injection of anti-CD25 mAb, PC61 (rat IgG1) (34) was made ascitic form in SCID mice and purified from the ascites by 40% ammonium sulfate precipitation twice. Purified rat IgG for control injections was purchased from Sigma (St. Louis, MO).

Preparation of T cell subpopulations

Spleen cells were depleted of CD4$^+$, CD8$^+$, or CD25$^+$ cells, as previously described (13, 14). Briefly, spleen cells were incubated at 5×10^6/ml for 45 min at 37°C with the culture supernatant of the hybridoma cells secreting anti-CD4 (RL172.4), anti-CD8 (3.155), or anti-CD25 (7D4) Ab, or the mixture of the supernatants, and rabbit complement (C) 1/10 diluted at the final concentration (Cedarlane Laboratories, Ontario, Canada). The treatment was repeated twice. To purify CD4$^+$ T cells, anti-CD8 plus C-treated cells were removed of B cells and adherent cells by panning on anti-mouse Ig-coated dishes, as previously described (13, 14). To purify CD25$^+$CD4$^+$ T cells, BALB/c spleen cells stained with PE-labeled anti-CD4 (H129.19) (PharMingen) and FITC-labeled anti-CD25 (7D4) were sorted by an EPICS ELITE cell sorter (Coulter Electronics, Miami, FL) with >90% purity, as previously described (16).

In vitro culture of lymphocytes and cytotoxicity assay

Spleen cells (5×10^6 cells in 2 ml) were cultured with or without 5×10^5 mitomycin C-treated tumor cells in DMEM containing 10% FCS in 24-well plates (Costar, Cambridge, MA). Five or seven days later, viable cells were harvested and used as effectors in the ^{51}Cr release cytotoxicity assay, in which target cells (1×10^6) were labeled with 3.7 MBq of sodium chromate (DuPont/NEN, Wilmington, DE) for 60 min at 37°C, washed three times, and incubated at 1×10^4 cells/well with various numbers of effector cells in 96-well round-bottom plates (Costar) for 4 or 6 h at 37°C. The mean percentage specific lysis of triplicate culture was calculated from the radioactivity of the supernatants: percent specific lysis = 100 × [(cpm experimental release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)]. Spontaneous release from the target cells incubated in medium alone was always <20% of the maximum release obtained by adding 1 N HCl to the labeled target cells.

To examine the activity of anti-CD4 (RL172.4), anti-CD8 (3.155), anti-α/β-TCR (H57-597), or anti-γ/δ-TCR Ab (GL3) to block the tumor killing mediated by cytotoxic cells, these mAbs as culture supernatants were added to the 4-h ^{51}Cr release assay described above.

In vitro measurement of cell proliferation and IL-2 activity

Spleen cells (5×10^5/well in 96-well flat-bottom plates (Costar)) were cultured for 3 to 10 days, and pulsed with [^{3}H]thymidine (37 kBq/well) (DuPont/NEN) for the last 6 h. IL-2 activity in the culture supernatants was assessed with the CTLL-2 cell line (35). Murine rIL-2 (3.89×10^6 U/mg) was a gift from Shionogi (Osaka, Japan).

To examine blocking of in vitro proliferation with anti-class II MHC Ab, graded concentrations of pan anti-class II MHC mAb CA4 (36) (a gift from Dr. O. Kanagawa, Washington University, St. Louis, MO) were added to the culture of CD25$^-$ spleen cells.

Histological and serological assessment of autoimmune disease

Tissues and organs (thyroid, lung, pancreas, stomach, adrenal gland, kidney, ovaries, or testes) were fixed in 10% Formalin and processed for hematoxylin and eosin staining. Gastritis was graded 0 to 2$^+$, depending on macroscopic and histological severity: 0 = the gastric mucosa was histologically intact; 1$^+$ = mild gastritis with histologically evident destruction of parietal cells and cellular infiltration of the gastric mucosa; 2$^+$ = severe destruction of the gastric mucosa accompanying the formation of giant rugae due to compensatory hyperplasia of mucous-secreting cells (see Ref. 8 for the giant rugae) (13, 14). Thyroiditis was also graded 0 to 2$^+$: 0 = the thyroid gland was histologically intact; 1$^+$ = mild thyroiditis with histologically evident destruction of the thyroid follicles and interstitial infiltration of inflammatory cells; 2$^+$ = severe destruction of the thyroid gland accompanying goiter formation (see Ref. 8 for the goiter) (13, 14). The ELISA (using alkaline phosphatase-conjugated secondary Ab and *p*-nitrophenyl disodium hexahydrate as the substrate) for detecting autoantibodies specific for the gastric parietal cell Ags or mouse thyroglobulins was previously described (13, 14).

Results

In vivo induction of effective tumor immunity by removing CD25$^+$4$^+$ T cells

To determine whether elimination of CD25$^+$4$^+$ cells is able to elicit primary immune responses to syngeneic tumors, we transferred to BALB/c athymic nude mice BALB/c splenic cell suspensions depleted of CD25$^+$ cells (hereafter designated to be CD25$^-$ cells (see the *insets* in Fig. 1*A* for cytofluorometric profiles)), and then, on the same day, s.c. transplanted BALB/c-derived RL♂1 leukemia cells (Fig. 1*A*). In the majority of mice, the tumors first grew and then regressed within 1 mo, allowing the hosts to survive a long-term (>80 days), whereas all the nude mice transferred with nondepleted spleen cells or the mixture of CD25$^-$ cells and CD4$^+$ T cells (10% of which were CD25$^+$ cells) in the ratio 3:1 died of tumor progression within 40 days (Fig. 1*B*). Upon rechallenge with larger doses of RL♂1, the CD25$^-$ cell-transferred nude mice rejected the tumor inocula more rapidly and vigorously than the primary rejection, indicating that they had become immune to the tumor (Fig. 1*A*, *middle panel*).

In the nude mice having rejected RL♂1, in vitro stimulation of their spleen cells with RL♂1 revealed two types of cytotoxic cells (type I and II in Fig. 2, *A* and *B*). The stimulated spleen cells from some mice specifically killed RL♂1, but not allogeneic tumor cells such as B6-derived B16 melanoma cells (type I), while similarly stimulated spleen cells from other mice killed not only RL♂1 but also B16 (type II). The former type required in vitro stimulation with RL♂1 to become detectable, whereas the latter did not. Furthermore, the former RL♂1-specific killing was mediated by CD8$^+$ CTLs: elimination of CD8$^+$ cells completely abolished the killing activity, but that of CD4$^+$ cells did not (Fig. 2*C*). On the other hand, the latter promiscuous killing activity appeared to be mainly mediated by CD4$^-$8$^-$ cells because elimination of both CD4$^+$ cells and CD8$^+$ cells did not abrogate the activity (Fig. 2*C*). In these nude mice having developed CD4$^-$8$^-$ killer cells, the retention of the killer activity after eliminating CD8$^+$ cells from the cultured spleen cells does not necessarily mean that they did not harbor CD8$^+$ CTLs, because tumor-specific CD8$^+$ CTL clones could be easily prepared in vitro from their spleens (29, and our unpublished data).

These results taken together indicate that elimination of CD25$^+$ T cells alone, without prior deliberate immunizations, can evoke potent immune responses to tumor cells in vivo by generating two

[3] Abbreviations used in this paper: bg, beige; C, complement; LAK, lymphokine-activated killer.

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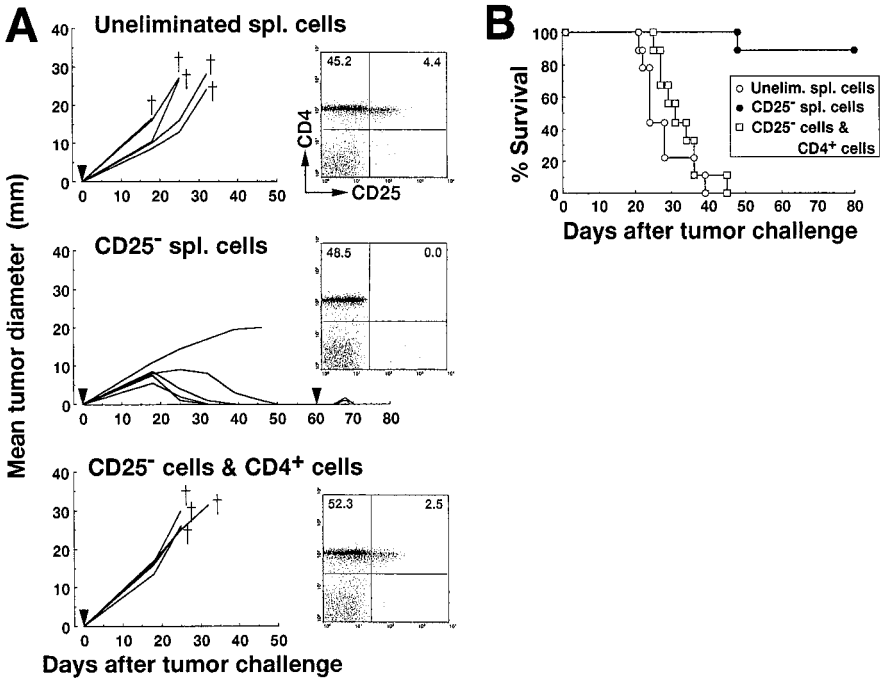

FIGURE 1. Eradication of tumor transplants in nude mice by transferring $CD25^+$ cell-depleted splenic cell ($CD25^-$ cell) suspensions. *A*, Tumor growth was monitored for individual BALB/c nude mice s.c. transplanted with 1.5×10^5 RL♂1 cells (arrow) immediately after i.v. transfer of 3×10^7 untreated spleen cells (*upper panel*), 3×10^7 $CD25^-$ spleen cells (*middle panel*), or mixtures of $CD25^-$ spleen cells (3×10^7) and $CD4^+$ spleen cells (1×10^7) (*lower panel*). The $CD25^-$ spleen cell-transferred nude mice having rejected the tumors were rechallenged on day 60 (arrow) with 10 times larger dose (1.5×10^6) of RL♂1 cells and monitored for tumor growth (*middle panel*). *Insets* show staining of each cell inoculum with FITC-labeled anti-CD4 Ab (ordinate) and PE-labeled anti-CD25 Ab (abscissa) (logarithmic scale). *B*, Percentage of mice surviving in each group ($n = 9$) on various days after tumor transplantation (total of three independent experiments).

types, at least, of effector cells, which are different in the cell surface phenotype, the specificity of tumor killing, and the requirement of Ag stimulation for their development.

In vivo induction of tumor immunity by administration of anti-CD25 mAb

To determine whether transient elimination of $CD25^+$ cells from normal mice can also elicit immunity to syngeneic tumors, we administered anti-CD25 mAb (PC61) twice (on 4 and 2 days before tumor inoculation) to BALB/c or B6 mice, and subsequently inoculated RL♂1 or B16 tumor cells, respectively (Fig. 3). In the majority (>90%) of PC61-treated BALB/c mice, the s.c. inoculated RL♂1 first grew and then regressed within 1 mo, whereas all of the BALB/c mice treated with normal rat Ig as a control died of tumor progression within 1 mo. Likewise, administration of PC61 to B6 mice significantly suppressed the growth of B16 when compared with control B6 mice treated with normal rat IgG, allowing the former to survive a longer term (>60 days) compared with the latter (<40 days). This PC61 treatment reduced the number of peripheral $CD25^+4^+$ T cells to a quarter of control mice for nearly 1 mo (our manuscript in preparation). Furthermore, this in vitro anti-CD25 treatment evoked rejection of various other syngeneic tumors in various other strains (S. Onizuka, et al., manuscript in preparation).

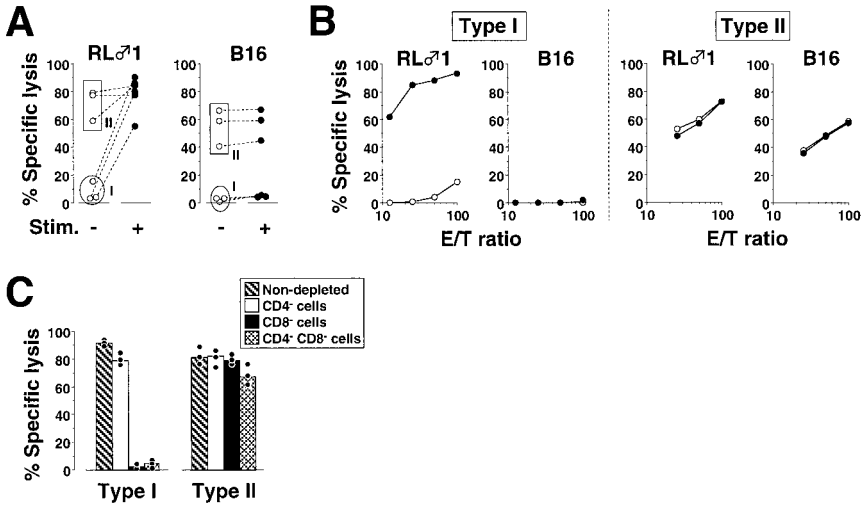

FIGURE 2. Two types of tumor-killing activity in the nude mice having rejected tumors after transfer of $CD25^-$ cells. *A*, Spleen cells from individual nude mice ($n = 6$) that had received $CD25^-$ cells and rejected RL♂1 (as shown in Fig. 1) were cultured for 7 days with or without RL♂1 (filled or open circles, respectively) and assessed for killing activity against RL♂1 or B16. The group of mice enclosed with circles or rectangles (designated I or II, respectively) in the *right* and *left figures* are the same. Dotted lines connect cytotoxic activities of RL♂1-stimulated or nonstimulated cultures from the same mice. *B*, Activity to kill RL♂1 or B16 in each representative mouse from the group enclosed with circles (type I) or rectangles (type II) in *A* is shown. Spleen cells from each mouse were cultured for 7 days with (●) or without RL♂1 (○). *C*, CD4/CD8 phenotype of cytotoxic cells. Individual mice from group I or II in *A* were assessed for cytotoxic activity at E:T ratio of 100 after depleting $CD4^+$ cells, $CD8^+$ cells, or both (designated $CD4^-$ cells, $CD8^-$ cells, or $CD4^-8^-$ cells, respectively) from the spleen cells cultured for 7 days with RL♂1, as shown in *B*.

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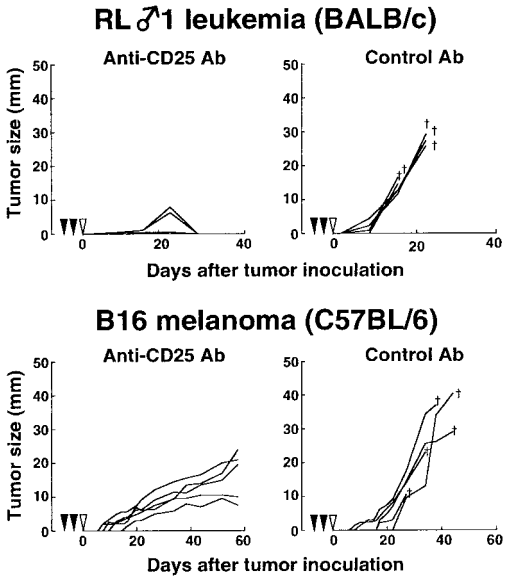

FIGURE 3. Induction of tumor immunity by administering anti-CD25 mAb to normal mice. Eight-week-old BALB/c or B6 mice were i.v. injected with 1 mg each of purified PC61 on 4 and 2 days (filled arrows) before s.c. inoculation of 1×10^5 RL♂1 or B16 cells (open arrow), and tumor growth was monitored for individual mice (five mice per group).

In vitro induction of cytotoxic cells from normal spleen cells by removing CD25$^+$4$^+$ T cells

To examine whether similar cytotoxic lymphocytes as detected in vivo (Figs. 1 and 2) can also be generated in vitro by simply eliminating CD25$^+$4$^+$ T cells, CD25$^-$ splenic cell suspensions prepared from tumor-unsensitized normal BALB/c mice were cultured for several days with or without RL♂1 cells and assessed for killing activity (Fig. 4). Cells harvested on day 5 from the RL♂1-stimulated culture indeed exhibited significant killing activity against RL♂1 cells (Fig. 4*A*). Interestingly, when CD25$^-$ spleen cells were cultured for 7 days, potent cytotoxic activity was detected even in the cultures not stimulated with RL♂1. By contrast, similarly cultured nondepleted spleen cells or the mixtures of CD25$^-$ cells and purified CD25$^+$CD4$^+$ T cells (in the ratio 25:1 (see the *inset* in Fig. 4*A*)) showed no cytotoxicity. Furthermore, the cells harvested from the 7-day culture killed not only RL♂1, which is NK sensitive (27), but also NK-resistant tumor cells (such as P815) and allogeneic ones (such as X5563 and B16) (Fig. 4*B*). This promiscuous killing activity could not be toward calf serum proteins adsorbed to the target cells during ^{51}Cr labeling, because tumor cells passaged in vivo and ^{51}Cr labeled in the medium containing normal mouse serum, instead of FCS, were lysed as well by CD25$^-$ cell-derived cytotoxic cells (data not shown). These findings were not confined to the BALB/c strain: CD25$^-$ cells from other strains (including B6 and C3H), when cultured for 1 wk without tumor cell stimulation, also gave rise to cytotoxic cells promiscuously killing a broad spectrum of syngeneic or allogeneic tumor cells (Fig. 4*B*).

Characterization of cytotoxic cells generated in vitro upon removal of CD25$^+$4$^+$ T cells

As shown in Fig. 4, there was no significant difference in the killing activity or the killing spectrum of target tumor cells between CD25$^-$ cells in vitro stimulated with tumor cells for 7 days and those nonstimulated (see the *inset* in Fig. 4*A*). We therefore analyzed in the following experiments the mechanism by which killer cells were generated from CD25$^-$ cells cultured for 1 wk without in vitro antigenic stimulation.

Fig. 5*A* shows that depletion of either CD4$^+$ cells or CD8$^+$ cells, or both, from the cell suspensions harvested from 1-wk culture of CD25$^-$ BALB/c spleen cells did not abrogate the cytotoxic activity against RL♂1 or B16. The result indicates that the promiscuous killing activity was mainly in the CD4$^-$8$^-$ population, although slight reduction of the killing activity by depleting CD8$^+$ cells suggests that tumor-nonspecific CD8$^+$ cytotoxic cells might also be generated in the culture.

We then examined whether the activity could be blocked by adding anti-CD4, CD8, α/β-TCR, or γ/δ-TCR Ab to the ^{51}Cr release assay (Fig. 5*B*). None of the Abs blocked the activity, in contrast to significant degrees of blocking exerted by anti-CD8 or anti-α/β-TCR Ab on allospecific CD8$^+$ CTLs generated in vitro by stimulating normal B6 spleen cells with BALB/c-derived RL♂1.

The lymphocytes harvested from 1-wk culture of B cell-depleted CD25$^-$ spleen cells prepared from B6 mice were composed of CD4$^+$ T cells (~60%), CD8$^+$ T cells (~30%), and CD4$^-$8$^-$ cells (~10%) (Fig. 5*C*). The CD4$^-$8$^-$ population contained α/β-TCR$^+$ cells (~50%), γ/δ-TCR$^+$ cells (~20%), and NK1.1$^+$ cells (~30%) (Fig. 5*C*). The population was mostly (>80%) B220$^+$, and contained CD122 (IL-2R β-chain)$^+$ cells (~70%), CD16$^+$

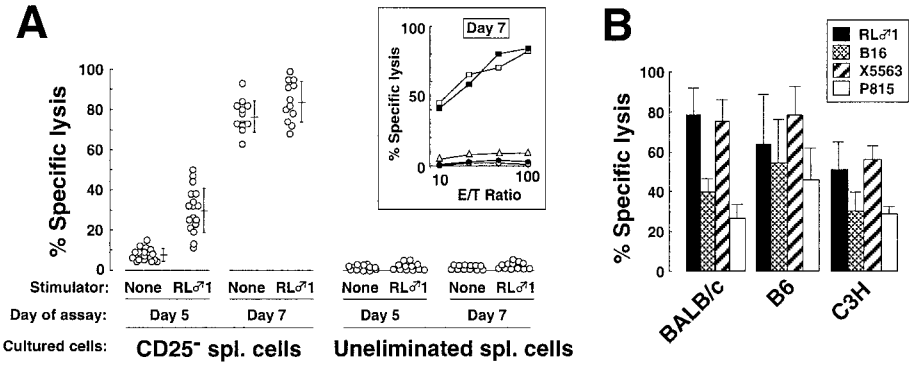

FIGURE 4. In vitro induction of cytotoxic lymphocytes by eliminating CD25$^+$4$^+$ T cells. *A*, CD25$^-$ or uneliminated spleen cells prepared from normal BALB/c mice were cultured with or without RL♂1 for 5 or 7 days and assessed for activity to kill RL♂1 at E:T ratio of 100. Each circle represents the activity of a single culture. Vertical bars are SDs of the mean. The *inset* shows a representative cytotoxic activity of CD25$^-$ (square) or uneliminated (circle) spleen cells, or CD25$^-$ cells mixed with purified CD25$^+$CD4$^+$ T cells (triangle), cultured for 7 days with (filled symbols) or without RL♂1 (open symbols). *B*, CD25$^-$ spleen cells from various strains were cultured for 7 days and assessed at E:T ratio of 100 for activity to kill various syngeneic or allogeneic tumor cells. Average activity of four independent experiments and SDs is shown.

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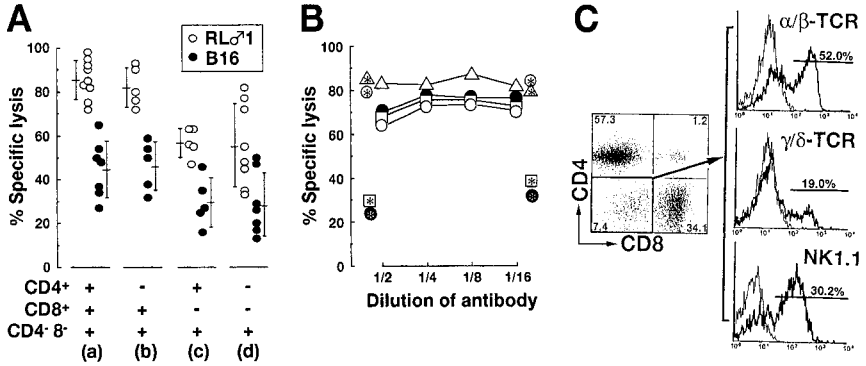

FIGURE 5. In vitro generation of TCR$^-$ CD4$^-$8$^-$ NK cells. *A*, BALB/c CD25$^-$ cells were cultured for 7 days without tumor stimulation and assessed for activity to kill RL♂1 (○) or B16 (●) after depleting CD4$^+$ cells, CD8$^+$ cells, or both, by treating cells with specific Ab and C: *a*, C treatment alone; *b*, anti-CD4 and C; *c*, anti-CD8 and C; *d*, anti-CD4, anti-CD8, and C. The results of five independent experiments are shown. *B*, Anti-CD4 (○), anti-CD8 (●), anti-α/β-TCR (□), or anti-γ/δ-TCR Ab (△) was added at various concentrations to the cytotoxicity assay with BALB/c CD25$^-$ cells cultured for 7 days and RL♂1 cells as the targets. As a positive control of blocking by anti-CD8 or anti-α/β-TCR Ab, Abs were added to the killing of RL♂1 cells by B6 spleen cells stimulated for 7 days with allogeneic RL♂1 cells (asterisks). A representative result of three independent experiments is shown. *C*, CD25$^-$ spleen cells from B6 mice were depleted of B cells, cultured for 7 days with APCs, and stained with FITC anti-CD4, PE anti-CD8, and biotinylated anti-α/β-TCR, anti-γ/δ-TCR, or anti-NK1.1 Ab with Cy5-streptavidin as the secondary reagent. Expression of α/β-TCR, γ/δ-TCR, or NK1.1 on CD4$^-$8$^-$ cells in the *left figure* is shown as histograms (abscissa is staining intensity in logarithmic scale; ordinate is the number of cells as arbitrary units). A representative staining of three independent experiments is shown.

cells (~5%), and CD24 (heat stable antigen (HSA))$^+$ cells (~5%) (data not shown).

Furthermore, culturing CD25$^-$ splenic cells prepared from B6-*bg/bg* mice, which are genetically defective in NK cell-killing capacity (37), failed to generate the promiscuous cytotoxic activity in the 1-wk culture, in contrast to intact generation of allo-specific CTL activity by B6-*bg/bg* mouse spleen cells stimulated with allogeneic RL♂1 cells (Fig. 6).

These functional and phenotypic studies collectively indicate that the promiscuous cytotoxic activity generated by culturing CD25$^-$ cells in vitro may be mainly in the TCR$^-$ CD4$^-$8$^-$ population, presumably in the NK1.1$^+$ NK cell population (38, 39).

The mechanism of spontaneous in vitro generation of CD4$^-$8$^-$ cytotoxic cells

In the course of this in vitro generation of cytotoxic cells, CD25$^-$ cells, especially CD25$^-$4$^+$ T cells, showed spontaneous and vigorous proliferation and secreted large amounts (~500 U/ml) of IL-2, whereas noneliminated spleen cells did not (Fig. 7, *A* and *B*). This response was not against xenogeneic proteins in the culture medium, because the use of normal BALB/c serum, instead of FCS, did not alter the response (data not shown). On the other

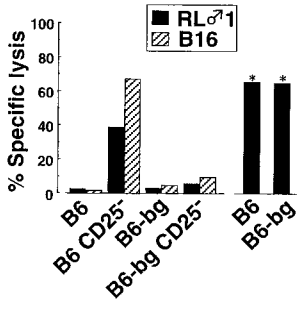

FIGURE 6. Absence of promiscuous killing activity of cultured CD25$^-$ cells in beige mice. CD25$^-$ or uneliminated spleen cells from B6-bg or B6 mice were cultured for 7 days, and assessed for killing activity against RL♂1 or B16 cells. Allospecific killing activity (asterisks) of uneliminated B6-bg or B6 spleen cells stimulated for 7 days with RL♂1 was also assessed against RL♂1 cells as the targets.

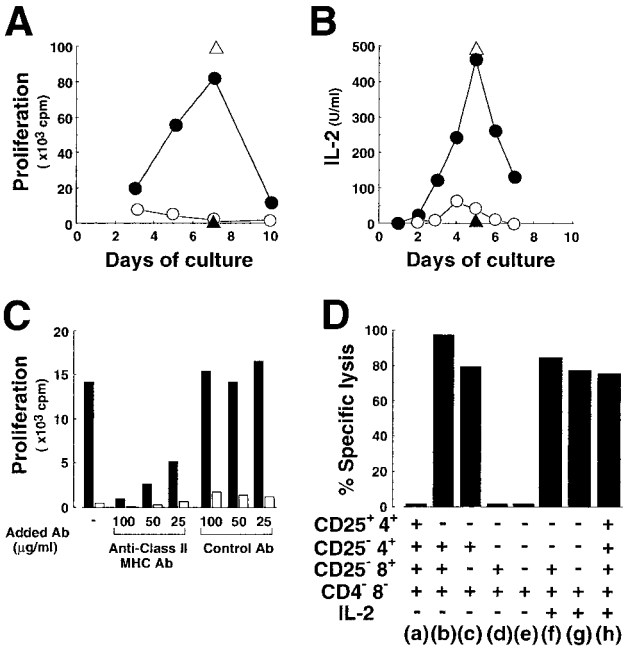

FIGURE 7. Requirement of CD25$^-$4$^+$ T cells or IL-2 for induction of CD4$^-$8$^-$ NK cells. *A* and *B*, CD25$^-$ or uneliminated BALB/c spleen cells (filled or open circle, respectively) were cultured for various days and assessed for proliferative activity (*A*) and IL-2 activity in supernatants (*B*). These activities in the culture of CD25$^-$ cells were also assessed after depleting CD4$^+$ cells or CD8$^+$ cells (filled or open triangle, respectively) on day 7 or 5 at the peak of proliferation or IL-2 secretion, respectively. *C*, Pan anti-class II MHC mAb (CA4) or control rat IgG was added at graded concentrations to 1-wk culture of CD25$^-$ or uneliminated BALB/c spleen cells (filled or open columns, respectively) and assessed for the effect on proliferation. *D*, BALB/c spleen cells were depleted of a particular cell population(s) by treatment with specific Ab and C, then cultured for 7 days and assessed for activity to kill RL♂1 at E:T ratio of 100. Treatments of spleen cells before culture were as follows: a, C treatment alone; b, anti-CD25 and C; c, anti-CD25, anti-CD8, and C; d, anti-CD25, anti-CD4, and C; e, anti-CD4, anti-CD8, and C. IL-2 (1000 U/ml) was added to the culture of spleen cells treated with: f, anti-CD25, anti-CD4 Ab plus C; g, anti-CD4, anti-CD8 Ab plus C; or h, C alone. A representative result of three independent experiments is shown.

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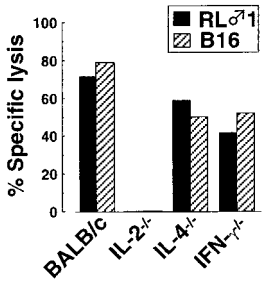

FIGURE 8. Inability of CD25$^-$4$^+$ T cells from IL-2-deficient mice to generate LAK/NK-like cells from normal CD4$^-$8$^-$ cells. CD25$^-$4$^+$ spleen cells from IL-2$^{-/-}$, IL-4$^{-/-}$, IFN-$\gamma^{-/-}$, or control BALB/c mice were mixed with an equal number of CD4$^-$8$^-$ spleen cells prepared from normal BALB/c mice, cultured for 7 days, and assessed for the activity to kill RL♂1 or B16 cells. A representative result of three independent experiments is shown.

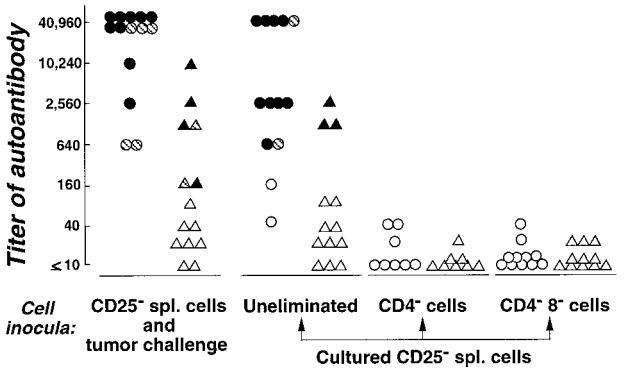

FIGURE 9. Development of autoimmunity in nude mice after inoculation of CD25$^-$ cells. BALB/c nude mice received CD25$^-$ BALB/c spleen cells and inoculation of RL♂1 cells, as shown in Fig. 1. Other groups of mice received uneliminated, CD4$^+$ cell-depleted (i.e., CD4$^-$), or CD4$^+$ cell- and CD8$^+$ cell-depleted (i.e., CD4$^-$8$^-$) cell suspensions (1×10^7 cells) prepared from CD25$^-$ cells cultured for 7 days. Three months after transfer, mice were assessed for histological development of gastritis (circle) and/or thyroiditis (triangle), and titers of autoantibodies specific for gastric parietal cells or thyroglobulins. Filled symbols mean grade 2 (severe) gastritis or thyroiditis; shaded symbols, grade 1 (mild) gastritis or thyroiditis; open symbols, intact gastric mucosa or thyroid glands.

hand, the proliferative response was blocked by adding anti-class II MHC Ab to the culture, indicating that the proliferating CD4$^+$ T cells were presumably responding to self peptides/class II MHC complexes on APCs (Fig. 7*C*).

The in vitro generation of CD4$^-$8$^-$ cytotoxic cells appeared to require the presence of these self-reactive CD25$^-$4$^+$ T cells, because depletion of CD4$^+$ T cells from the CD25$^-$ population before culture abrogated the generation (Fig. 7*D*, treatment d), whereas removal of CD8$^+$ T cells did not (Fig. 7*D*, treatment c). On the other hand, addition of IL-2 (1000 U/ml) to the culture of such CD4$^+$ cell-depleted CD25$^-$ cells (including CD25$^-$8$^+$ cells and CD4$^-$8$^-$ cells) or CD4$^-$8$^-$ cells (Fig. 7*D*, treatment f and g, respectively) generated marked cytotoxic activity, which was comparable with the activity generated by culturing uneliminated spleen cells with the same dose of IL-2 (Fig. 7*D*, treatment h).

To further examine the key role of IL-2 secreted by CD4$^+$ T cells in generating CD4$^-$8$^-$ cytotoxic cells, CD25$^-$4$^+$ T cells prepared from IL-2$^{-/-}$, IL-4$^{-/-}$, or IFN-$\gamma^{-/-}$ mice on BALB/c background were mixed with an equal number of CD4$^-$8$^-$ cells from normal BALB/c mice and then assessed for cytotoxic activity after 1-wk culture (Fig. 8). CD25$^-$4$^+$ T cells from IL-2$^{-/-}$ mice failed to generate the activity, whereas those from IL-4$^{-/-}$ or IFN-$\gamma^{-/-}$ mice generated nearly equivalent levels of killer activity as with CD25$^-$4$^+$ T cells from normal BALB/c mice.

Thus, elimination of CD25$^+$ cells leads to spontaneous activation/proliferation of CD25$^-$4$^+$ self-reactive T cells; and large amounts of IL-2 produced by them may be responsible for the generation of CD4$^-$8$^-$ cytotoxic cells, which are similar to LAK cells (or IL-2-activated NK cells (Fig. 7*D*, treatment h)) in their promiscuous cytotoxic activity and the critical dependency of their generation upon IL-2 (38–42).

T cell subpopulations required for autoimmunity

The CD25$^-$ cell-transferred nude mice that had rejected tumor transplants (as shown in Fig. 1*A*) developed histologically evident autoimmune diseases, including gastritis (100% incidence, n = 14), thyroiditis (42.9%), and oophoritis (64.3%) accompanying respective autoantibodies (such as those specific for gastric parietal cells, thyroglobulins, or oocytes (8, 13, 14)) (Fig. 9). Transfer of in vitro cultured CD25$^-$ cells also produced similar autoimmune diseases at similar incidences, whereas transfer of CD4$^-$8$^+$ or CD4$^-$8$^-$ cells, or both, prepared by eliminating CD4$^+$ cells from the cultured CD25$^-$ cells, did not. Thus, CD4$^+$ T cells were indispensable for producing these organ-specific autoimmune diseases; and CD8$^+$ or CD4$^-$8$^-$ cells alone were unable to cause the autoimmunity.

In contrast to these CD25$^-$ cell-inoculated BALB/c nude mice, normal BALB/c or B6 mice treated with PC61 (Fig. 3) did not develop histologically or serologically evident autoimmunity (S.Y., J.S., S.S., unpublished data).

Discussion

The main conclusion in this study is that removal of immunoregulatory CD25$^+$4$^+$ T cells can break immunological unresponsiveness to syngeneic tumor cells in vivo and in vitro. The result indicates that tumor immunity and autoimmunity share a common regulatory basis, because removal of the population also elicits autoimmunity in vivo (Fig. 9) and in vitro (Fig. 7*A*).

It has been postulated that one of the elements impeding effective tumor immunity in tumor-bearing hosts may be concomitant development of a T cell population suppressing the generation or the action of tumor-killing effector cells (43–45). Although some of such suppressor T cells were shown to be CD4$^+$, they have been elusive from further characterization because of the lack of reliable markers specific for them (46). Our results indicate that these suppressive T cells, at least in part, can be CD25$^+$4$^+$ T cells. The CD25$^+$4$^+$ immunoregulatory T cells, however, bear several characteristics distinct from the suppressor T cells to date reported in tumor immunity (43, 44). First, removal of CD25$^+$4$^+$ T cells before tumor implantation was effective in evoking specific tumor immunity in vivo (Figs. 1 and 3) and in generating cytotoxic cells in vitro (Fig. 4). Second, their removal elicited not only tumor immunity, but also various autoimmunities in vivo (Fig. 9) and self-reactivity in vitro (Fig. 7). The removal also enhanced immune responses to allografts or xenogeneic proteins (13). Third, they are continuously produced by the normal thymus and already functional in the thymus, as previously shown (14, 16, 17); and their depletion from thymocyte suspensions indeed generated CD4$^-$8$^-$ NK-like killer cells in vitro (our unpublished data) and autoimmunity in vivo (17). Furthermore, these CD25$^+$4$^+$ T cells/thymocytes are naturally anergic (nonproliferative) to antigenic stimulation in vitro, and, upon stimulation, potently suppress the activation/proliferation of other CD4$^+$ or CD8$^+$ T cells in an Ag-nonspecific manner through cell to cell interactions on APCs (16,

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17). These findings when taken together indicate that the $CD25^+4^+$ immunoregulatory T cells present in normal naive mice may be engaged in continuously up-regulating the activation thresholds of other T cells, thereby impeding effective generation of tumor immunity while inhibiting autoimmunity (16–19). To further elucidate how they suppress tumor immunity (and autoimmunity), the natural ligands physiologically stimulating them must be characterized. Judging from their activated phenotype (e.g., being $CD25^+$, $CD45RB^{low}$, $CD44^{high}$, and $CD5^{high}$ (8, 9, 11, 13, 16, 17)) in normal naive mice, the $CD25^+4^+$ immunoregulatory T cells might be inherently reactive with, and continuously stimulated by self Ags in the normal internal environment, although they themselves may be harmless because of their anergic property (16, 17).

Removal of $CD25^+4^+$ immunoregulatory cells led to the activation of not only tumor-specific $CD8^+$ CTLs (and presumably tumor-specific $CD4^+$ Th cells), but also tumor-nonspecific $CD4^-8^-$ effector cells in vivo, and predominantly the latter in vitro. It also led to in vitro vigorous proliferation of $CD4^+$ self-reactive T cells presumably responding to self peptides/class II MHC expressed on APCs, as in the autologous or syngeneic mixed lymphocyte reaction (47, 48). For the following reasons, it is the large amounts of IL-2 formed by such $CD4^+$ self-reactive T cells that may be responsible for the generation of the $CD4^-8^-$ effector cells, at least in vitro. First, similar cytotoxic cells could be generated by adding an equivalent amount of IL-2 to the culture of normal spleen cells or $CD4^-8^-$ cells. Second, $CD25^-4^+$ T cells from IL-2-deficient mice failed to generate the cytotoxic cells from the $CD4^-8^-$ population in normal mice. Third, the TCR^- $CD4^-8^-$ cytotoxic cells generated by culturing $CD25^-$ cells killed not only NK-sensitive target cells (such as RL♂1 (27)), but also NK-resistant ones (such as P815), as IL-2-induced LAK cells can kill a broader spectrum of tumors than NK cells (49). The majority of such $CD25^-$ cell-derived killer cells indeed appeared to be NK cells in terms of their cell surface phenotype (e.g., $NK1.1^+$, $B220^+$), marked IL-2 dependency in their generation, and the lack of tumor-killing activity in beige mice (38–42). Taken together, these results indicate that active NK cells as LAK cells can be easily generated in vitro by simply eliminating a T cell subpopulation, without exogenous IL-2.

It remains to be determined, however, how in vivo removal of $CD25^+4^+$ T cells leads to the generation of two distinct types of effector cells (tumor-specific $CD8^+$ CTLs and tumor-nonspecific $CD4^-8^-$ NK-like cells (Fig. 2)), or whether $CD4^+$ self-reactive T cells play key roles in the in vivo generation of tumor immunity as well by secreting large amounts of IL-2. By in vivo administration of anti-CD25 Ab (Fig. 3), $CD4^-8^-$ LAK/NK-like cells indeed appeared within 1 wk, followed by dominant development of tumor-specific $CD8^+$ CTLs, which seemed to play main roles in ultimate tumor rejection (our manuscript in preparation). The result suggests that in vivo removal of $CD25^+4^+$ cells may first activate LAK/NK cells by activating $CD4^+$ self-reactive T cells and instigating their IL-2 secretion; and tumor-nonspecific killing by such LAK/NK cells and consequent release of tumor Ags, together with the IL-2 secreted by $CD4^+$ self-reactive T cells, may contribute to the subsequent development of tumor-specific $CD8^+$ CTLs. Alternatively, although mutually not exclusive, the removal of $CD25^+4^+$ cells may enhance the development of both LAK/NK cells (through activating $CD4^+$ self-reactive T cells) and $CD8^+$ CTLs (and tumor-specific $CD4^+$ Th cells) at the same time, because the immunoregulatory $CD25^+4^+$ T cells can directly suppress the activation/proliferation of both $CD4^+$ T cells and $CD8^+$ T cells, at least in vitro (16, 17). Further analysis of the in vivo interplay of the two types of effector cells in tumor immunity may contribute to our devising effective ways to generate them in vitro, especially tumor-specific $CD8^+$ CTLs.

A critical issue in our study is how tumor immunity can be evoked without autoimmunity when the $CD25^+4^+$ immunoregulatory T cells are manipulated. The present and previous studies by us and others have shown that, upon introduction of abnormal control of self-reactive T cells, the intensity and the range of the autoimmune responses (i.e., which self Ags are more prone to be aggressed, or which self-reactive clones are more prone to be activated) depend on the effector T cell subpopulations involved, the degree and duration of depleting $CD25^+4^+$ T cells, and, most importantly, the host genetic background, which includes MHC as well as non-MHC genes (7, 13, 18, 50–53). For example, when $CD4^+$ T cells are depleted from in vitro cultured $CD25^-$ cells and the remaining cells, including $CD8^+$ or $CD4^-8^-$ cytotoxic cells, are adoptively transferred, no histologically and serologically evident autoimmune disease developed in the recipients (Fig. 9), presumably because $CD4^+$ effector T cells are required for the development of this organ-specific autoimmunity as in many other models (8–14, 50–54). Furthermore, in BALB/c mice, which are genetically prone to develop organ-specific autoimmune diseases (in particular autoimmune gastritis (13, 14, 18, 50–53)), limiting the period or the degree of depleting $CD25^+$ T cells, for example, by in vivo administration of anti-CD25 Ab for a short period, could evoke effective tumor immunity without autoimmunity (Fig. 3, and our manuscript in preparation). In genetically autoimmune-resistant B6 mice (18, 50–53), on the other hand, complete depletion of $CD25^+4^+$ T cells from the cell inocula (as shown in Fig. 1*A*) led to tumor rejection, but failed to cause any autoimmune disease in B6 nude mice (our unpublished result). To further differentiate tumor immunity from autoimmunity, it is required to elucidate the molecular basis of the $CD25^+$ T cell-mediated immunoregulation and the host genes determining the susceptibility/resistance to autoimmunity.

Thus, with a caveat of possible autoimmunity in certain situations (see discussion above), the present findings in mice may help to devise a novel immunotherapy for cancer in humans or to make the current immunotherapies more effective. For example, administration of anti-CD25 Ab to cancer-bearing hosts for a limited period may evoke or enhance tumor immunity. Removal of $CD25^+4^+$ T cells from PBL or tumor-infiltrating T cells before their in vitro culture with IL-2 may lead to production of more potent or a larger number of cytotoxic cells, including CTLs and LAK/NK cells (40, 45, 55). Furthermore, such in vivo or in vitro elimination of the $CD25^+4^+$ immunoregulatory T cells may enhance tumor immunity when combined with the current attempts to augment immunogenicity of tumor cells, for example, by cytokine gene transduction in tumor cells, or vaccination with tumor Ags/peptides or Ag-pulsed dendritic cells (56, 57).

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